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Immunomodulatory, anti-inflammatory and wound healing properties of Echinacea species

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LIST OF ABBREVIATIONS

cAMP	3'-5'-cyclic adenosine monophosphate
CB2	cannabinoid receptor 2
CFU	colony forming unit
Con A	concanavalin A
COX-2	cyclooxygenase-2
Cr	Chromium
EA	*Echinacea angustifolia*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	*Echinacea purpurea*
EPA	*Echinacea pallida*
EtOH	Ethanol
FBS	fetal bovine serum
FWD	food and water deprived
GC	Glucocorticoids
GM-CSF	granulocyte monocyte colony stimulating factor
HBSS	hank's balanced salt solution
HPA axis	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography
ICAM-1	intercellular adhesion molecule 1 (CD54)
IFN-γ	interferon-γ
Ig	Immunoglobulin
IκB-α	NF-κB inhibitor alpha
IL	Interleukin

iNOS	inducible nitric oxide synthase
ip	intraperitoneal(ly)
iv	intravenous(ly)
LB	Luria-Bertani
LPS	Lipopolysaccharide
MIP-1α	macrophage inflammatory protein-1α
MOI	multiplicity of infection
NF-κB	nuclear factor kappa B
NK cell	natural killer cell
NO	nitric oxide
PBS	phosphate buffed saline
PBST	PBS/0.1% Tween-20
PE	Phycoerythrin
PECs	peritoneal exudate cells
PFC	plaque forming cell
PGE2	prostaglandin E2
RST	Restraint
SAM	sympathetic-adrenal mendullary axis
SNP	sodium nitroprusside
sRBC	sheep red blood cells
STATs	signal transducers and activators of transcription
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
UV	Ultraviolet

ABSTRACT

Herbal or botanical supplements have multiple health benefits. Public interest in these supplements has increased greatly in recent years aimed at modulation of the immune system to combat immune-related diseases. Historically, *Echinacea* has long been used as phytotherapy for wound healing, pain relief and treatment of the common cold. Although the non-specific immunostimulating properties of *Echinacea* species have been widely investigated, academic knowledge of the adaptive immune-modulating activity, the anti-inflammatory activity and the mechanistic basis for these immunomodulatory properties remain elusive. In this dissertation, we focused our research interest in these less explored fields, with an emphasis on the herbal anti-inflammatory activity in both *in vitro* macrophage cell line and *in vivo* oral administration models. *In vivo*, alcohol extracts of *Echinacea* exhibited multiple immune-modulating effects. In addition to the non-specific, innate immunomodulation, they enhance B cell and T cell function (determined by increased production of antibodies and cytokines). These modulating effects of *Echinacea* were more robust when host immune functions were disturbed by mild stress. *In vitro*, alcohol extracts of *Echinacea* suppressed activated macrophages (RAW 264.7 cells) so they produced lower amounts of inflammatory mediators including nitric oxide (NO). The decreased NO production might be relative to decreased inducible nitric oxide synthase (iNOS) enzyme expression and increased arginase activity, suggesting that *Echinacea* could switch macrophage to alternate activation. In a cutaneous wound healing model, we demonstrated that alcohol extract of *E. pallida* helped improve restraint stress-delayed wound healing. These data provided novel evidence to support the multiple immunomodulatory properties of *Echinacea* and will help define the mechanisms behind the success of traditional use of *Echinacea* for pathogenic infections and inflammatory diseases.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Plants are a rich source of natural chemicals whose importance to human beings is self-evident. Plants provide us not only food and building materials, but also medicines. Plants harbor great treasures of chemicals with medicinal value. The plants with medicinal purposes are regarded as medicinal herbs. Human use of herbal medicine has a long history, whose record can date back to about 60,000 years ago (Solecki, 1975). Today science and technology diversify medical services and medicines, however, it is estimated that as many as 80% of the world population still depend on herbal medicines to meet their primary healthcare (van Wijk, 2000). In the far East, Chinese medicines enjoy an important place in health care, more than 80 % of them originate from plants (Lee, 2000). In the Western world, the birthplace of "modern" medicine, although the importance of herbal medicine has been replaced by synthesized pure drugs, herbal medicine is still playing an essential role in the health care system and scientific community. Moreover, medicinal herbs are an important source for new drug development. About 40% of all current medical drugs derive their chemistry from medicinal herbs. In contrast to modern pure single drugs, herbal medicine has multiple health benefits. In the US, herbal medicine is classified as dietary supplements, which are consumed without prescription. Surveys indicate that over 30% of Americans are using herbal products for health purposes, spending about $4 billion annually (O'Hara et al., 1998; Bent and Ko, 2004). Notable examples of the botanical dietary supplements used widely in the US today include *Echinacea*, garlic, *ginkgo biloba*, ginseng, and St. John's wort.

Echinacea is a purple coneflower native to North America. Plains Indians first used it to relieve pain and to treat various conditions such as snakebites and wounds (Borchers et al., 2000). Early European settlers learned of its medicinal effects, and expanded its uses in treatment of infectious diseases. Since then, *Echinacea* has become one of the popular herbal

medicines in North America and Europe. Today, *Echinacea* products are mainly used for reducing the duration and/or the symptoms associated with the common cold and other upper respiratory infections. It is estimated that Americans spend about $300 million per year on *Echinacea* products and their sales make up 10% of the total US herbal market (Islam and Carter, 2005). There are nine known species of *Echinacea*, three of them, *E. angustifolia* (EA), *E. palllida* (EPA) and *E. purpurea* (EP) are considered useful medicinal plants and, therefore, widely used in phytotherapy. They have similar, but differential, chemical profiles and biological properties. Active principles may include hydrophobic alkamides, water-soluble caffeic acid derivatives and high molecular weight polysaccharides. Pharmacokinetics studies indicate that at least alkamides are readily bioavailable (Matthias et al., 2005a; Woelkart et al., 2005a). The biological activities of *Echinacea* are largely relative to its modulation of the immune system. In addition to the three commonly used species, other *Echinacea* species may have biological activities (Senchina et al., 2006; McCann et al., 2007).

Echinacea is closely linked to human use and health care. Its huge herbal market evokes the need to understand the plant itself and its biological nature. In recent years, hundreds of papers that pertain to *Echinacea* have been published, however, this is only the first step to understand the biochemical utility of the herb. Fortunately, comprehensive and interdisciplinary studies of *Echinacea* are underway to define the genetics, phytophysiology, phytochemistry and immunopharmacology of this herb. This dissertation is an inseparable part of a larger effort on *Echinacea*. We explored its immunomodulation and anti-inflammatory activity. It can be asserted that the data derived from these studies will enrich our understanding of the herbal biological properties, and help develop novel therapeutic strategies and plant-based drugs. The significance of studying *Echinacea* and other medicinal herbs can be illustrated by a successful example. After all, facts speak louder than words. *Artemisia annua* or Qinhao was long used as a Chinese medicinal herb in the treatment of fevers. Laboratory study of Qinhao led to isolation and purification of the active principle artemisinin

or qinghaosu (essence of Qinghao) in the 1970s. The work on modification of artemisinin structure resulted in emergence of potent classes of novel semi-synthesized derivative, such as artemether and artesunate. Artemisinin and its derivatives are not only currently widely used to treat multidrug-resistant malaria and cerebral malaria, but also found to have new uses such as antitumor, antiangiogenesis, antiviral and immunomodulation. Today, synthesis of artemisinin derivatives and analogs and study of their biological properties are a hot topic and represent a promising field in drug discovery (Li and Wu, 2003; Lee, 2007).

Dissertation Organization

This dissertation is organized in six chapters in order of general introduction, original research papers and general conclusion. Original research papers are arranged in chapter 2-5 which have been published by or will be submitted to their respective journals. Each paper was designed to address a specific and self-explanatory research interest. Four papers as a whole reflect continuity of our laboratory experiments under this dissertation topic.

Chapter 1 is a general introduction to the dissertation. It provides background information on the (non-specific) immunomodulatory properties of three medicinally used *Echinacea* species, mainly identified by laboratory work so far. In this review, we provide some necessary information related to our research in alternative macrophage activation and wound healing.

In chapter 2, we determined the immunoenhancing properties of *Echinacea* species in an *in vivo* gavage model, with an emphasis of their positive effects on adaptive immunity. We reported an "unexpected" finding that *Echinacea* exhibited an anti-inflammatory potential. This formed the basis for our subsequent research in alternative macrophage activation

In Chapter 3, we preliminarily determined the nitric oxide (NO) inhibiting activity of *Echinacea* alcohol extracts in both *in vitro* macrophage cell line and *in vivo* gavage models. Since NO is a key mediator involved in both host defense and inflammation, the potentially compromised macrophage function (phagocytosis and bacterial killing) was evaluated in the

parallel experiments.

Chapter 4 is an extension of chapter 3. We determined the mechanistic basis for the NO inhibiting activity of *Echinacea* species in macrophage cell line. We investigated the effect of *Echinacea* alcohol extracts on macrophage activation switching (between inflammatory activation and alternative activation), and explored the respective role of five fractions of alcohol extracts in macrophage activation.

In chapter 5, we applied our research to an *in vivo* model and determined the activity of EPA in a skin wound healing model. Chronic restrain stress was introduced in this model to further understand the immunomodulatory (immune homeostasis regulation) properties of *Echinacea*.

This dissertation ends in chapter 6 with a general discussion on the four original experimental papers. Additionally, it suggests certain recommendations for future research.

Literature Review

The genus *Echinacea* enjoys a long history as an herbal remedy. It first attracted academic attention in the 1930s (Cundell et al., 2003). In the past two decades, a large number of papers pertaining to *Echinacea* have been published in German, English and other languages, and the number of the papers is still rising rapidly. As we carried out experiments for this dissertation, several papers closely related to our research were already published. Since clinical data provided little information about the possible action of mechanism of the herb, in this review we discuss the biological activities and the immunomodulatory properties of *Echinacea* based on the collected data from *in vitro* assays and animal studies, and published in English.

Bearing in mind that most of the *Echinacea* preparations used in laboratory studies are a mixture of many naturally occurring compounds. The biological activities of a given preparation are determined by its specific chemical properties, which are subject to both

internal and external factors, such as species, parts of the plant selected, phase of plant development at harvest, soil type in which the plant is grown, preparation method, preparation form, experimental model, and administration route and dose (Percival, 2000; Hermann et al., 2003; Islam and Carter, 2005). These factors are interdependent and largely determine the diversities of *Echinacea* preparations. Precisely because of this, several aspects should be addressed at the beginning of this review. First, although it is more accurate to cite each *Echinacea* study with its preparation methods or active principles, in most cases we still used *Echinacea* as a general term instead of a "specific" preparation for the sake of a simplified review. Note that this is true for inductive reasoning (from a specific preparation to *Echinacea*) but not vice versa (it may be wrong to draw a conclusion from *Echinacea* to a specific preparation). Secondly, some study results, particularly those using crude preparations, might be difficult to compare and reproduce in the laboratory because of a lack of chemical analysis. Lastly, unlike single pure drugs which usually target certain tissues or molecules, *Echinacea* preparations may target many tissues or cell components and then elicit a wider range of biological activities because they contain indefinite bioactive metabolites.

Echinacea has multiple biological activities, most of them determined from in vitro and animal studies

Historically, *Echinacea* was used as a cure by Native Americans for various conditions and ailments. From current viewpoints, we believe most of the early uses of *Echinacea* lacked sufficient scientific support. However, *Echinacea* has stood the test of time. Today, *Echinacea* is most widely used as a preventive and treatment for upper respiratory infections including colds and influenza. In order to evaluate its effectiveness for these conditions, a number of human studies have been conducted. Unfortunately, the results are rather inconsistent. While some have validated its antiviral effects, others have refuted it. Nevertheless, the most consistent results indicate that *Echinacea* possesses an ability to inhibit the development and alleviate the symptoms of colds (Barrett, 2003; Barnes et al., 2005; Woelkart et al., 2008).

In contrast to a conflicting clinical standpoint, a majority of laboratory investigations suggested that *Echinacea* plays a positive role in the context of its medicinal uses including infection-fighting capacity. Note that some laboratory studies only provided indirect evidence due to the use of a blend of *Echinacea* and other herbs. Until now, *Echinacea* has been found to have multiple antimicrobial activities, such as antiviral (Thompson, 1998; Binns et al., 2002; Kapadia et al., 2002), antibacterial (Roesler et al., 1991b; Steinmuller et al., 1993), antifungal (Roesler et al., 1991b; Steinmuller et al., 1993; Binns et al., 2000; Morazzoni et al., 2005), insecticidal (Clifford et al., 2002), and anti-parasitic activity (Steinmuller et al., 1993; Parnham, 1996). *Echinacea* harbors the anti-infective activities mainly through the enhancement of host immune system, rather than direct killing of pathogens (Awang, 1999). A subsequent section will address the immunoenhancing properties of *Echinacea*.

Echinacea has antioxidant and anti-inflammatory activities. These activities clearly reflect another nature of *Echinacea*, that is, it may possess an opposite modulation of host physiological and immune responses. Use of *in vitro* free radical generation systems shows *Echinacea* has strong antioxidant activities which can be attributed to its chemical constituents - caffeic acid derivatives (Hu and Kitts, 2000; Sloley et al., 2001; Mishima et al., 2004; Pellati et al., 2004). Caffeic acid and its derivative echinacoside protect hydroxyl radical-induced degradation of type III collagen in skin photodamage (Facino et al., 1995). Of the three medicinally used *Echinacea* species, EPA exhibits the highest reducing power and greatest antioxidant activity, consistent with the highest echinacoside concentration in this plant (Cervellati et al., 2002; Zaporozhets et al., 2004). Alkamides, another class of bioactive phytochemicals in *Echinacea* are now recognized as largely responsible for the anti-inflammatory properties that will be discussed later.

The dual capacities of *Echinacea* as both immunostimulant and anti-inflammatory agent guarantee its wide range of biological activities. Other miscellaneous activities of *Echinacea* include antitumor (Currier and Miller, 2002; Kapadia et al., 2002), modulation of apoptosis (Di

Carlo et al., 2003; Chicca et al., 2007; Smalinskiene et al., 2007), and anti-ageing (Brousseau and Miller 2005; Miller, 2005).

Note that most of the biological activities of *Echinacea* have been demonstrated only by *in vitro* and/or animal studies, but not through use in humans. As one of the dietary supplements, safety from potential side-effects and interactions with other therapies should be a first concern when consuming *Echinacea* for treatment of some immune-mediated diseases, i.e. cancer, allergy, multiple sclerosis and AIDS (Bielory, 2004; Quimby, 2007). For example, as an immunostimulant, *Echinacea* is supposed to help people with HIV/AIDS develop a stronger immune system, however, it may further weaken their immune system to control HIV (Hosein, 1999).

Echinacea enhances overall immune function though it acts more strongly on innate immunity

A growing body of evidence supports that most of *Echinacea*'s biological effects are relevant to its immunomodulatory properties. Of the two main immune branches, *Echinacea* primarily targets the non-specific innate immunity. The effects of *Echinacea* on innate immunity appear to be contradictory. In some studies *Echinacea* can stimulate an immune response, but other studies indicate it can also suppress the same response. Whether the effect of *Echinacea* is immunoenhancing or immunosuppressive (anti-inflammatory) might be dependent on both the chemical profiles in a given herbal preparation and the host and/or immune cell functional state. In this section, we focus on the immunoenhancing properties. In the next section, the anti-inflammatory activities will be discussed.

Animal studies demonstrate *Echinacea* increases the number of circulating leukocytes, stimulates the proliferation of phagocytes in the spleen and bone marrow, and alters the percentage of white blood cells and splenocytes (Roesler et al., 1991a; Sun et al., 1999; O'Neill et al., 2002; Cundell et al., 2003; Brousseau and Miller, 2005). Cundell et al. (2003) observed a sustained increase in the percentage of circulating lymphocytes and monocytes associated with

a remarkable decrease in the percentage of circulating neutrophils in aging rats fed with aerial parts of *Echinacea* (without indicating species). The decreased percentage of neutrophils might be due to increases in granulocyte migration into the tissues (Roesler et al., 1991b; O'Neill et al., 2002). *Echinacea*-induced increase in total white blood cell count usually occurs synchronously with the activation in the function of these immune cells.

Macrophages and neutrophils are the main cell components that are influenced by *Echinacea*. Purified polysaccharides from *Echinacea* stimulated the phagocytosis and production of inflammatory mediators by macrophages and neutrophils in both *in vitro* assays and animal models (Stimpel et al., 1984; Luettig et al., 1989; Steinmuller et al., 1993). *In vitro*, human macrophages were activated by commercial preparations of EP (fresh pressed juice) to produce oxygen radicals and proinflammatory cytokines (Burger et al., 1997). Oral administration of *Echinacea* preparations including alkamides, polysaccharides and cichoric acid, to normal rats stimulated production of many cytokines, i.e. tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, interferon (IFN)-β and IL-10 by alveolar macrophages and/ spleen cells (Goel et al., 2002a, 2002b). Hwang et al. (2004) found water soluble EP extracts able to stimulate IL-6, IL-10, macrophage inflammatory protein (MIP)-1α and TNF-α from non-adherent murine splenocytes. *In vitro* alkamides of *Echinacea* has also been found to mediate cytokine gene expression within human peripheral leukocytes. It induces *de novo* synthesis of TNF-α mRNA in human macrophages, T and B cells, but no measurable increase in TNF-α protein (Gertsch et al., 2004).

Natural killer (NK) cells play a critical role in clearing viral infections through the processes of cytotoxicity, and production of cytokines such as IFN-γ and granulocyte monocyte colony stimulating factor (GM-CSF) (Gan et al., 2003). Studies with *Echinacea* have demonstrated its enhancing effect on NK cell activity *in vitro* and *in vivo* (Miller, 2005). Dried, ground preparation of fresh EP increased NK cytotoxic function *in vitro* in human peripheral blood lymphocytes of both healthy individual and patients with chronic fatigue syndrome or

acquired immunodeficiency syndrome (See et al., 1997). Currier and Miller (2000 and 2001) observed that aging mice, fed with a diet containing *Echinacea* ingredients, had a significant enhancement of NK cell cytotoxic activity and an increase in NK cell numbers. Gan et al. (2003) further found that *Echinacea* extracts could modulate NK cells at multiple levels including the maturation and increased cytotoxic potential of NK cells. Miller (2005) suggested that increased NK activity is partially associated with macrophage activation by *Echinacea*.

While many studies have emphasized the innate immune stimulation of *Echinacea*, its effects on adaptive immune functions are less known. Freier et al. (2003) observed an increase in immunoglobulin (Ig) M response against sheep red blood cells (sRBC) in mice orally treated with glycerine extracts of aerial parts of EP plant following sRBC immunization. Several other studies observed an increase of humoral immunity (antibodies against sRBC) *in vivo* but only provided indirect information because of the use of a blend of *Echinacea* with other herbs (Rehman et al., 1999; Bodinet et al., 2002a; Bodinet et al., 2004).

In addition, *Echinacea* products may possess the ability to modulate T cell function. A recent investigation with EP dried powder administrated intraperitoneally (ip) found an increase in CD4+ T-cells in mouse peripheral blood but implied it is a secondary effect in association with macrophage activation (Mishima et al., 2004). Another study reported that an EA root extract with a high content of polysaccharides and echinacoside enhances T-cell function *in vitro* by stimulating IFN-γ production (Morazzoni et al., 2005). However, in Jurkat cells, a human T-cell line, alkamides inhibit IL-2 production (Sasagawa, 2006) and have a mixed effect on the transcriptional factor nuclear factor kappa B (NFκB) expression (Matthias et al., 2008).

Echinacea is well known as a non-specific immunostimulant. This concept may partly come from its positive effects on the nonadaptive cell immunity, but more importantly originate from its ability to boost the whole immune system. As time goes on, there is some evidence to support the herbal capacity for modulation of adaptive immune branch. On the

other hand, the concept of *Echinacea* as a nonspecific immunostimulant is based on a sufficiently large number of empirical observations on crude preparations of *Echinacea* or a mixture of many natural chemicals, which combinationally exert either direct or indirect modulating effect on different immune components.

Echinacea harbors anti-inflammatory properties that may represent an active research field in future Echinacea study

The collective evidence mentioned above supports the immunoenhancing properties of *Echinacea*. The up-regulation of immune responses is consistent with an immune activated antimicrobial effect (Stimpel et al., 1984; Steinmuller et al., 1993; Burger et al., 1997). However, *Echinacea* exhibits an opposite pharmacological activity, too. In this case, it acts as an anti-inflammatory agent that actually suppresses some immune function. In recent years, the anti-inflammatory activity of *Echinacea* has attracted increasing attention not only because is has been used for centuries for wound healing benefits, but more importantly, there is an increasing concern about the possible negative effects caused by excessive inflammation. Robust immune function is beneficial for host defense in response to various infections and injuries, but can also lead to inflammation. Inflammation is a double-edged sword. It has important physiological significance, but excessive inflammation is detrimental in various health conditions including cancer, diabetes, asthma, Alzheimer's disease, rheumatoid arthritis and the normal aging (Caruso et al., 2004; Ware, 2005). At present, *Echinacea* is commonly termed as an immunomodulant instead of the previous nomenclature of immunostimulant.

Experiments with mice demonstrated that topical application of a polysaccharide fraction from EA roots or oral administration of EP root dry powder significantly inhibited carrageenan-induced paw edema formation (Tubaro et al., 1987; Raso et al., 2002), and this effect was in part related to the down-regulation of cyclooxygenase (COX)-2 expression in peritoneal macrophages (Raso et al., 2002). Echinacoside was proposed to have

hyaluronidase inhibiting activity and thus increase the amount of hyaluronan available to the extracellar matrix required for scarless wound repair (Speroni et al., 2002; Rousseau et al., 2006). When one dose of alcohol extracts of EP or EPA was applied topically to abraded skin or excised wounds on rats for 72 h, EPA extract exhibited more robust anti-inflammatory and enhanced wound healing effects than EP extract. The authors attributed the effects of EPA extract to its constituent echinacoside (Speroni et al., 2002).

More recent studies suggested that from both pharmacological and bioavailability standpoints, it is the lipophilic alkamides of *Echinacea* that play a more important role in the herbal anti-inflammatory activities (Woelkart and Bauer, 2007). *In vitro*, polyunsaturated alkamides from EA inhibited microsomal cyclooxygenase and leukocyte 5-lipoxygenase activity, two enzymes responsible for production of local proinflammatory hormones and regulation of fibroblast wound closure (Muller-Jakic et al., 1994; Clifford et al., 2002).

In the light of the role of macrophages in inflammatory response, studies with macrophage cell line RAW 264.7 cells demonstrated that *Echinacea* alcohol extracts and their alkamides inhibited activated macrophages to produce NO, prostaglandin E2 (PGE2) and TNF-α (Chen et al., 2005, Lalone et al., 2007; Zhai et al., 2007). Such decreased production of inflammatory mediators might be relative to downregulated NF-κB activity by certain individual alkamides (Matthias et al., 2007). However, a recent study showed certain alkamides from EA roots inhibited PGE2 formation by human neuroglioma cells through decreased cyclooxygenase-2 (COX-2) activity, but not COX-2 mRNA and protein expression. On the contrary, alkamides increased COX-2 mRNA and protein expression (Hinz et al., 2007).

As matters stand now, research work on the anti-inflammatory activity of *Echinacea* may be of significance in both understanding of the biological activity of *Echinacea* and new drug discovery. Firstly, individual phytochemicals, i.e. alkamides, are receiving unprecedented attention. Unlike polysaccharides which occur in every plant, certain

alkamides and caffeic acid derivatives are unique to *Echinacea* species (Bone, 1997). They may represent important natural sources for new drug discovery. Second, an in-depth study of the anti-inflammatory activity of individual phytochemicals will facilitate finding potential molecular target candidates, especially those that play a key role in regulation of signal transduction pathways and inflammatory responses.

Echinacea contains many bioactive metabolites that determine its wide spectrum of biological activities

Echinacea depends on its phytochemical profiles for specific pharmacological activities. The three medicinal *Echinacea* species have similar phytochemical profiles with slight variation in amounts of individual compounds. Several widely accepted active compounds can be divided into three classes: high molecular weight polysaccharides, lipophilic alkamides and polar caffeic acid derivative (Barnes et al., 2005). The typical biological activities, mechanistic basis and physical properties of these three classes of phytochemicals are listed in table 1.

Polysaccharide constituents. Polysaccharides are common natural compounds in the plant kingdom. There are two types, PS1 (a methylglucuronoarabinoxylan) and PS2 (an acidic arabinorhamnogalactan) that are relative to the biological activity of *Echinacea* (Bone, 1997; Barnes et al., 2005). Some studies on *Echinacea* polysaccharides used a purified component derived from tissue cultures of *Echinacea*. Other studies used polysaccharides-containing *Echinacea* materials such as water extracts or fresh pressed juice. *Echinacea* polysaccharides received early scientific attention because of their antimicrobial and immune stimulating properties. They were shown to stimulate macrophages and neutrophils to produce inflammatory cytokines and reactive oxygen species (ROS) (Stimpel et al., 1984; Luettig et al., 1989; Burger et al., 1997; Rininger et al., 2000), and protect immunocompromised mice from lethal fungal and bacterial infection (Roesler et al., 1991; Steinmuller et al., 1993). Polysaccharides from EP stimulated both classical and alternative pathways of complement activation (Alban et al., 2002).

The absorption of polysaccharides in gastrointestinal tract is uncertain. In animal models, polysaccharides were usually administered via ip or intravenous (iv) injection, thus, bypassing digestion and absorption (Luettig et al., 1989; Roesler et al., 1991). Topical application of polysaccharides was found to have anti-inflammatory effects in the carrageenan paw edema and the Croton oil ear test models (Tubaro et al., 1987; Tragni et al., 1988). For studies of water-soluble polysaccharides, especially with *in vitro* cell culture or injection into animals, special attention should be paid to endotoxin contamination, which may give rise to false-positive results via activation of toll-like receptor (TLR)-4 expressed on the membranes of certain immune cell components, i.e. macrophages. The specific medicinal value of polysaccharides from *Echinacea* is doubtful because they are common components of plant cell wall and have an oral bioavailability problem (Bone, 1997).

Alkamide constituents. Lipophilic alkamides are predominantly present in the roots of EA and EP, but largely absent in EPA. They are composed of a highly unsaturated fatty acid and an amide. The unsaturated fatty acid may be reflective of the true active form (Bone, 1997). Compared to the fading interest in polysaccharides, alkamides have received more attention recently as they may represent a group of natural compounds with immunomodulating and anti-inflammatory properties when administered *in vivo* and are recognized to be a prime choice of biological marker for activity of *Echinacea* preparations.

In vitro, alkamides inhibited metabolic enzymes involved in inflammatory response (Muller-Jakic et al., 1994; Clifford et al., 2002) and suppress macrophage production of inflammatory mediators (Chen et al., 2005, Lalone et al., 2007; Zhai et al., 2007). The inhibitory effect of alkamides on NF-κB has been preliminarily demonstrated (Matthias et al., 2007).

Alkamides have a structural similarity with anandamide, an endogenous cannabinoid (CB) receptor ligand. Thus, they may modulate immune responses through their interaction with CB2 receptors (Gertsch et al., 2004; Woelkart et al., 2005b). Alkamides have been

demonstrated to significantly inhibit LPS-induced inflammation in human whole blood via both CB2-dependent and -independent pathways (Raduner et al., 2006). CB2 receptors belong to the family of G protein-coupled receptors, which are mainly expressed on immune cells and in the spleen (Raduner et al., 2006). CB2 receptors are thought of as an important molecular target in drug discovery since they take part in various pathophysiological processes. The finding of the interaction of *Echinacea*-derived alkamides with CB2 receptors may be one of the biggest achievements in *Echinacea* studies so far since this group of natural occurring compounds may provide parental structures for the design and development of novel drugs that target CB2 receptors (Gertsch et al., 2006; Matovic et al., 2007).

In addition to the anti-inflammatory activity, alkamides may enhance macrophage function similarly to *Echinacea*-derived polysaccharides. In a healthy rat model, oral administration of a mixture of alkamides, polysaccharides and cichoric acid significantly increased the phagocytosis and production of NO and cytokines by LPS-stimulated alveolar macrophages and/or spleen macrophages (Geol et al., 2002a, 2002b). Such a macrophage enhancing effect may be attributable to alkamides from the viewpoint of bioavailability.

Intestinal absorption studies using Caco-2 monolayers, a model for the intestinal epithelial barrier, found alkamides diffused rapidly through the Caco-2 monolayers, suggesting that alkamides can easily reach the bloodstream without being modified by the digestive process (Jager et al., 2002; Matthias et al., 2004; Matthias et al., 2005b). The results from the Caco-2 model have been confirmed in human pharmacokinetic studies (Matthias et al., 2005a, 2005b; Woelkart et al., 2005a).

Caffeic acid derivatives. Polar caffeic acid derivatives include a wide range of phytochemicals such as echinacoside, cichoric acid, caftaric acid, chlorogenic acid and cynarin. Echinacoside occurs in EA and EPA. EP contains high levels of cichoric acid, but lacks echinacoside. Cynarin is a characteristic compound of EA. Caffeic acid and its derivatives

received attention in recent years for their antioxidant, anti-inflammatory, and cicatrizing (forming scar tissue) activities (Speroni et al., 2002).

Laboratory investigations show that caffeic acid and its derivatives have strong antioxidant activities and free radical scavenging abilities in cell-free free radical generation systems (Facino et al., 1995; Hu et al., 2000; Sloley et al., 2001; Mishima et al., 2004; Pellati et al., 2004), and the antioxidant potential could be enhanced by alkamides and polysaccharides (Dalby-Brown et al., 2005). *In vitro*, caffeic acid derivatives have antihyaluronidase activity (Facino et al., 1993). This biochemical nature of echinacoside has been functionally linked to the anti-inflammatory and wound healing properties of *Echinacea* (Speroni et al., 2002; Rousseau et al., 2006). The immunomodulating effects of the above-mentioned individual caffeic acid derivatives are unknown, but they may elicit a wide range of biological responses (Verma and Hansch, 2004; Jiang et al., 2005).

Pharmacokinetic studies of naturally occurring or synthetic caffeic acid derivatives (e.g. caftaric acid, chlorogenic acid and echinacoside) showed that they are quickly absorbed in the rat stomach, but eliminated rapidly from the blood circulation (Jia et al., 2006; Lafay et al., 2006; Vanzo et al., 2007). Matthias et al. (2004) suggested a low bioavailability for caffeic acid derivatives of *Echinacea* based on the observations that they permeate poorly through the Caco-2 monolayer model. Human studies indicated undetectable parent caffeic acid derivatives or their metabolites in plasma samples of healthy volunteers after *Echinacea* tablet ingestion (Matthias et al., 2005a, 2005b).

Other bioactive constituents. In addition to the above-mentioned three mainly recognized classes of phytochemicals, there might be other plant metabolites that are responsible for the herbal biological activities. For examples, glycoproteins are purported to be active components (Classen et al., 2000; Thude and Classen, 2005). Ketones are lipophilic compounds that usually concomitantly appear in alkamide fractions of EPA. Like alkamides, ketones are easily absorbed by the intestine (Chicca et al., 2008). The interaction between

ketones and alkamides is unknown. Ketones have been suggested to have antifungal activity (Binns et al., 2000) and direct cytotoxicity on cancer cells (Pellati et al., 2006; Chicca et al., 2008). In addition, melanin from *Echinacea* was suggested to be an effective mucosal immune modulator. It activates NF-κB in monocytes *in vitro* through a TLR-2-dependent process. *In vivo* melanin enhances production of IFN-γ by spleen cells and IgA and IL-6 production by Peyer's patch cells (Pugh et al., 2005).

It should be noted that different classes of chemicals may have some biological activities in common. For examples, polysaccharides show anti-inflammatory activities in addition to immunomodulation, and alkamides showed immunostimuating activities in addition to anti-inflammation. This may indicate that each chemical class has pharmacological diversities. In addition, individual metabolites within each chemical class may also have distinct biological activities.

Natural bioactive chemicals of *Echinacea* may work not only individually, but also additively, synergistically or antagonistically, endowing the herb with multiple biological activities, and therefore could account for the diverse effects observed in studies using extracts of *Echinacea*. On the current herbal market, *Echinacea* products are mainly crude preparations and often blended with other herbs in various forms. Interestingly, it has been shown that isolated phytochemicals do not mimic the biological effects of whole plant extracts (Currier et al., 2003) and in animal experiments a combination of *Echinacea* extracts demonstrated a higher activity than extracts of a single plant (Bodinet et al., 2002a). It appears that the biological activity of a combination of its phytochemical components is more robust than one individual compound (Bodinet et al., 2002a; Currier et al., 2003; Randolph et al., 2003). Studies of the interactive effects of individual chemicals may have important clinical implications.

Perspective and research interests related to this dissertation

Although *Echinacea* has a long history as an herbal medicine, its precise mechanism

has not been completely identified. Mechanistic understanding of *Echinacea* is of both potential medicinal significance and of important academic value. Almost all of the known biological activities of *Echinacea* are, to a large extent, linked to its immunomodulating nature. A typical example is that *Echinacea* modulates production of the endpoint products of inflammatory macrophages *in vitro* and *ex vivo*. Macrophages as one of the important immune components are usually used to screen and evaluate the efficacy of new *Echinacea* preparations including fractions and individual compounds. Although *Echinacea* preparations can modulate production of inflammatory cytokines and free radicals by activated macrophages, the mechanistic basis for these observed phenomena remains unclear. Fortunately, certain individual alkamides have been found to modulate NF-κB activities and/or bind CB2 receptor (Gertsch et al., 2004; Raduner et al., 2006; Matthias et al., 2007), providing a molecular basis for understanding of the herbal anti-inflammatory activities.

It is worthwhile to note that macrophages have dual immune functions and can be either classically activated (pro-inflammatory) or alternatively activated (anti-inflammatory). A given macrophage may switch from an activated state to another upon an external stimulus (Porcheray et al., 2005). Two types of activated macrophages are characterized by specific activation phenotypes and distinct biochemical pathways. It is interesting to determine *Echinacea*-mediated changes in the intracellular and extracellular molecular markers, and biochemical metabolic processes by using immunological, biochemical, cell and molecular biology techniques. For examples, L-arginine can be metabolized to produce either NO or L-ornithine (Morris et al., 1998; Meurs et al., 2003). Macrophages undergo classical activation to activate inducible nitric oxide synthase (iNOS) and produce NO as well as other mediators that cause excessive inflammation. In addition macrophages undergo alternative activation to activate arginase leading to L-ornithine production as well as other mediators resulting in wound healing. Studies of the effects of *Echinacea* on these cross-regulated pathways may provide a new viewpoint for understanding of *Echinacea*.

Compared to *in vitro* assays, animal experiments may be more appropriate to study the true efficacy of *Echinacea*. To that end wound healing may be used as model to evaluate the effectiveness of various *Echinacea* preparations and to understand its anti-inflammatory mechanism. Wound healing is a complex process (Park et al., 2004). Wounding initiates migration of neutrophils and macrophages to the wound site at the earliest stage of tissue repair. These two immune cell types play an integral role in wound healing through release of cytokines, growth factors and mediators. Inflammatory mediators i.e. TNF-α , NO and ROS are indispensable for excisional wound repair (Kondo and Ohshima, 1996). However, to accomplish successful wound repair, these inflammatory mediators are tightly regulated by a set of complex mechanisms through which the body makes full use of its positive regulation but controls its negative effect. In this case, *Echinacea* may be used to improve wound healing due to its immunomodulatory properties. Although *Echinacea* had been studied in several animal inflammatory models, they lacked a systemic determination of the dynamic pattern of wound healing as well as proinflammatory and anti-inflammatory mediators throughout wound healing process with exposure to *Echinacea*.

It should be noted that *Echinacea* has multiple functional influences on the immune system. The combined effect of several active compounds may be synergistic or antagonistic, and the type of action initiated largely relies on the host condition. If *Echinacea* is beneficial for wound healing, it would be interesting to know what effect of *Echinacea* could have if the wound healing process is perturbed by other factors, i.e. stress. Chronic psychological or physical stressors activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to elevated plasma levels of glucocorticoids. The pharmacological level of glucocorticoids usually inhibit many components of inflammatory response, i.e. down-regulated production of proinflammatory cytokines and decreased phagocytosis of activated neutrophils and macrophages, which in turn delay cellular recruitment and proliferation at the wound site (Dhabhar, 2002; Sheridan et al., 2004; Paus et al., 2006). It has been well demonstrated in

murine model of cutaneous healing that chronic restraint stress can inhibit cellular infiltration, delay wound healing and increase the susceptibility to wound infection (Padgett et al., 1998;

Table 1.1. Bioactive metabolites of *Echinacea* and their properties

Properties	Bioactive Metabolites[1,2]		
	Polysaccharides	**Alkamides**	**Caffeic acid derivatives**
Chemical distribution	Every plant	Mainly in EA and EP (EPA has unique ketones)	Echinacoside occurs in EA and EPA; EP has rich cichoric acid; Cynarin is unique to EA
Chemical structure	Polymers of simple sugars	Condensation of an unsaturated fatty acid and an amine	Caffeic acid monomer or polymer
Physical nature	Hydrophilic	Lipophilic	Water-soluble
Oral bioavailability	Low	High	Low
Common administration route in animal studies	i,p, iv, or topically	Orally	Topically
Biological activities	Immunostimulatory i.e. increase phagocytosis and production of inflammation mediators	Anti-inflammatory i.e. inhibition of production of inflammatory mediators; Bind CB2 receptor	Anti-oxidant and free radical scavenging

1. Structure-activity relationship is undefined
2. The biological activity of a combination of multiple phytochemicals is more robust than single individual compound

Rojas et al., 2002; Sheridan et al., 2004; Head et al., 2006). In this case, it is of interest to determine the effect if *Echinacea* on stress-induced inhibition of wound healing. It is highly likely that *Echinacea* exerts multiple immunomodulatory effects, i.e. enhancement of immune function in the earlier stages, and a switch to alternate macrophage activation in the later stages in order to allow wound tissue regeneration.

Conclusion

Echinacea is a laboratorily proven effective herbal medicine. It shows many pharmacological

activities mainly through modulation of the immune system in response to pathogenic challenge or mitogenic stimulation. One of the potential advantages with the use of *Echinacea* may be that many natural chemicals in a given herbal preparation act together, leading to ideal biological effects that are of health benefit via a dynamic homeostasis of the host's physiological and immune functions. Polysaccharides, alkamides and caffeic acid derivatives are three widely accepted classes of medicinal phytochemicals in *Echinacea*. They, however, unlike crude preparations with additional miscellaneous activities, exhibit strikingly distinct biological properties; they are immunostimulating, anti-inflammatory and antioxidant, respectively. Some of these identified chemicals and other potential yet unidentified chemicals may represent important chemical structures for novel drug discovery. Mechanistic study of *Echinacea*'s effects is underway and some biomacromolecules, i.e. CB2 receptor, have been suggested to be therapeutic target candidates. Study of the structure-activity relationship of individual effective natural chemicals may help to not only understand the biological nature of *Echinacea*, but also make new plant-based drugs possible.

References

Alban S, Classen B, Brunner G, Blaschek W. 2002. Differentiation between the complement modulating effects of an arabinogalactan-protein from *Echinacea purpurea* and heparin. Planta Med 68:1118-1124.

Awang, DVC. 1999. Immune stimulants and antiviral botanicals: *Echinacea* and ginseng. p. 450–456. In: J. Janick (ed.), Perspectives on new crops and new uses. ASHS Press, Alexandria, VA.

Barnes J, Anderson LA, Gibbons S, Phillipson JD. 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt.,*Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol 57:929-954.

Barrett B. 2003. Medicinal properties of *Echinacea*: A critical review. Phytomed 10:66-86.

Bielory L. 2004. Complementary and alternative interventions in asthma, allergy, and immunology. Ann Allergy Asthma Immunol 93:S45-54.

Bent S, Ko R. 2004. Commonly used herbal medicines in the United States: a review. Am J Med 116: 478-485.

Binns SE, Hudson J, Merali S, Arnason JT. 2002. Antiviral activity of characterized extracts from *Echinacea spp*. (Heliantheae: Asteraceae) against herpes simplex virus (HSV-I). Planta Med 68:780-783.

Binns SE, Purgina B, Bergeron C, Smith ML, Ball L, Baum BR, Arnason JT. 2000. Light-mediated antifungal activity of *Echinacea* extracts. Planta Med 66:241-244.

Bodinet C, Lindequist U, Teuscher E, Freudenstein J. 2002a. Effect of an orally applied herbal immunomodulator on cytokine induction and antibody response in normal and immunosuppressed mice. Phytomedicine 9:606-613.

Bodinet C, Mentel R, Wegner U, Lindequist U, Teuscher E, Freudenstein J. 2002b. Effect of oral application of an immunomodulating plant extract on Influenza virus type A infection in mice. Planta Med 68:896-900.

Bodinet C, Lindequist U, Teuscher E, Freudenstein J. 2004. Influence of peroral application of a herbal immunomodulator on the antibody production of Peyer's patches cells. Arzneimittelforschung 54:114-118.

Bone K. 1997. *Echinacea*: what makes it work? Alt Med Rev 2:87-93.

Borchers AT, Keen CL, Stern JS, Gershwin ME. 2000. Inflammation and Native American medicine: the role of botanicals. Am J Clin Nutr 72:339-347.

Brousseau M, Miller SC. 2005. Enhancement of natural killer cells and increased survival of aging mice fed daily *Echinacea* root extract from youth. Biogerontology 6:157-163.

Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG. 1997. *Echinacea*-induced cytokine production by human macrophages. Int J Immunopharmacol 19:371-379.

Caruso C, Lio D, Cavallone L, Franceschi C. 2004. Aging, longevity, inflammation, and cancer. Ann N Y Acad Sci 1028:1-13.

Cervellati R, Renzulli C, Guerra MC, Speroni E. 2002. Evaluation of antioxidant activity of some natural polyphenolic compounds using the Briggs-Rauscher reaction method. J Agric Food Chem 50:7504-7509.

Chicca A, Adinolfi B, Martinotti E, Fogli S, Breschi MC, Pellati F, Benvenuti S, Nieri P. 2007. Cytotoxic effects of *Echinacea* root hexanic extracts on human cancer cell lines. J Ethnopharmacol 110:148-153.

Chicca A, Pellati F, Adinolfi B, Matthias A, Massarelli I, Benvenuti S, Martinotti E, Bianucci AM, Bone K, Lehmann R, Nieri P. 2008. Cytotoxic activity of polyacetylenes and polyenes isolated from roots of *Echinacea pallida*. Br J Pharmacol Jan 14 [Epub ahead of print]

Classen B, Witthohn K, Blaschek W. 2000. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the β-glucosyl Yariv reagent. Carbohydr Res 327:497-504.

Clifford LJ, Nair MG, Rana J, Dewitt DL. 2002. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. Phytomedicine 9:249-253.

Cundell DR, Matrone MA, Ratajczak P, Pierce JD Jr. 2003. The effect of aerial parts of *Echinacea* on the circulating white cell levels and selected immune functions of the aging male Sprague-Dawley rat. Int Immunopharmacol 3:1041-1048.

Currier NL, Miller SC. 2002. The effect of immunization with killed tumor cells, with/without feeding of *Echinacea purpurea* in an erythroleukemic mouse model. J Altern Complement Med 8:49-58.

Currier NL, Miller SC. 2000. Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. Exp Gerontol 35:627-639.

Currier NL, Miller SC. 2001. *Echinacea purpurea* and melatonin augment natural-killer cells in leukemic mice and prolong life span. J Altern Complement Med 7:241-251.

Currier NL, Lejtenyi D, Miller SC. 2003. Effect over time of in-vivo administration of the polysaccharide arabinogalactan on immune and hemopoietic cell lineages in murine spleen and bone marrow. Phytomedicine 10:145-153.

Dalby-Brown L, Barsett H, Landbo AK, Meyer AS, Molgaard P. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. J Agric Food Chem 53:9413-9423.

Dhabhar FS. 2002. Stress-induced augmentation of immune function - the role of stress hormones, leukocyte trafficking, and cytokines. Brain Behav Immun 16:785-798.

Di Carlo G, Nuzzo I, Capasso R, Sanges MR, Galdiero E, Capasso F, Carratelli CR. 2003. Modulation of apoptosis in mice treated with *Echinacea* and *St. John's wort*. Pharmacol Res 48:273-277.

Facino RM, Carini M, Aldini G, Marinello C, Arlandini E, Franzoi L, Colombo M, Pietta P, Mauri P. 1993. Direct characterization of caffeoyl esters with antihyaluronidase activity in crude extracts from *Echinacea angustifolia* roots by fast atom bombardment tandem mass spectrometry. Farmaco 48:1447-1461.

Facino RM, Carini M, Aldini G, Saibene L, Pietta P, Mauri P. 1995. Echinacoside and caffeoyl conjugates protect collagen from *Echinacea* extracts in the prevention of skin photodamage. Planta Med 61:510-514.

Freier DO, Wright K, Klein K, Voll D, Dabiri K, Cosulich K, George R. 2003. Enhancement of the humoral immune response by *Echinacea purpurea* in female Swiss mice. Immunopharmacol Immunotoxicol 25:551-560.

Gan XH, Zhang L, Heber D, Bonavida B. 2003. Mechanism of activation of human peripheral blood NK cells at the single cell level by *Echinacea* water soluble extracts: recruitment of lymphocyte-target conjugates and killer cells and activation of programming for lysis. Int Immunopharmacol 3:811-824.

Gertsch J, Schoop R, Kuenzle U, Suter A. 2004. *Echinacea* alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS Lett 577:563-569.

Gertsch J, Raduner S, Altmann KH. New natural noncannabinoid ligands for cannabinoid type-2 (CB2) receptors. 2006. J Recept Signal Transduct Res 26:709-730.

Goel V, Chang C, Slama JV, Barton R, Bauer R, Gahler R, Basu TK. 2002a. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. Int Immunopharmacol 2:381-387.

Goel V, Chang C, Slama J, Barton R, Bauer R, Gahler R, Basu T. 2002b. *Echinacea* stimulates macrophage function in the lung and spleen of normal rats. J Nutr Biochem 13:487.

Head CC, Farrow MJ, Sheridan JF, Padgett DA. 2006. Androstenediol reduces the anti-inflammatory effects of restraint stress during wound healing. Bain Behav Immun 20:590-596.

Hermann JR, Honeyman MS, Zimmerman JJ, Thacker BJ, Holden PJ, Chang CC. 2003. Effect of dietary *Echinacea purpurea* on viremia and performance in porcine reproductive and respiratory syndrome virus-infected nursery pigs. J Anim Sci 81:2139-2144.

Hinz B, Woelkart K, Bauer R. 2007. Alkamides from *Echinacea* inhibit cyclooxygenase-2 activity in human neuroglioma cells. Biochem Biophys Res Commun 360:441-446.

Hosein SR. 1999. Are *Echinacea* and HIV not a good mix? TreatmentUpdate95, 11(1).

Hu C, Kitts DD. 2000. Studies on the antioxidant activity of *Echinacea* root extract. J Agric Food Chem 48:1466-1472.

Hwang SA, Dasgupta A, Actor JK. 2004. Cytokine production by non-adherent mouse splenocyte cultures to *Echinacea* extracts. Clin Chim Acta 343:161-166.

Islam J, Carter R: 2005. Use of *Echinacea* in upper respiratory tract infection. South Med J 98:311-318.

Jager H, Meinel L, Dietz B, Lapke C, Bauer R, Merkle HP, Heilmann J. 2002. Transport of alkamides from *Echinacea* species through Caco-2 monolayers. Planta Med 68:469-471.

Jia C, Shi H, Wu X, Li Y, Chen J, Tu P. 2006. Determination of echinacoside in rat serum by reversed-phase high-performance liquid chromatography with ultraviolet detection and its application to pharmacokinetics and bioavailability. J Chromatogr B Analyt Technol Biomed Life Sci 844:308-313.

Jiang RW, Lau KM, Hon PM, Mak TC, Woo KS, Fung KP. 2005. Chemistry and biological activities of caffeic acid derivatives from Salvia miltiorrhiza. Curr Med Chem 12:237-46.

Kapadia GJ, Azuine MA, Tokuda H, Hang E, Mukainaka T, Nishino H, Sridhar R. 2002. Inhibitory effect of herbal remedies on 12-O-tetradecanoylphorbol-13-acetate-promoted Epstein-Barr virus early antigen activation. Pharmacol Res 45:213-220.

Kondo T, Ohshima T. 1996. The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. Int J Legal Med 108:231-236.

Lafay S, Gil-Izquierdo A, Manach C, Morand C, Besson C, Scalbert A. 2006. Chlorogenic

acid is absorbed in its intact form in the stomach of rats. J Nutr 136:1192-1197.

Lalone CA, Hammer KD, Wu L, Bae J, Leyva N, Liu Y, Solco AK, Kraus GA, Murphy PA, Wurtele ES, Kim OK, Seo KI, Widrlechner MP, Birt DF. 2007. *Echinacea* species and alkamides inhibit prostaglandin E(2) production in RAW 264.7 mouse macrophage cells. J Agric Food Chem 55:7314-7322.

Lee KH. 2000. Research and future trends in the pharmaceutical development of medicinal herbs from Chinese medicine. Public Health Nutr 3:515-522.

Lee S. 2007. Artemisinin, promising lead natural product for various drug developments. Mini Rev Med Chem 7:411-422.

Li Y, Wu YL**.** 2003. An over four millennium story behind qinghaosu (artemisinin)--a fantastic antimalarial drug from a traditional Chinese herb. Curr Med Chem 10:2197-2230.

Luettig B, C Steinmuller, G. E Gifford, H Wagner, M. L Lohmann Matthes. 1989. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. J Nat Cancer Institute 81:669–675.

McCann DA, Solco A, Liu Y, Macaluso F, Murphy PA, Kohut ML, Senchina DS. 2007. Cytokine- and interferon-modulating properties of *Echinacea* spp. root tinctures stored at -20 degrees C for 2 years. J Interferon Cytokine Res 27: 425-436.

Matovic N, Matthias A, Gertsch J, Raduner S, Bone KM, Lehmann RP, Devoss JJ. 2007. Stereoselective synthesis, natural occurrence and CB(2) receptor binding affinities of alkylamides from herbal medicines such as *Echinacea* sp. Org Biomol Chem 5:169-174.

Matthias A, Blanchfield JT, Penman KG, Toth I, Lang CS, De Voss JJ, Lehmann RP. 2004. Permeability studies of alkylamides and caffeic acid conjugates from *Echinacea* using a Caco-2 cell monolayer model. J Clin Pharm Ther 29:7-13.

Matthias A, Addison RS, Penman KG, Dickinson RG, Bone KM, Lehmann RP. 2005a. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. Life Sci 77:2018-2029.

Matthias A, Penman KG, Matovic NJ, Bone KM, De Voss JJ, Lehmann RP. 2005b. Bioavailability of *Echinacea* constituents: Caco-2 monolayers and pharmacokinetics of the alkylamides and caffeic acid conjugates. Molecules 10:1242-1251.

Matthias A, Banbury L, Stevenson LM, Bone KM, Leach DN, Lehmann RP. 2007. Alkylamides from *Echinacea* modulate induced immune responses in macrophages. Immunol Invest 36:117-130.

Matthias A, Banbury L, Bone KM, Leach DN, Lehmann RP. 2008. *Echinacea* alkylamides modulate induced immune responses in T-cells. Fitoterapia 79:53-58.

Meurs H, Maarsingh H, Zaagsma J. 2003. Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness. Trends Pharmacol Sci 24:450-455.

Miller SC. 2005. *Echinacea*: a miracle herb against aging and cancer? Evidence *in vivo* in mice. Evid Based Complement Alternat Med 2:309-314.

Mishima S, Saito K, Maruyama H, Inoue M, Yamashita T, Ishida T, Gu Y. 2004. Antioxidant and immuno-enhancing effects of *Echinacea purpurea*. Biol Pharm Bull 27:1004-1009.

Morazzoni P, Cristoni A, Di Pierro F, Avanzini C, Ravarino D, Stornello S, Zucca M, Musso T. 2005. *In vitro* and *in vitro* immune stimulating effects of a new standardized *Echinacea angustifolia* root extract (Polinacea™). Fitoterapia 76:401-411.

Morris SM Jr, Kepka-Lenhart D, Chen LC. 1998. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol 275:E740-747.

Muller-Jakic B, Breu W, Probstle A, Redl K, Greger H, Bauer R. 1994. *In vitro* inhibition of cyclooxygenase and 5-lipoxygenase by alkamides from *Echinacea* and *Achillea* species. Planta Med 60:37-40.

O'Hara M, Kiefer D, Farrell K, Kemper K. 1998. A review of 12 commonly used medicinal herbs. Arch Fam Med 7:523-536

O'Neill W, McKee S, Clarke AF. 2002. Immunological and haematinic consequences of feeding a standardised *Echinacea* (*Echinacea angustifolia*) extract to healthy horses. Equine Vet J 34:222-227.

Padgett DA, Marucha PT, Sheridan JF. 1998. Restraint stress slows cutaneous wound healing in mice. Brain Behav Immun 12:64-73.

Parnham MJ. 1996. Benefit-risk assessment of the squeezed sap of the purple coneflower (*Echinacea purpurea*) for long-term oral immunostimulation. Phytomedicine 3:95-102.

Paus R, Theoharides TC, Arck PC. 2006. Neuroimmunoendocrine circuitry of the 'brain-skin connection'. Trends Immunol 27:32-39.

Pellati F, Calò S, Benvenuti S, Adinolfi B, Nieri P, Melegari M. 2006. Isolation and structure elucidation of cytotoxic polyacetylenes and polyenes from *Echinacea pallida*. Phytochemistry 67:1359-1364.

Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F. 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea spp*. J Pharm Biomed Anal 35:289-301.

Percival SS. 2000. Use of *Echinacea* in medicine. Biochem Pharmacol 60:155-158.

Pillai S, Pillai C, Mitscher LA, Cooper R. 2007. Use of quantitative flow cytometry to measure *ex vivo* immunostimulant activity of *Echinacea*: the case for polysaccharides. J Altern Complement Med 13:625-634.

Porcheray F, Viaud S, Rimaniol AC, Léone C, Samah B, Dereuddre-Bosquet N, Dormont D, Gras G. 2005. Macrophage activation switching: an asset for the resolution of inflammation. Clin Exp Immunol 142:481-489.

Pugh ND, Balachandran P, Lata H, Dayan FE, Joshi V, Bedir E, Makino T, Moraes R, Khan I, Pasco DS. 2005. Melanin: dietary mucosal immune modulator from *Echinacea* and other botanical supplements. Int Immunopharmacol 5:637-647.

Quimby EL. 2007. The use of herbal therapies in pediatric oncology patients: treating symptoms of cancer and side effects of standard therapies. J Pediatr Oncol Nurs 24:35-40.

Raduner S, Majewska A, Chen JZ, Xie XQ, Hamon J, Faller B, Altmann KH, Gertsch J. 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. J Biol Chem 281:14192-14206.

Randolph RK, Gellenbeck K, Stonebrook K, Brovelli E, Qian Y, Bankaitis-Davis D, Cheronis J. 2003. Regulation of human immune gene expression as influenced by a commercial blended *Echinacea* product: preliminary studies. Exp Biol Med (Maywood) 228:1051-1056.

Raso GM, Pacilio M, Di Carlo G, Esposito E, Pinto L, Meli R. 2002. In-vivo and in-vitro anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perfor*atum. J Pharm Pharmacol 54:1379-1383.

Rehman J, Dillow JM, Carter SM, Chou J, Le B, Maisel AS. 1999. Increased production of antigen-specific immunoglobulins G and M following in vivo treatment with the medicinal plants *Echinacea angustifolia* and *Hydrastis canadensis*. Immunol Lett 68:391-395.

Roesler J, Emmendorffer A, Steinmuller C, Luettig B, Wagner H, Lohmann-Matthes ML. 1991a. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to test subjects mediates activation of the phagocyte system. Int J Immunopharmacol 13: 931-941.

Roesler J, Steinmuller C, Kiderlen A, Emmendorffer A, Wagner H, Lohmann-Matthes ML. 1991b. Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with *Listeria monocytogenes* and *Candida albicans*. Int J Immunopharmacol 13:27-37.

Rojas IG, Padgett DA, Sheridan JF, Marucha PT. 2002. Stress-induced susceptibility to bacterial infection during cutaneous wound healing. Brain Behav Immun 16:74-84.

Rousseau B, Tateya I, Lim X, Munoz-del-Rio A, Bless DM. 2006. Investigation of anti-hyaluronidase treatment on vocal fold wound healing. J Voice 20:443-451.

Sasagawa M, Cech NB, Gray DE, Elmer GW, Wenner CA. 2006. *Echinacea* alkylamides inhibit interleukin-2 production by Jurkat T cells. Int Immunopharmacol 6:1214-1221.

See DM, Broumand N, Sahl L, Tilles JG. 1997. *In vitro* effects of *Echinacea* and ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. Immunopharmacology 35:229-235.

Senchina DS, Wu L, Flinn GN, Konopka del N, McCoy JA, Widrlechner MP, Wurtele ES, Kohut ML. 2006. Year-and-a-half old, dried *Echinacea* roots retain cytokine-modulating capabilities in an in vitro human older adult model of influenza vaccination. Planta Med 72:1207-1215.

Sheridan JF, Padgett DA, Avitsur R, Marucha PT. 2004. Experimental models of stress and wound healing. World J Surg 28:327-330.

Sloley BD, Urichuk LJ, Tywin C, Coutts RT, Pang PK, Shan JJ. 2001. Comparison of chemical components and antioxidants capacity of different *Echinacea* species. J Pharm Pharmacol 53:849-857.

Smalinskiene A, Lesauskaite V, Ryselis S, Abdrakhmanov O, Kregzdyte R, Sadauskiene I, Ivanov L, Savickiene N, Zitkevicius V, Savickas A. 2007. Assessment of the effect of *Echinacea purpurea* (L.) Moench on apoptotic and mitotic activity of liver cells during intoxication by cadmium. Ann N Y Acad Sci 1095:574-584.

Solecki R. 1975. Shanidar IV, a Neanderthal flower burial in northern Iraq. Science 190:880-881.

Speroni, E., Govoni, P., Guizzardi, S., Renzulli, C. & Guerra, M. C. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. J Ethnopharmacol 79:265-272.

Steinmuller C, Roesler J, Grottrup E, Franke G, Wagner H, Lohmann-Matthes ML. 1993. Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. Int J Immunopharmacol 15:605-614.

Stimpel M, Proksch A, Wagner H, Lohmann-Matthes ML. 1984. Macrophage activation and induction of macrophage cytotoxicity by purified polysaccharide fractions from the plant *Echinacea purpurea*. Infect Immun 46:845-849.

Sun LZ, Currier NL, Miller SC. 1999. The American coneflower: a prophylactic role involving nonspecific immunity. J Alt Compl Med 5:437-446.

Thompson KD. 1998. Antiviral activity of Viracea against acyclovir susceptible and acyclovir resistant strains of herps simplex virus. Antiviral Res 39:55-61.

Thude S and Classen B. 2005. High molecular weight constituents from roots of *Echinacea pallida*: an arabinogalactan–protein and an arabinan, Phytochemistry 66:1026–1032.

Tragni E, Galli CL, Tubaro A, Del Negro P, Della Loggia R. 1988. Anti-inflammatory activity of *Echinacea angustifolia* fractions separated on the basis of molecular weight. Pharmacol Res Commun 20:S87-S90.

Tubaro A, Tragni E, Del Negro P, Galli CL, Della Loggia R. 1987. Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. J Pharm Pharmacol 39:567-569.

Vanzo A, Cecotti R, Vrhovsek U, Torres AM, Mattivi F, Passamonti S. 2007. The fate of trans-caftaric acid administered into the rat stomach. J Agric Food Chem 55:1604-1611.

Verma RP, Hansch C. 2004. An Approach towards the quantitative structure-activity relationships of caffeic acid and its derivatives. Chembiochem 5:1188-1195.

Ware WR. 2005. Inflammation - a double-edged sword. International Health News Issues 155-157. (www.yourhealthbase.com/inflammation.htm)

Woelkart K, Bauer R. 2007. The role of alkamides as an active principle of *Echinacea*. Planta Med 73:615-623.

van Wijk J. 2000. Phytobusiness requires social chemistry. Phytochemistry 55:93-95.

Woelkart K, Koidl C, Grisold A, Gangemi JD, Turner RB, Marth E, Bauer R. 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. J Clin Pharmacol 45:683-689.

Woelkart K, Xu W, Pei Y, Makriyannis A, Picone RP, Bauer R. 2005b. The endocannabinoid system as a target for alkamides from *Echinacea angustifolia* roots. Planta Med 71:70-705.

Woelkart K, Linde K, Bauer R. 2008. *Echinacea* for preventing and treating the common cold. Planta Med 74:633-637.

Zaporozhets OA, Krushynska OA, Lipkovska NA, Barvinchenko VN. 2004. A new test method for the evaluation of total antioxidant activity of herbal products. J Agric Food Chem 52:21-25.

Zhai Z, Liu Y, Wu L, Senchina DS, Wurtele ES, Murphy PA, Kohut ML, Cunnick JE. 2007. Enhancement of innate and adaptive immune functions by multiple *Echinacea* species. J Med Food 10:423-434.

CHAPTER 2. ENHANCEMENT OF INNATE AND ADAPTIVE IMMUNE FUNCTIONS BY MULTIPLE *ECHINACEA* SPECIES

Modified from a paper to be published in *Journal of Medicinal Food*

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Abstract

Echinacea preparations are commonly used as non-specific immunomodulatory agents. Alcohol extracts from three widely used *Echinacea* species, *E. angustifolia*, *E. pallida* and *E. purpurea*, were investigated for immunomodulating properties. The three *Echinacea* species demonstrated a broad difference in concentrations of individual lipophilic amides and water soluble caffeic acid derivatives. Mice were gavaged once/day (7 days) with one of the *Echinacea* extracts (130 mg/kg) or vehicle and immunized with sheep red blood cells (sRBC) 4 days prior to collection of immune cells for multiple immunological assays. The three herb extracts induced similar, but differential changes in the percentage of immune cell populations and their biological functions, including increased percentages of CD49+ and CD19+ lymphocytes in spleen, and natural killer cell cytotoxicity. Antibody response to sRBC was significantly increased equally by extracts of all three *Echinacea* species. Concanavalin A stimulated splenocytes from *E. angustifolia*- and *E. pallida*-treated mice demonstrated significantly higher T cell proliferation. In addition, the *Echinacea* treatment significantly

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altered the cytokine production by mitogen-stimulated splenic cells. The three herbal extracts significantly increased interferon-γ production, but inhibited the release of tumor necrosis factor-α and interleukin (IL)-1β. Only *E. angustifolia*- and *E. pallida*-treated mice demonstrated significantly higher production of IL-4 and increased IL-10 production. Taken together, these findings demonstrated that *Echinacea* is a wide-spectrum immunomodulator that modulates both innate and adaptive immune responses. In particular, *E. angustifolia* or *E. pallida*, may have more anti-inflammatory potential.

Introduction

Echinacea is an herbaceous plant genus, consisting of nine species. Three species, *E. angustifolia*, *E. pallida* and *E. purpurea*, have been used medicinally in the US and Europe and are being introduced into other regions due to the increasing popularity in alternative herbal remedies. *Echinacea* has a wide range of pharmacological activities. Besides its common function as an supportive therapy in the treatment of the common cold and upper respiratory tract infections, in which it reduces the duration and/or the severity of the symptoms, [1,2] it has been shown to possess palliative effects on wound damage,[3] inflammation, [3,4] and tumor growth.[5]

As a widely used over-the-counter and self-prescribed herbal medicine, it is important to elucidate its mode of action in order to enhance clinical benefits and minimize toxicity. The most consistent result identified by the majority of studies so far indicates that *Echinacea* has non-specific immunomodulatory properties through the activation of innate immune cells.[6,7] For example, in animals *Echinacea* showed profound effects on immune cell number,[8] granulocyte migration,[9,10] macrophage phagocytosis,[10,11] natural killer (NK) cell cytotoxicity,[12,13] and cytokine production.[8,11] In addition, an arabinogalactan-protein isolated from *E. purpurea* stimulates both the classical and alternative pathways of complement activation.[14]

While the bulk of studies have emphasized the innate immune properties of *Echinacea*, few studies investigated its adaptive immune modulation. One study demonstrated an increase in immunoglobulin (Ig) M response against sheep red blood cells (sRBC) in the mice treated with a glycerine extract of *E. purpurea*.[15] Other studies demonstrated an increase of humoral immunity *in vivo* but only provided indirect information due to the use of a blend of *Echinacea* and other herbs.[16,17] Data regarding the effect of *Echinacea* on T cells are more limited. A previous study with *E. purpurea* found an increase in CD4+ T-lymphocytes in mouse peripheral blood but implied it is a secondary effect in association with macrophage activation.[18] Morazzoni *et al*.[19] recently reported that *E. angustifolia* extract enhances T cell function *in vitro* by stimulating interferon (IFN)-γ production in anti-CD3-treated murine T-cell cultures. These experimental results suggest that *Echinacea* may modulate adaptive immune responses.

However, the evidence for *Echinacea* effects is confusing and inconsistent since most studies have used different *Echinacea* preparations, and these preparations are largely influenced by many factors, such as phytochemical variability due to growing conditions and lack of extraction standardization.[2,7] Moreover, bacteria-derived endotoxin contamination during the extraction processes may give rise to false positive results by way of the stimulation of the immune system, especially with *in vitro* cell culture experiments or injection of the extracts into animals. Many previous reports have neglected this confounding factor of endotoxin, although some touched upon it.[19-22]

Isolation and characterization of bioactive phytochemicals in *Echinacea* extracts used in studies is essential. A few widely accepted active compounds of *Echinacea* include lipophilic alkamides and polar caffeic acid derivatives (cichoric acid and echinacoside).[23,24] The in-depth investigation of these active compounds seems to be helpful for the better understanding of the biological nature of *Echinacea*. However, a purified phytochemical does not mimic the immunological effects of whole plant extracts. It appears that the immunopharmacological

activities of *Echinacea* depend on a combination of several active compounds,[25] but not any single individual constituent. In addition, extracts of *Echinacea* are commercially available and economical, but commercially available purified compounds are expensive and not approved for human consumption. Thus, studies using the *Echinacea* extracts are still of great value. Animal experiments have show that a combination of *Echinacea* extracts demonstrated greater effects than extracts of single plants (reviewed in 17), but using a mixture of plants makes it difficult to distinguish the biological significance of each plant or identify redundancy in effects.

Our preliminary studies using oral administration of *Echinacea* found that the dry powder or alcohol extract of *E. purpurea* root could stimulate splenic T cell proliferation and NK cell cytotoxicity. Based on the experimental data accessible to date on *Echinacea*, the purpose of the present study was (1) to provide a comparison of the immunomodulatory activity of three species of *Echinacea*, (2) to clarify *Echinacea*'s activities on specific acquired immunity, and (3) identify differences in immune activation based on differences in high performance liquid chromatography (HPLC) profiles. Alcohol extracts of the three *Echinacea* species were prepared and administered in order to compare their effects on multiple immune parameters, i.e. NK cell activity, plaque forming cell (PFC) response against sRBC, B- lymphocyte and T-lymphocyte proliferation as well as T cell and macrophage cytokine production in healthy BALB/c mice. We found many functional immune assays were affected by *Echinacea* preparations, suggesting that *Echinacea* not only stimulates innate immunity, but also enhances adaptive immunity.

Materials and Methods

Echinacea preparation

E. angustifolia, *E. pallida* and *E. purpurea* were harvested in the USDA North Central Regional Plant Introduction Station (Ames, IA) in October 2003 with identification numbers PI

631285 (*E. angustifolia*), PI 631293 (*E. pallida*), and PI 631307 (*E. purpurea*). Extracts from the dried roots of these plants were prepared as follows: ground root powder was placed into Whatman 43 mm × 123 mm cellulose extraction thimble (Whatman International Ltd., Maidstone, England), covered with glass wool on the top, and refluxed with 250 ml different organic solvents, 100% ethanol, 95% ethanol, chloroform, and hexane, for 6 h using Soxhlet extraction device. The extract was evaporated to dryness with a rotary evaporator (Büchner-Brinkman, R-114, Switzerland) at < 30°C under reduced pressure, and the residue was stored in a red glass bottle to preserve it from photoxidation [3] at -20°C. The average efficiency of Soxhlet extraction of the three species is 23% (230 mg residue/gram starting material).

Before animal treatment, the extracts were dissolved in 95% ethanol and then diluted in Nanopure water to a final suspension containing 14.7 mg/ml extracts in 5% ethanol. Ultrasonic treatment was used during the dilution process to improve solubility. After a homogenized suspension was obtained, aliquots were stored at -20°C and thawed for gavage or other analyses with each aliquot used once. The endotoxin level was evaluated in aliquots of the three *Echinacea* preparations using the Bio-Whitaker QCL 1000 kit (Cambridge, MA) and was below the limit of detection (0.1 EU/ml).

Phytochemical analysis

The phytochemical analysis was performed to detect amides and caffeic acid derivatives in the *Echinacea* preparations with the use of high performance liquid chromatography (HPLC).[26] Before analysis, dimethyl sulfoxide was added in order to obtain fully dissolved samples. 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 3,5-dimethoxy-4-hydroxy-cinnamic acid ($C_{11}H_{12}O_5$) each were added as internal standards for quantification of lipophilic chemicals and hydrophilic chemicals, respectively. Fifteen microliters of each sample was injected into a Beckman Coulter HPLC with a 508 autosampler, 126 pump control and 168 UV-photodiode array detector controlled by 32 Karat ™ software (Version 5.0), and a YMC-Pack ODS-AM RP C18

(250 × 4.6 mm, 5 μm) analytical column (Waters, MA). Solvent system for lipophilic constituents was acetonitrile/H_2O at a flow rate of 1.0 ml/min following a linear gradient of 40-80% acetonitrile over 45 min. Solvent system for hydrophilic constituents consisted of acetonitrile/H_2O and 0.01% formic acid, at a flow rate of 1.0 ml/min following a linear gradient of 10-35% acetonitrile over 25 min. Online UV spectra were collected between 190-400 nm. The lipophilic chemicals were quantified based on the internal standard with the limit of HPLC detection at approximately 0.02 μg/ml.

Animals

Animal care and experimental procedures were approved by the Iowa State University Committee on Animal Care. Male BALB/c mice at 8 weeks of age were obtained from Harlan Laboratories (Indianapolis, IN) and allowed to acclimate to new environment for 2 -3 weeks. The mice were housed three/cage and provided free access to food and water. The animal room was maintained on a reverse 12 h light /dark cycle (lights on at 8PM).

Echinacea administration

This study consisted of two independent experiments with an identical study design. After acclimation, the mice were randomly assigned to five groups. Groups 1-3 were gavaged with one of the three *Echinacea* preparations. Group 4 was gavaged with an equal volume of 5% ethanol as vehicle control. Group 5 served as a no gavage control (no treatment group). The vehicle control and the no gavage control were established to control for the effects of vehicle as well as handling stress. The *Echinacea* preparations were orally administered to the animals at 130mg/kg body weight once daily for 7 consecutive days using an animal feeding needle. This dosage and regimen was chosen based on an extrapolation of the dose recommended for humans (4 g powder/day for an average 65 kg human ×1 week).[27] We extrapolated the dose by using a 10-fold increase for mice according to skin to body ratio difference for mice and humans. We multiplied the dose by 23%, as this

was the average efficiency of Soxhlet extraction. The gavage dose volume was 0.18 ml extract suspension per 20 g mouse body weight. On the fourth day, all mice were injected intraperitoneally with 0.5 ml of 20% washed sRBC (Remel, Lenexa, KS) in Hank's balanced salt solution (HBSS; GIBCO, Invitrogen Corporation) to induce an immune response. Our preliminary observation found that sRBC-injected mice did not display significantly immunological changes as measured in this study when compared to those animals receiving an injection of HBSS except that there were somewhat of changes in the percentage of leukocyte compositions in the spleen. Thus, in this study all mice were treated equally with sRBC injection to maintain the same experimental condition.

Sample collection

The mice were weighed at the beginning and the end of the experimental treatments. Twelve to fifteen hours after the last gavage, the mice were euthanized by CO_2 asphyxiation and immune samples collected immediately. Blood was collected by heart puncture using a heparin-containing syringe. An aliquot of blood was taken to perform hematological assay by using Hemavet 850 Hematology Analyzer (Drew Scientific, Inc., Oxford, CT). The remaining blood was diluted 1:10 with AIM-V media, supplemented with 2 mM glutamine, 25 mM Hepes buffer and 50 μg/ml gentamicin (AIM-V media), for use in a whole blood proliferation assay.

The spleens were aseptically removed from the mice and placed in AIM-V media. The spleen weight was recorded to assess spleen/body weight ratio. The spleen was then dissociated into a single cell suspension by gently grinding between the frosted ends of two sterile microscope slides. The splenocytes were enumerated and diluted to 5×10^6 cells/ml in RPMI 1640 (GIBCO, Invitrogen Corporation) supplemented with 2 mM glutamine, 25 mM Hepes buffer, 50 μg/ml gentamicin and 10% heated-inactivated fetal bovine serum (FBS) (complete media, CM) to use in immune function assays. A separate aliquot of splenocytes was diluted in

HBSS containing 25 mM Hepes buffer, 50 μg/ml gentamicin and 6% FBS (HBSS-6% FBS) for antibody staining for CD19+ and CD49+ subsets.

Flow cytometric assay of lymphocyte subsets

Fluorescent-labeled monoclonal antibodies were used to identify lymphocyte subsets in the spleen. Freshly isolated splenocytes from mice were simultaneously stained with phycoerythrin (PE)-labeled anti-mouse CD49b mAb (pan NK cells) and biotin-labeled anti-mouse CD19 mAb (B cells) (Pharmingen, San Diego, CA). PE rat IgM kappa and biotinylated rat IgG2α kappa (Pharmingen) were used for the negative isotypic controls. Strepavidin-cychrome (Pharmingen) was used as a fluorescent tag for biotinylated antibodies. Splenocytes (5×10^5 cells) in HBSS-6%FBS were diluted with 100 μl of ice-cold phosphate buffered saline/0.1% NaN_3 (PBS/Azide). One microgram of anti-CD49b or 0.2 μg of anti-CD19 as well as 4 μl of normal mouse serum for blocking were added. An equal amount of isotypic antibodies and normal mouse serum were added to the corresponding control tubes. After incubation for 40 min in ice water bath, red blood cells were lysed using ice cold ammonium chloride solution. Cells were washed with PBS/Azide and Strepavidin-cychrome was then added to tubes containing biotinylated antibodies. The cells were incubated again in ice water bath. After one more wash the cells were fixed with PBS containing 1% formaldehyde. The cells were analyzed within 72 h using Beckman-Coulter Epics XL-MCL flow cytometer (Fullerton, CA).

NK cell cytotoxicity

Splenic NK cell cytotoxicity was assessed by the chromium (^{51}Cr) release assay as previously described,[28] but with a small modification. Briefly, recombinant human-IL-2 (5 ng, Sigma) and splenocytes were plated to flat-bottom 96-well plate (Corning Incorporation, Corning, NY) and incubated at 37°C in 7% CO_2 incubator overnight. YAC-1 cells (American Type Culture Collection, Manassas, VA) were used as targets and were labeled with 200 μCi

of ^{51}Cr as sodium chromate (PerkinElmer, Boston, MA) at 37°C for 70 min. Washed target cells were plated at 10^4/well in all experimental, spontaneous and maximum wells and the plates incubated for additional 4.5 h at 37°C. Three effector/target cell ratios, 25:1, 50:1 and 100:1 were assessed in triplicate. Total incubation time including IL-2 activation and target killing was 24 h. Following addition of trichloroacetic acid (10%) to maximum release wells to lyse the targets, the plate was centrifuged at 500 rpm for 5 min. An aliquot of cell-free supernatant was removed and counted in a gamma Trac 1191 counter (TM Analytic, Inc., Elk Grove Village, IL). Percent cytotoxicity of experimental samples was calculated using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100\%$$

PFC assay

B-cells making antigen specific antibodies to sRBC's were quantitated using a PFC assay. Guinea pig complement (Cedarlane Laboratories Limited, Ontario, Canada) adsorbed against packed sRBC, 4% sRBC, and mouse splenocytes were mixed in equal volume. An aliquot (40 µl) was immediately transferred into two sides of a Cunningham chamber.[29] Slide chambers were sealed with melted paraffin and incubated for 1 h at 37°C in a humidified incubator. Plaques were counted under low power on a microscope.

Cell proliferation assay

Mitogen-induced proliferation assay was performed using whole spleen cells and whole blood cells as described previously. Concanavalin A (Con A) and *E. coli* lipopolysaccharide (LPS) (Sigma, St. Louis, MO) were used as non-specific T-lymphocyte mitogen and B-lymphocyte mitogen, respectively.

Splenocyte proliferation was performed using 5×10^5 cells/well in triplicate in a 96-well Costar plate and a final concentration of LPS at 10 µg/ml and Con A at 1 and 3 µg/ml. After co-incubation for 28 h, all wells were pulsed with [^{3}H]-thymidine (1 µCi/well) (PerkinElmer, Boston, MA) and continued to incubate at 37°C, 7% CO_2. Following 48 h of total incubation, the cultures were harvested using Skatron Cell Harvester (Sterling, VA), and the ^{3}H-thymidine incorporation was determined by using a liquid scintillation analyzer (Mode Tri-carb 2100TR, Packard Instrument Company, Inc., Downers Grove, IL).

The blood cell proliferation assay was conducted by using a similar protocol as described in the splenocyte proliferation assay. Diluted blood was incubated without or with LPS (10 µg/ml) or Con A (20 µg/ml) for 52 h, all wells were then pulsed with 1 µCi/well of [^{3}H]-thymidine and incubated for 20 h. The cultures were harvested and the ^{3}H-thymidine incorporation was determined.

Enzyme-linked immunosorbent assay (ELISA) of cytokine production

The levels of cytokines were measured in the cultures of mitogen-stimulated mouse splenocytes *ex vivo*. Five hundred microliters of spleen cells were added to a 24- well flat-bottom Costar plate containing 500 µl of media or mitogen (LPS or Con A, 20 µg/ml) and incubated for 24-72 h at 37°C, 7% CO_2. Following incubation, the culture supernatants were harvested and frozen at -20°C for later analysis by enzyme-linked immunosorbent assay (OptEIA ELISA mouse kits, BD Biosciences, San Diego, CA). ELISA's for IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40), IFN-γ and TNF-α were conducted according to the manufacturer's protocol with the exception that the plates were read at 655 nm in a plate reader (Bio-Rad, Hercules, CA) 30 min after the addition of substrate. The cytokine levels were determined by comparison to a standard curve generated from serial dilutions of purified recombinant mouse cytokines.

Statistical analysis

Statistix software (version 8.0, Analytical Software, Tallahasee, FL) was used for the statistical analysis. Differences between *Echinacea* groups or the no gavage control and the vehicle control were tested by two-way analysis of variance (group × experiment) with subsequent *a priori* contrasts using to compare all three *Echinacea* treatments to the vehicle control. For analysis of NK data a factor for effector/target ratio was included in the model. For the PFC cell assay the model contained a factor for covariance based on the percentage of CD19+ cells and for the NK cell activity the model contained a factor for covariance based on the percentage of CD49+ cells. A value $p<0.05$ was considered statistically significant.

Results

HPLC analysis

The HPLC profiles of alcohol extracts of three *Echinacea* species are shown in Fig.2.1. The levels of phytochemical constituents quantified using HPLC are listed in Table 2.1. Amounts and types of identifiable amides and caffeic acid derivatives were quite distinct between the three species. Total identifiable constituents in the preparations of *E. angustofolia*, *E. pallida* and *E. purpurea* were 107.7, 29.5 and 87.4 µg/ mg extract, respectively. Most phytochemicals detected were lipophilic amides, especially in *E. angustifolia* and *E. purpurea* preparations in which amides represents approximately 94.5% and 91.2% of all phytochemicals, respectively. A minor part of the phytochemicals was caffeic acid derivatives. Echinacoside was found to be the main caffeic acid derivative in both preparations of *E. angustifolia* and *E. pallida*, whereas no echinacoside was detected in the *E. purpurea* preparation. Cichoric acid was measurable in both *E. pallida* and *E. purpurea* preparations and was the main caffeic acid derivative of the *E. purpurea* preparation. Cynarin was detected in the *E. angustofolia* preparation, chlorogenic acid in the *E. angustifolia* and *E. pallida*

preparations and caftaric acid in the *E. purpurea* and *E. pallid*a preparations. None of the phytochemical constituents was detectable in the vehicle.

General animal observation

Adverse effects or obvious changes in behavior of the animals were not seen throughout the 7-day treatment course. No difference in body weight of the animals was found between the vehicle control and any of other groups before or after experimental treatment. The mean body weights of five groups before and after treatment were 25.8 ± 2.0 g and 25.6 ± 1.8 g, respectively.

Peripheral blood and spleen cell subpopulations

Peripheral blood hematological parameters were measured following the 7-day *Echinacea* treatment. No significant changes to leukocyte number, red blood cell number, hemoglobin level or other hematological parameters were associated with oral administration of any of the three *Echinacea* preparations as compared to the vehicle control and the no gavage control (data not shown). However, the three *Echinacea* preparations significantly increased the percentage of lymphocytes in peripheral blood over the vehicle control group and the no gavage control (62.7% vs 58.0%; p =0.001). For other leukocyte subpopulations (neutrophil, monocyte, eosinophil and basophil), no significant difference was observed between the *Echinacea* treatment groups and the two control groups.

There were no significant differences in spleen weight, spleen-to-body weight ratio, and total spleen cell number per mouse between the vehicle control and any of the other four groups. However, *Echinacea* treatment groups demonstrated a significant increase in the percentage of the lymphocyte subpopulation when compared to the vehicle control plus the no gavage control (83.3% vs 81.1%; p = 0.004). Meanwhile, the *Echinacea* treatment induced a marginal decrease in the percentage of splenic neutrophils as compared to the vehicle and no

gavage controls (10.1% vs 11.5%; p=0.084). There was no significant change in any of the other splenocyte subpopulations measured.

Noting an increase in the general percentage of splenic lymphocytes, specific lymphocyte subsets were further analyzed by flow cytometry for the expression of the CD49 and CD19 markers (Fig 2.2). After 7 days of oral administration of vehicle (5% ethanol), there was a significant decrease in the percentages of both CD49+ and CD19+ splenocytes when compared with the no gavage control (p=0.002 and p=0.017, respectively). However, groups which received *Echinacea* preparations by gavage did not exhibit the decrease in these subpopulations, as the *Echinacea* treatments are not significantly different than the no gavage control. Individual comparisons of the *Echinacea* treatment groups demonstrated that *E. purpurea* treatment group was significantly increased in the percentage of both CD49+ and CD 19+ cells over the vehicle control. *E. angustifolia* group was significantly increased in the percentage of CD49+ cells, but not CD19+ cells when compared with the vehicle control. No significant effect of *E. pallida* on splenic CD49+ and CD19+ subsets was seen.

NK cell killing

The capacity of NK cells to lyse the target YAC-1 tumor cells *in vitro* was performed by ^{51}Cr- release assay. Since *Echinacea* treatment altered the CD49+ subset in spleen, NK cell cytotoxicity analysis was covaried for the percentage of CD49+ cells measured in each individual (Fig 2.3). No difference was seen between the three *Echinacea* treatment groups and the no gavage control. When compared to the vehicle control, both the *Echinacea* treatment and the no gavage control showed a higher NK cell killing ($p < 0.03$ and $p < 0.035$, respectively). If the three *Echinacea* treatment groups were compared to the vehicle control separately, only the *E. pallida* group demonstrated significant increase in NK cell cytotoxicity ($p < 0.035$).

PFC response against sRBC

B-cells specific for sRBC were enumerated using a PFC assay. Since the *Echinacea* treatment groups displayed significant effects on the percentage of B cells as measured by CD19+ splenocytes, statistical analysis of PFC was adjusted by covariance with the percentage of CD19+ cells (Fig 2.4). No significant difference in antibody response against sRBC was found between the two control groups. Nor was there a significant difference among the three *Echinacea* treatment groups. However, the PFC counts of three *Echinacea* treatment groups were significantly higher than those of the two control groups ($p = 0.003$). Individual comparisons demonstrated that all three *Echinacea* species increased PFC response to sRBC (p values < 0.035).

Mitogen-stimulated lymphocyte proliferation

Splenic lymphocyte and blood lymphocyte proliferation was performed by ^{3}H-thymidine incorporation in the presence or in the absence of LPS or Con A. For splenic lymphocytes, the two mitogens produced a significant increase in cell proliferation (p values < 0.0001) (Fig 2.5A). In the absence of mitogen, there was no significant difference among the five groups for splenic lymphocyte proliferation. In the presence of LPS, splenic lymphocyte proliferation was not affected by any of the three *Echinacea* treatments. With Con A at a low concentration of 1 μg/ml, the three *Echinacea* treatments demonstrated a marginal enhancement of splenic lymphocyte proliferation ($p = 0.065$). Individual comparisons demonstrated that *E. pallida* had a significant enhancing effect on splenic lymphocyte proliferation when compared with the vehicle control ($p = 0.046$). When the Con A concentration was increased to 3 μg/ml in the cultures, the three *Echinacea* treatments were significantly increased over the vehicle control ($p = 0.034$), which was due to both *E. angustifolia* and *E. pallida* having significantly higher splenic lymphocyte proliferation than the vehicle control ($p = 0.032$ and $p = 0.015$,

respectively). Under all conditions, no difference in lymphocyte proliferation was observed between the vehicle control and the no gavage control.

In blood cultures (Fig 2.5B), there was a significant stimulation of baseline blood lymphocyte proliferation by the *Echinacea* treatment in comparison to both controls (p = 0.014). *E. angustifolia* and *E. pallida* were both significantly different than controls (p values < 0.043), while *E. purpurea* was only marginally different (p = 0.091). However, in the presence of Con A and LPS, *Echinacea* did not enhance blood lymphocyte proliferation. For the blood cultures there was no difference in lymphocyte proliferation between the vehicle control and the no gavage control.

Cytokine production

Cytokines are recognized as immune transmitters that regulate interactive effects among immune cells. A specialized cytokine can stimulate certain types of immune cells, but inhibits others. Cytokine concentrations were assessed in cultures of unstimulated and mitogen-stimulated splenocytes derived from the gavage and no gavage treatment groups.

T_H2 cells secrete a number of cytokines, such as IL-4, IL-6 and IL-10 that drive humoral immunity, but inhibit macrophages and are considered anti-inflammatory. *E. angustifolia* and *E. pallida* showed increased IL-4 and IL-10 production over the vehicle control in Con A-stimulated spleen cells, however the enhancement reached significance only for the increased IL-4 production (p = 0.046) (Fig 2.6). The increase in IL-10 was only marginally significant for cultures from *E. angustifolia* and *E. pallida* when compared to the vehicle control group (p = 0.057). For spleen cells without mitogen stimulation, *E. angustifolia* significantly increased IL-4 production compared to the vehicle control group (p = 0.013). The three *Echinacea* preparations had no effect on IL-6 release.

T_H1 cells secrete IL-2 and IFN-γ that activate cell mediated immunity, including NK cells and macrophages. The *Echinacea* treatment significantly increased IL-2 and IFN-γ production in baseline cultures of splenocytes (p values < 0.035) and IFN-γ production by Con A-

stimulated splenocytes ($p = 0.005$) (Fig 2.7). Individual comparison of *Echinacea* treatment groups showed that only *E. angustifolia* produced a significant increase in IL-2 production in Con A-stimulated splenocytes ($p = 0.037$). It is of interest to note that IFN- γ production in the vehicle control group was suppressed in comparison to the no gavage control group ($p = 0.054$).

Macrophages produce numerous inflammatory mediators, among them the cytokines TNF-α, IL-1β and IL-12. In comparison to the vehicle control group, the three *Echinacea* preparations significantly decreased the production of IL-1β ($p = 0.007$) and TNF-α ($p = 0.004$) by LPS-stimulated splenocytes, but had no significant effect on IL-12 production (Fig 2.8). It is interesting to note that the vehicle control group demonstrated a significantly enhanced production of the inflammatory cytokine TNF-α compared to the no gavage group ($p = 0.004$), while IL-1β demonstrated a non-significant trend towards an increase. However, for baseline spleen cells without mitogen stimulation, *E. purpurea* induced a significant increase in IL-1β production compared to the vehicle control ($p = 0.006$).

Discussion

According to accumulated data, *Echinacea* exerts its pharmacological action via the modulation of non-specific innate immune parameters such as macrophage phagocytosis and pro-inflammatory cytokine production.[6,7] Thus, *Echinacea* is thought of as a non-specific immunomodulator. "Non-specific" may also mean that the herb promotes overall immune system function. The results from the present study provide information supporting the later concept as oral administration of *Echinacea* resulted in multiple immunological changes, including the activation of NK cell activity, the enhancement of B-cell response to sRBC, increased T cell proliferation in response to mitogens, and increased production of some T cell cytokines. Also, this study demonstrates that the preparations from three common *Echinacea* species did not display opposing influences on immune system function.

For the experimental animals, the 7-day orogastric gavage and daily handling is doubtlessly a stressor that leads to stress induced changes of immunity.[30,31] In addition, the vehicle of 5% ethanol could also contribute to some immune changes. It has been shown that a short term consumption of low dose dietary ethanol (4-7%) for 1-2 weeks could inhibit cell-mediated immune responses and sensitize the host to infection.[32,33] In order to control these "stress" effects and highlight the effects of *Echinacea* on the immune system, both the vehicle control and the no gavage control were tested in this study. The observed differences in the vehicle control from the no gavage control reflect the immunological changes induced by stress and vehicle. Under the same stress condition, the difference between the *Echinacea* treatment groups and the vehicle control mirrors the immunological changes caused by *Echinacea*. We found that as compared with the no gavage control, *Echinacea* treatment groups did not differ in all of the immune parameters we measured, but it did differ significantly from the vehicle control. In comparison to the no gavage control, the vehicle control displayed a weakened immune capacity as evidenced by a decrease in splenic CD19+ cells and CD49+ cells, as well as, NK cell cytotoxicity. Conversely, the gavage with vehicle induced an increase in TNF-α and IL-1β production as compared to the no gavage control, suggesting that the 7-day handling and gavage with 5% ethanol altered host immune responsiveness. Interestingly, all these altered immune parameters could be returned to their normal levels by treatment with *Echinacea*. These results are of particular significance under clinical aspects, since the weakened immune system ranks high among the main indications for herbal immunomodulants.[17] For this purpose, the immunostimulating effects of *Echinacea* have been investigated in several non-pathogenic, immunosuppressed animal models (i.e. old age or hydrocortisone-treated animals).[8,12,13,17,34] In this respect, mild handling stress- and/or low dose ethanol- induced immunosuppression is likely another ideal model to observe the immunological potential of *Echinacea* with greater similarities to daily human stressful conditions.

Previous research demonstrated that *Echinacea* increases the numbers of circulating leukocytes, including total cell count and subpopulations (i.e. neutrophil, NK cell, and T lymphocyte).[8,10,18] We found an increased percentage of lymphocytes not only in peripheral blood but also in the spleens of mice following 7-day oral treatment with our *Echinacea* preparations so that extends the previous findings. Cundell *et al*.[8] observed a sustained, significant increase in the percentage of circulating mononuclear cells (lymphocytes and monocytes) associated with a significant decrease in the percentage of circulating neutrophils in aging rats fed with *Echinacea* for 8 weeks. In healthy horses *Echinacea* increases the number of peripheral lymphocytes, but decreases neutrophil count on day 35 during a 42- day feeding with *E. angustifolia* extract.[10] The present study observed a significant increase in the percentage of total lymphocytes in animals gavaged with *Echinacea* compared to vehicle treated animals, suggesting that other leukocyte types must be decreased. Although the percentage of neutrophils in both blood and spleen was not significantly different among the animals from *Echinacea* and vehicle treated groups, this population exhibited a small, non-significant, decrease in *Echinacea* treated animals. Other studies have suggested that the decrease in the percentage of neutrophils may be due to increases in granulocyte migration into the tissues.[9,10] As lymphocytes consist of NK cells, T cells and B cells, *Echinacea*-induced changes in the percentage of lymphocyte subpopulations indicated *Echinacea* might modulate both innate and adaptive immune functions.

NK cells play a critical role in clearing viral infections through the processes of cytotoxicity and production of cytokines, such as IFN-γ. It has been demonstrated that *Echinacea* increased NK cytotoxic function *in vitro* in human peripheral blood lymphocytes of both healthy individuals and patients with chronic fatigue syndrome or acquired immunodeficiency syndrome.[34] Currier and Miller[12,13] observed a significant activation of NK cells and an increase in NK cell numbers in aging mice fed with a diet containing commercially prepared root extract of *E. purpurea*. Our present study found the three *Echinacea* preparations

induced similar effects on NK cell activity. In comparison to the no gavage control, gavage with vehicle resulted in a decrease in the NK cell cytotoxicity, which is likely due to both stress effects of daily handling and 5% ethanol. It is interesting that *Echinacea* could attenuate the decreased NK cell activity. Moreover, analysis of data using covariance indicated that *Echinacea* increased NK cell activity over a simple increase in the cell count.

Changes in B cell responses by a glycerine extract of *E. purpurea* have been noted.[15] To discern an effect on B cells we measured PFC response against sRBC, the percentage CD19+ cells in spleen, and B cell proliferation in LPS-stimulated spleen cell. Gavage with *Echinacea* extracts led to a significant increase in PFC response against sRBC. *Echinacea* also affected the percentage of lymphocytes as well as the percentage of CD19+ cells in the spleen. As with the effects on NK cell activity, the vehicle control decreased the number of CD19+ cells in spleen cells when compared with the no gavage control. However, the vehicle-induced decrease in CD19+ cells was attenuated in animals receiving *Echinacea*, with a greater increase in animals gavaged with *E. purpurea*, suggesting that *Echinacea* may affect B lymphocyte development or migration *in vivo*.

It is well known that both subsets of T_H cells are usually activated in immune response to complex antigens. In response to viral infection, antigen-specific T-helper and T-cytotoxic cells proliferate, secrete cytokines and the T-cytotoxic cells kill virally infected cells. Historical use of *Echinacea* in the treatment of viral infection has lead to the reasoning that *Echinacea* acts on T cells.[22] Morazzoni et al.[19] observed anti-CD3-treated murine T cell proliferation by LPS-free *E. angustifolia* root extract and suggested *Echinacea* activity on the immune system involves the interaction with T cells. Our results demonstrated that Con A induced splenic T cell proliferation could be enhanced by *in vivo* treatment with *E. angustifolia* or *E. pallida*. In addition, it was found that *E. angustifolia* administered *in vivo* stimulated lymphocyte proliferation in the absence of mitogens. The effects of *Echinacea* on T cell cytokine production were measured, including both T_H1 cytokines, IFN-γ and IL-2 and T_H2 cytokines,

IL-4, IL-6 and IL-10. *Echinacea* extracts were found to have enhancing effects on IFN-γ and IL-2 production and IL-4 and IL-10 levels showed 3-4-fold increase by spleen cells from animals gavaged with *E. angustifolia* and *E. pallida*. These results indicate that *E. angustifolia* and *E. pallida* modulate both the T_H1- and T_H2-cell immune function. *E. purpurea*, in contrast to other two *Echinacea* species, showed a relatively weaker effect on the T_H1 cell cytokine productions.

T_H1 cell activation will in turn activate macrophages that protect against intracellular pathogens. The effects of *Echinacea* on phagocytosis and cytokine production by macrophages have been extensively investigated *in vitro* and *in vivo*, but the results were rather inconsistent (see review [6,7]). Macrophages are important as a first line of defense against infections. Upon activation, they may secrete many pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-12 and IL-6. However, inflammatory processes subsequently need to be downregulated to allow healing. These divergent and at times, seemingly contradictory effects reflect the dichotomy of macrophages as both pro- and anti-inflammatory effectors in response to host environmental changes. Recently one study[25] reported opposing effects of *Echinacea* on cytokine gene expression when used *in vitro* vs oral administration *in vivo.* The *in vitro* data found a short-term (6 h) exposure of human monocytic cell line THP-1 cells to *E. purpurea* stimulates the expression of inflammation-related genes, such as IL-1β, TNF-α, IL-8, ICAM-1 and Cox-2. However, oral administration of *Echinacea in vivo* induced a reduction in the expression of inflammation-related genes, but increases the IFN-α expression in healthy individuals.[25] The effects of *Echinacea* on cytokine production vary considerably, depending on the experimental conditions used (e.g. animal model, cell culture model, and the treatment scheme). Our animal model in this study demonstrated *Echinacea* exerted a strong inhibition on the TNF-α and IL-1β production by macrophages in the presence of LPS, suggesting that *Echinacea* has anti-inflammatory activity, as demonstrated previously.[3,4] The down-regulation of these two important inflammatory mediators might be associated with increased production of IL-4. IL-4

supports the differentiation of CD4+ into T_H2-typed cells and simultaneously suppresses the development of T_H1-typed cells. On macrophages, IL-4 acts in an anti-inflammatory manner to inhibit the production of pro-inflammatory cytokines, i.e. IL-1β and TNF-α.[35,36]

Undoubtedly, the observed differential effects of three *Echinacea* species on certain immune parameters are associated with their variation in phytochemical composition. Among phytochemicals, amides, echinacoside and cichoric acid are thought of as the main active compounds responsible for the immunomodulatory action of alcohol extracts of *Echinacea*.[24,37,38] *E. purpurea* has been reported to have a mix of constituents different from the other two species.[24,38] Chromatographic analysis of our preparations showed that *E. purpurea* lacks echinacoside, but contains cichoric acid. Most, though not all, amides were present in the three *Echinacea* preparations and occupied a major part of all identifiable phytochemicals, especially for *E. angustifolia* and *E. purpurea* preparations. Among the three *Echinacea* species, *E. purpurea* is believed to have the strongest potency on the immune system.[39] It is unexpected that *E. purpurea* displayed a weaker potential to stimulate T_H2- and T_H1-type cytokine production than *E. angustifolia* and *E. pallida,* especially since the HPLC results demonstrate that the *E. purpurea* extract contained high levels of amides and cichoric acid, the latter proven to have stronger immunostimulatory effects than echinacoside.[38] So in this study, other phytochemicals, but not amides and cichoric acid in the *E. angustifolia* and *E. pallida* preparations, may be responsible for the strong immunomodulatory effects on T lymphocytes. Echinacoside and chlorogenic acid were the main caffeic acid derivatives in both *E. angustifolia* and *E. pallida* preparations we used. Echinacoside has been studied for its antioxidant, anti-inflammatory and cicatrizing activities.[3,4,23,37] Data on the immunomodulatory effect of chlorogenic acid and cynarin, a characteristic component of *E. angustifolia*, are few. Given the use of the crude extracts in the present study and minor proportion of measurable constituents in total extracts (< 10.8%), we could not answer which

chemical makes the major contribution to the modulatory effect on immune function and if there are other undetected active phytochemicals in the extracts.

In conclusion, the present oro-gastric administration studies with three different *Echinacea* species have proven them to be effective immunomodulators. We found that three different species of *Echinacea* exhibit multiple modulating effects on immune function. They stimulate not only non-specific, innate immune response, but also specific, adaptive immune function, suggesting that *Echinacea* possesses an immunomodualting potential for the overall immune system. The effects of *Echinacea* were more robust in immune responses that were suppressed by the daily handling in the vehicle control group as compared to the no gavage group. To our knowledge, this is the first study that demonstrates the relevance of *Echinacea*'s immune enhancing effects in conjunction with a mild stress.

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References

1. Caruso TJ, Gwaltney JM Jr: Treatment of the common cold with *Echinacea*: a structured review. Clin Infect Dis 2005;40:807-810.
2. Islam J, Carter R: Use of *Echinacea* in upper respiratory tract infection. South Med J 2005;98:311-318.

3. Speroni E, Govoni P, Guizzardi S, Renzulli C, Guerra MC: Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. J Ethnopharmacol 2002;79:265-272.
4. Raso GM, Pacilio M, Di Carlo G, Esposito E, Pinto L, Meli R: *In-vivo* and *in-vitro* anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*. J Pharm Pharmacol 2002;54:1379-1383.
5. Currier NL, Miller SC: The effect of immunization with killed tumor cells, with/without feeding of *Echinacea purpurea* in an erythroleukemic mouse model. J Altern Complement Med 2002;8:49-58.
6. Barrett B: Medicinal properties of *Echinacea*: A critical review. Phytomed 2003;10:66-86.
7. Percival SS: Use of *Echinacea* in medicine. Biochem Pharmacol 2000;60:155-158.
8. Cundell DR, Matrone MA, Ratajczak P, Pierce JD Jr: The effect of aerial parts of *Echinacea* on the circulating white cell levels and selected immune functions of the aging male Sprague-Dawley rat. Int Immunopharmacol 2003;3:1041-1048.
9. Roesler J, Steinmuller C, Kiderlen A, Emmendorffer A, Wagner H, Lohmann-Matthes ML: Application of purified polysaccharides from cell cultures of the plant *Echinacea purpurea* to mice mediates protection against systemic infections with *Listeria monocytogenes* and *Candida albicans*. Int J Immunopharmacol 1991;13:27-37.
10. O'Neill W, McKee S, Clarke AF: Immunological and haematinic consequences of feeding a standardised *Echinacea* (*Echinacea angustifolia*) extract to healthy horses. Equine Vet J 2002;34:222-227.
11. Goel V, Chang C, Slama J, Barton R, Bauer R, Gahler R, Basu T: *Echinacea* stimulates macrophage function in the lung and spleen of normal rats. J Nutr Biochem 2002;13:487-492.
12. Currier NL, Miller SC: Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. Exp Gerontol 2000;35:627-639.
13. Currier NL, Miller SC: *Echinacea purpurea* and melatonin augment natural-killer cells in leukemic mice and prolong life span. J Altern Complement Med 2001;7:241-251.

14. Alban S, Classen B, Brunner G, Blaschek W: Differentiation between the complement modulating effects of an arabinogalactan-protein from *Echinacea purpurea* and heparin. Planta Med 2002;68: 1118-1124.
15. Freier DO, Wright K, Klein K, Voll D, Dabiri K, Cosulich K, George R: Enhancement of the humoral immune response by *Echinacea purpurea* in female Swiss mice. Immunopharmacol Immunotoxicol 2003;25:551-560.
16. Rehman J, Dillow JM, Carter SM, Chou J, Le B, Maisel AS: Increased production of antigen-specific immunoglobulins G and M following *in vivo* treatment with the medicinal plants *Echinacea angustifolia* and *Hydrastis canadensis*. Immunol Lett 1999;68:391-395.
17. Bodinet C, Lindequist U, Teuscher E, Freudenstein J: Effect of an orally applied herbal immunomodulator on cytokine induction and antibody response in normal and immunosuppressed mice. Phytomed 2002;9:606-613.
18. Mishima S, Saito K, Maruyama H, Inoue M, Yamashita T, Ishida T, Gu Y: Antioxidant and immuno-enhancing effects of *Echinacea purpurea*. Biol Pharm Bull 2004;27:1004-1009.
19. Morazzoni P, Cristoni A, Di Pierro F, Avanzini C, Ravarino D, Stornello S, Zucca M, Musso T: *In vitro* and *in vivo* immune stimulating effects of a new standardized *Echinacea angustifolia* root extract (Polinacea™). Fitoterapia 2005;76:401-411.
20. Stimpel M, Proksch A, Wagner H, Lohman-Matthes ML: Macrophage activation and induction of macrophage cytotoxicity by purified fractions from the plant *Echinacea purpurea*. Infect Immun 1984;46:845-849.
21. Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG: *Echinacea*-induced cytokine production by human macrophages. Int J Immunopharmacol 1997;19:371-379.
22. Senchina DS, McCann DA, Asp JM, Johnson JA, Cunnick JE, Kaiser MS, Kohut ML: Changes in immunomodulatory properties of *Echinacea spp*. root infusions and tinctures stored at 4 degrees C for four days. Clin Chim Acta 2005;355:67-82.
23. Sloley BD, Urichuk LJ, Tywin C, Coutts RT, Pang PK, Shan JJ: Comparison of chemical components and antioxidants capacity of different Echinacea species. J Pharm Pharmacol 2001;53:849-57.

24. Hu C, Kitts DD: Studies on the antioxidant activity of *Echinacea* root extract. J Agric Food Chem 2000;48:1466-1472.

25. Randolph RK, Gellenbeck K, Stonebrook K, Brovelli E, Qian Y, Bankaitis-Davis D, Cheronis J: Regulation of human immune gene expression as influenced by a commercial blended *Echinacea* product: preliminary studies. Exp Biol Med(Maywood) 2003;228:1051-1056.

26. Wu L, Bae J, Kraus G, Wurtele ES: Diacetylenic isobutylamides of *Echinacea*: synthesis and natural distribution. Phytochem 2004;65:2477-2484.

27. Zink T, Chaffin J: Herbal 'health' products: what family physicians need to know. Am Fam Physician 1998;58:1133-1140.

28. Cunnick JE, Sakamoto K, Chapes SK, Fortner GW, Takemoto DJ: Induction of tumor cytotoxic immune cells using a protein from the bitter melon (*Momordica charantia*). Cell Immunol 1990;126:278-289.

29. Cunningham AJ, Szenberg A: Further improvements in the plaque technique for detecting single antibody-forming cells. Immunol 1968;14:599-600.

30. Balcombe JP, Barnard ND, Sandusky C: Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 2004;43:42-51.

31. Hale KD, Weigent DA, Gauthier, DK, Hiramoto RN, Ghanta VK: Cytokine and hormone profiles in mice subjected to handling combined with rectal temperature measurement stress and handling only stress. Life Sci 2003;72:1495-1508.

32. Jayasinghe R, Gianutsos G, Hubbard AK: Ethanol-induced suppression of cell-mediated immunity in the mouse. Alcohol Clin Exp Res 1992;16:331-335.

33. Saad AJ, Domiati-Saad R, Jerrells TR: Ethanol ingestion increases susceptibility of mice to *Listeria monocytogenes*. Alcohol Clin Exp Res 1993;17:75-85.

34. See DM, Broumand N, Sahl L, Tilles JG: *In vitro* effects of *Echinacea* and ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatigue syndrome or acquired immunodeficiency syndrome patients. Immunopharmacol 1997;35:229-235.

35. Etter H, Althaus R, Eugster HP, Santamaria-Babi LF, Weber L, Moser R: IL-4 and IL-13 downregulate rolling adhesion of leukocytes to IL-1 or TNF-alpha-activated endothelial cells by limiting the interval of E-selectin expression. Cytokine 1998;10:395-403.

36. D'Andrea A, Ma X, Aste-Amezaga M, Paganin C, Trinchieri G: Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. J Exp Med 1995;181:537-546.

37. Dalby-Brown L, Barsett H, Landbo AK, Meyer AS, Molgaard P. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. J Agric Food Chem 2005;53:9413-9423.

38. Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F: Analysis of phenolic compounds and radical scavenging activity of *Echinacea spp*. J Pharm Biomed Anal 2004;35:289-301.

39. Bodinet C, Willigmann I, Beuscher N: Host-resistance increasing activity of root extracts from *Echinacea* species. Planta Med 1993;59(Suppl):A672-673.

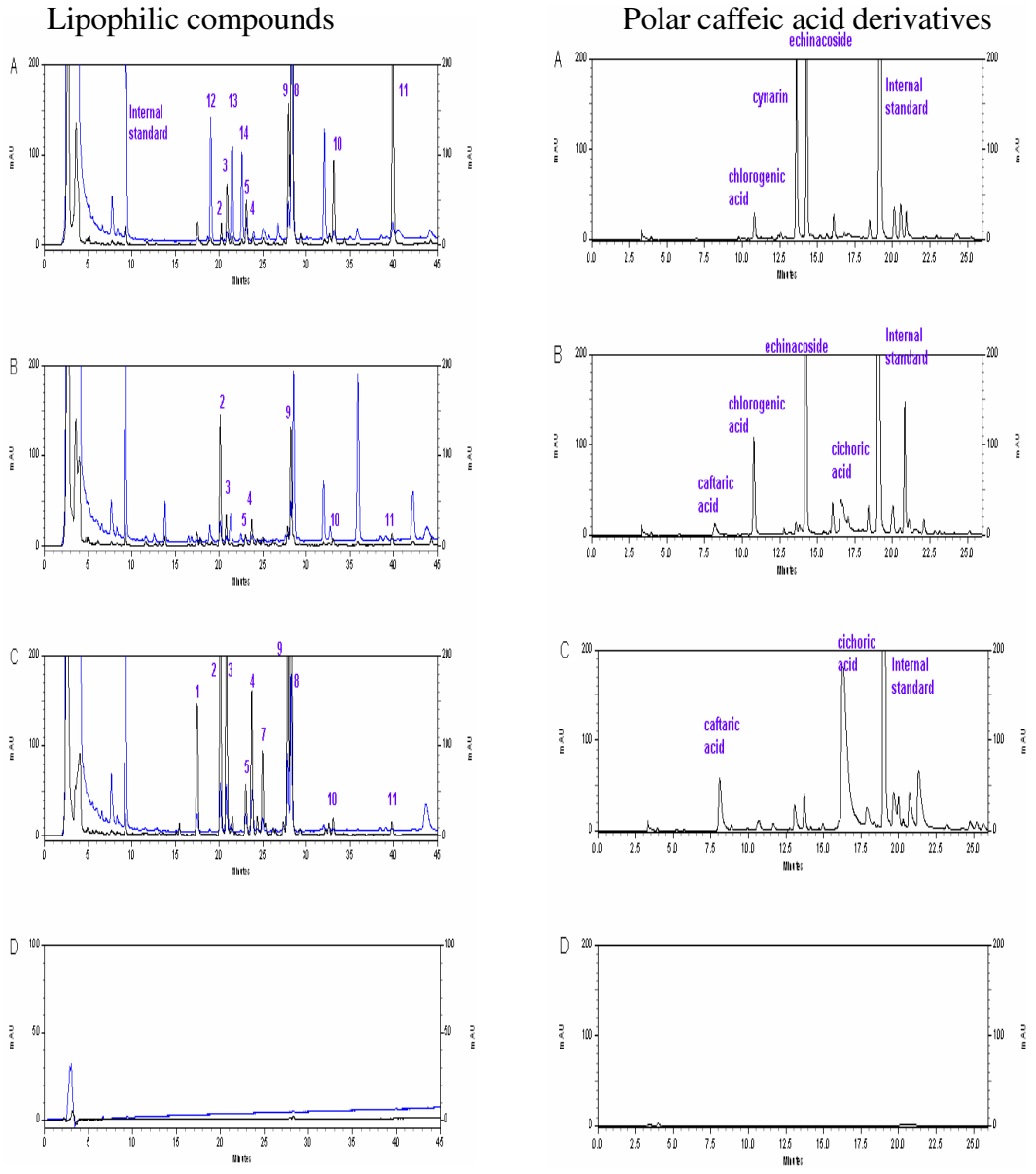


Fig. 2.1. HPLC chromatograms of the lipophilic amides and hydrophilic caffeic acid derivatives in *Echinacea* preparations. (A) *E. angustifolia*, (B) *E. pallida*, (C) *E. purpurea*, (D) vehicle. HPLC conditions see Materials and Methods. UV absorbance at 210 nm (upper line) and 260 nm (lower line) for lipophilic chromatography. Peak 1-14 represent amides as shown in table 1. UV absorbance at 330 nm for hydrophilic chromatography.

Table 2.1. Concentration and percentage of known amides and caffeic acid derivatives in *Echinacea* preparations

	E. angustifolia			*E. pallid*			*E. purpurea*		
	µg/mg extract[a]	**% metabolites[b]**	**µg/d/mouse[c]**	**µg/mg extract**	**% metabolites**	**µg/d/mouse**	**µg/mg extract**	**% metabolites**	**µg/d/mouse**
amide 1	1.620	1.50	5.447				6.708	7.68	22.550
amide 2	1.161	1.08	3.904	6.443	21.83	21.660	12.471	14.27	41.921
amide 3	3.319	3.08	11.158	1.626	5.51	5.466	12.532	14.34	42.127
amide 4	0.488	0.45	1.639	1.403	4.75	4.715	6.184	7.08	20.789
amide 5	2.531	2.35	8.507	0.704	2.38	2.367	2.483	2.84	8.347
amide 7	0.000	0.00	0.000	0.000	0.00	0.000	3.619	4.14	12.167
amide 8	55.425	51.47	186.319	0.000	0.00	0.000	21.573	24.69	72.521
amide 9	7.685	7.14	25.835	6.885	23.32	23.146	12.477	14.28	41.942
amide 10	5.334	4.95	17.931	0.431	1.46	1.449	0.905	1.04	3.042
amide 11	13.306	12.36	44.730	0.713	2.42	2.398	0.782	0.90	2.630
amide 12	4.055	3.77	13.631	0.000	0.00	0.000	0.000	0.00	0.000
amide 13	3.834	3.56	12.888	0.000	0.00	0.000	0.000	0.00	0.000
amide 14	3.011	2.80	10.121	0.000	0.00	0.000	0.000	0.00	0.000
Total amides	101.769	94.499	342.110	18.206	61.673	61.200	79.734	91.256	268.036
caftaric acid	0.000	0.00	0.000	0.543	1.84	1.824	1.184	1.36	3.980
chlorogenic acid	0.473	0.44	1.590	1.357	4.60	4.563	0.000	0.00	0.000
cynarin	1.929	1.79	6.483	0.000	0.00	0.000	0.000	0.00	0.000
echinacoside	3.523	3.27	11.843	8.350	28.29	28.069	0.000	0.00	0.000
cichoric acid	0.000	0.00	0.000	1.064	3.60	3.577	6.456	7.39	21.702
Total caffeic acid derivatives	5.924	5.50	19.916	11.314	38.33	38.033	7.640	8.74	25.682
Total metabolites	107.694	100	362.025	29.520	100	99.234	87.374	100	293.719

. Average of duplicate determinations.

b. % metabolites = (concentration of individual metabolite / concentration of total metabolites) × 100.

c. Intake of individual metabolite for each mouse on each day of oral *Echinacea* administration.

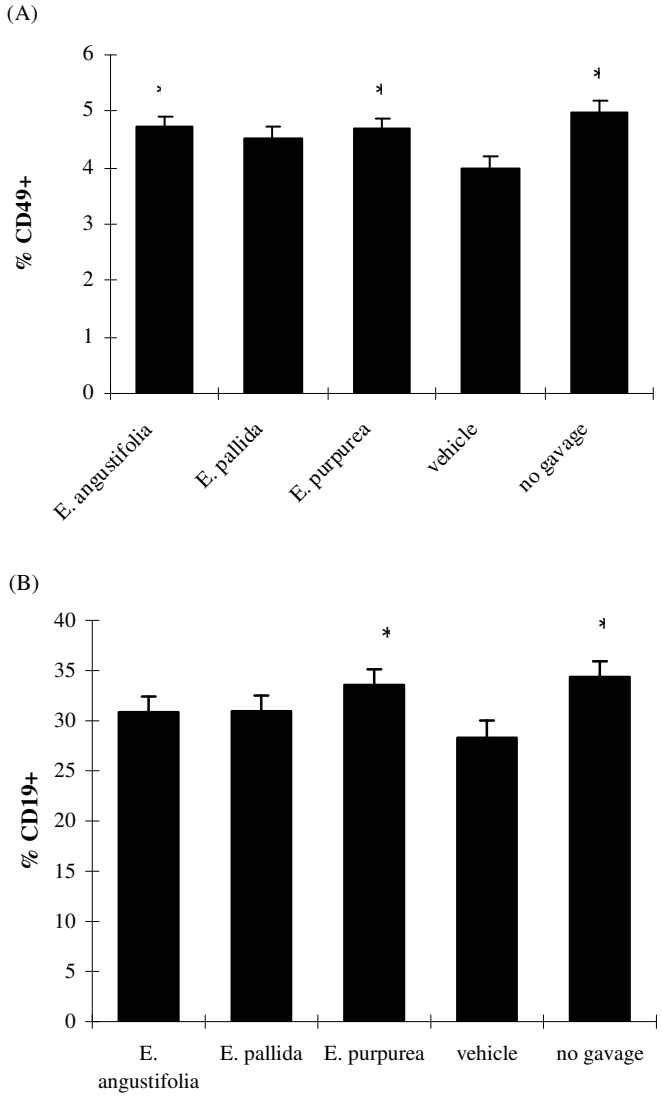


Fig. 2.2. Effect of *Echinacea* preparations on the percentages of splenic (A) CD49+ subset and (B) CD19+ subset. Male BALB/c mice were orally administered one of three *Echinacea* preparations 130mg/kg daily for consecutive 7 days. The vehicle control mice received equally volumetric vehicle of 5% ethanol. Spleen cells were isolated to analyze the expression of the CD49 (NK cell subset) and CD19 (B-cell subset) markers by flow cytometry as described in experimental methods. Results were presented as the mean ±standard error of two independent experiments (N=6). *Indicates a significant group difference from the vehicle control at $p<$ 0.05.

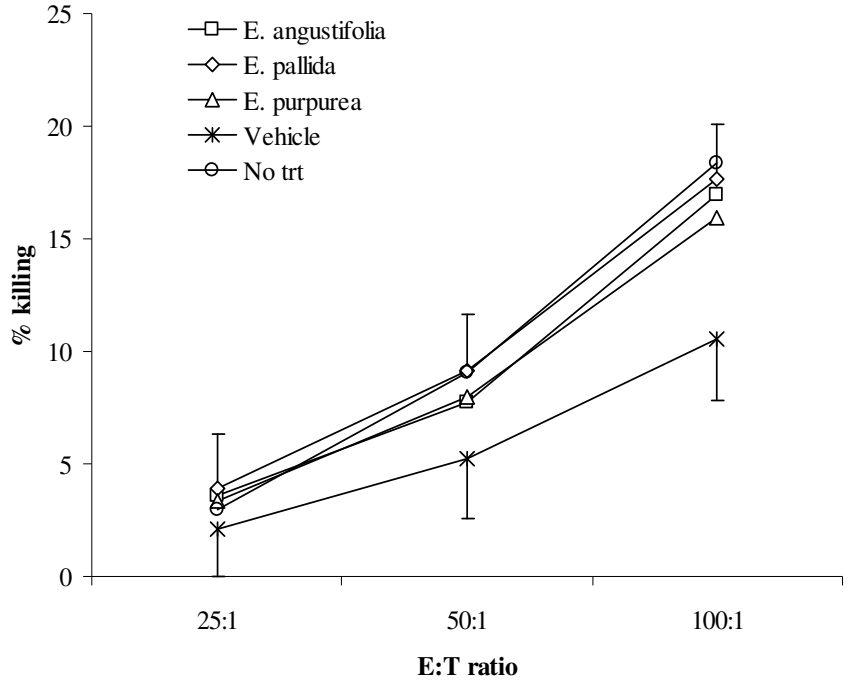


Fig. 2.3. Effect of *Echinacea* preparations on NK cytotoxity after covarying for the percentage of CD49+ splenocytes in each animal. NK cell cytotoxicity was measured as described in experimental methods and expressed as percentage cytolysis of target cells. Results were presented as the mean ±standard error of two independent experiments (N=6).

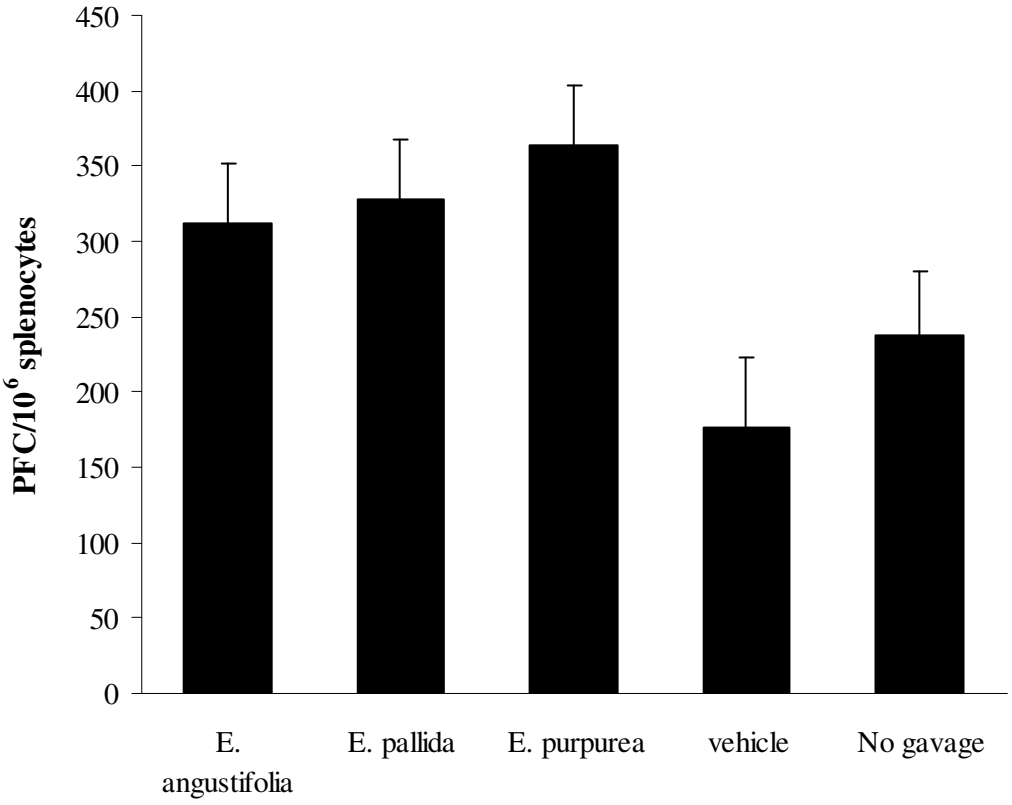


Fig. 2.4. Effect of *Echinacea* preparations on splenic plaque forming cell (PFC) response after covariance for the percentage of CD19+ splenocytes in each animal. PFC response was assayed as described in experimental methods and expressed as PFC per 10^6 splenocytes. Results were presented as the mean ±standard error of two independent experiments (N=6).

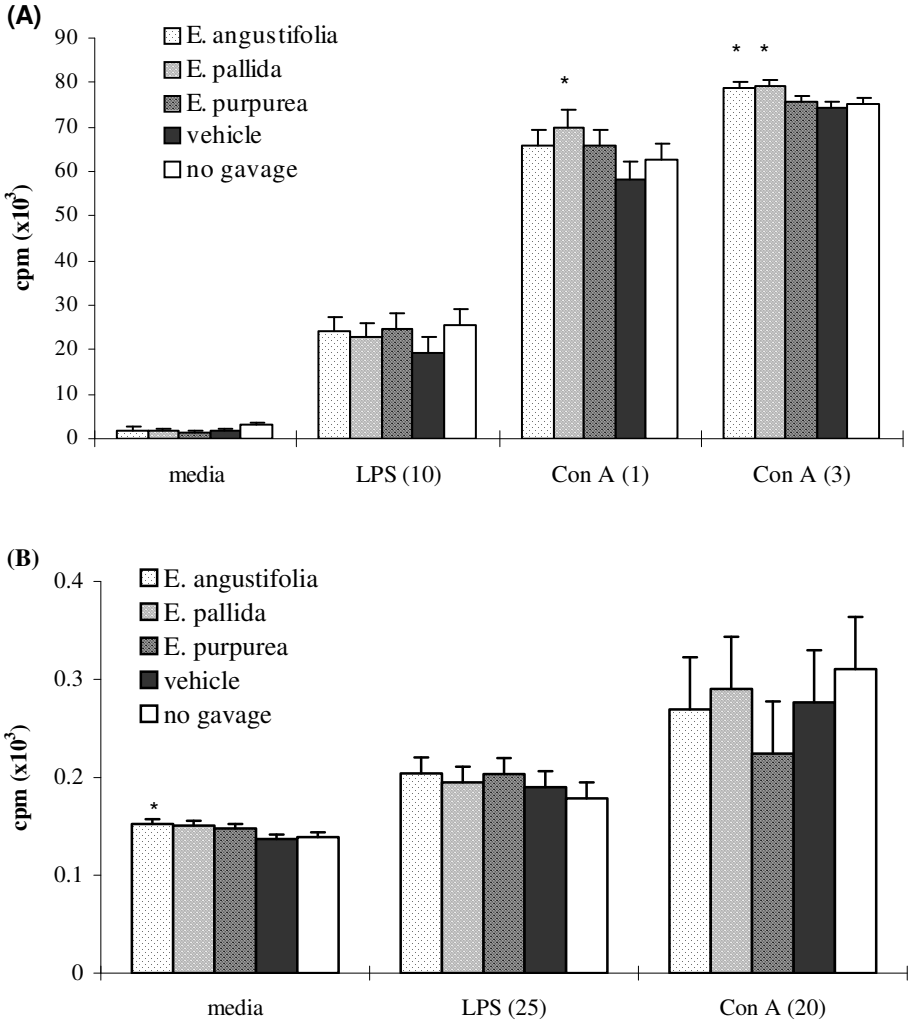
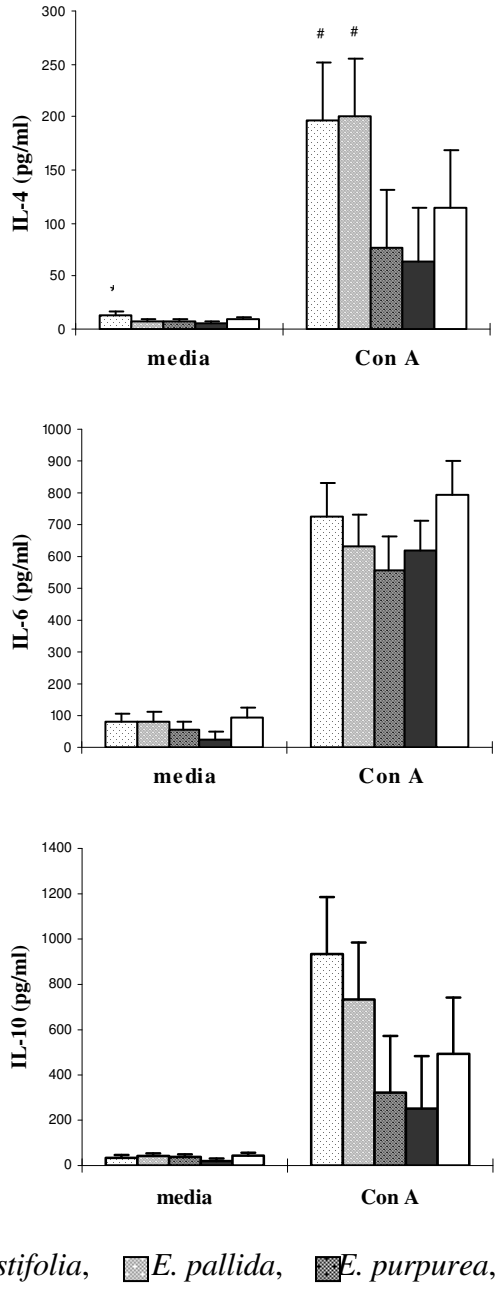


Fig. 2.5. Effect of *Echinacea* preparations on (A) splenic lymphocyte proliferation and (B) blood lymphocyte proliferation. Lymphocyte proliferation assay was conducted as described in experimental methods and was expressed as cpm (^{3}H-incorporation) $\times 10^3$. Results were presented as the mean ±standard error of two independent experiments (N=6). The value in the parenthesis means the concentration of mitogen (μg/ml). * Indicates a significant difference from the vehicle control at $p<0.05$.



E. angustifolia, *E. pallida*, *E. purpurea*, vehicle, no gavage

Fig. 2.6. Effect of *Echinacea* preparations on T_H2 cytokine production by mouse splenocytes stimulated *in vitro* without or with mitogen. Spleen cells were incubated without or with Con A at 10 μg/ml for 72 h. Cytokine levels were determined as described in experimental methods. Results were presented as the mean ± standard error of two independent experiments (N=6). * Indicates a significant difference from the vehicle control at $p<0.05$. # Indicates a significant difference of combination of corresponding *Echinacea* treatment groups with the vehicle control

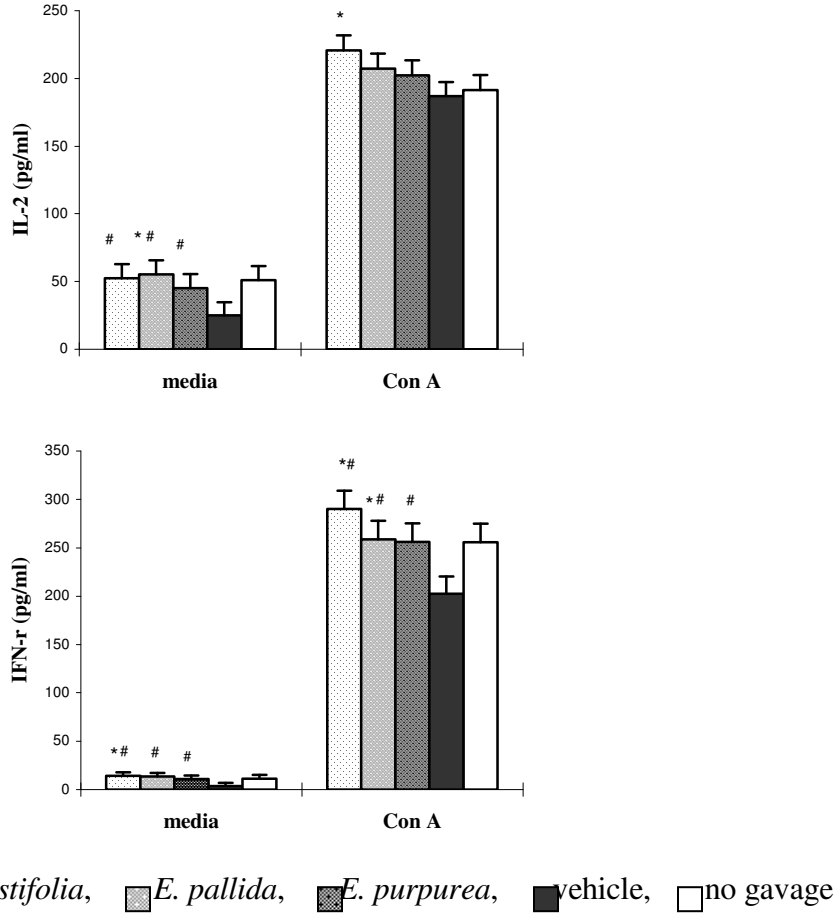


Fig. 2.7. Effect of *Echinacea* preparations on T_H1 cytokine production by mouse splenocytes stimulated *in vitro* without or with mitogen. Spleen cells were incubated without or with Con A at 10 μg/ml for 48h. Cytokine levels were determined as described in experimental methods. Results were presented as the mean ± standard error of two independent experiments (N=6). * Indicates a significant difference from the vehicle control at $p<0.05$. # Indicates a significant difference of combination of corresponding *Echinacea* treatment groups with the vehicle control

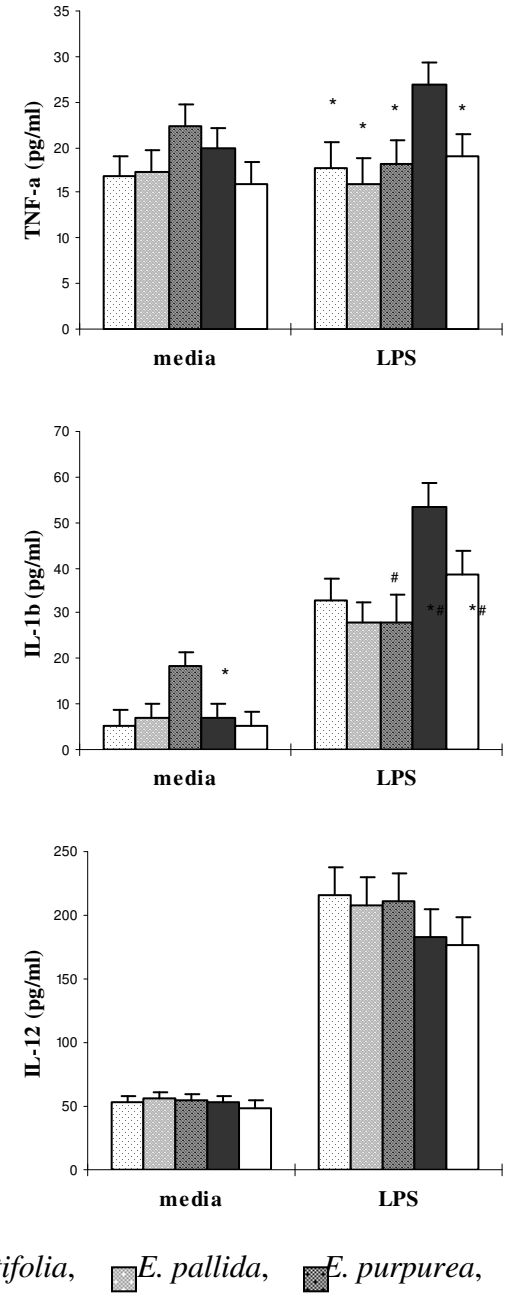


Fig. 2.8. Effect of *Echinacea* preparations on macrophage cytokine production *in vitro* without or with mitogen. Spleen cells were incubated without or with LPS at 10 μg/ml for 24 at 37°C in a 7% CO_2 incubator. Cytokine levels were determined as described in experimental methods. Results were presented as the mean ± standard error of two independent experiments (N=6). * Indicates a significant difference from the vehicle control at $p<0.05$. # Indicates a significant difference of combination of corresponding *Echinacea* treatment groups with the vehicle control.

CHAPTER 3. ALCOHOL EXTRACTS OF *ECHINACEA* INHIBIT PRODUCTION OF NITRIC OXIDE AND TUMOUR NECROSIS FACTOR-ALPHA BY MACROPHAGES *IN VITRO*

A paper published in *Food and Agricultural Immunology*[1]

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Abstract

Echinacea has been suggested to have anti-inflammatory activity *in vivo*. Nitric oxide (NO), tumour necrosis factor-alpha (TNF-α), and interleukin-1beta are important mediators in the inflammatory response. The effects of alcohol extracts of *E. angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP) on production of these inflammatory mediators in both LPS-stimulated RAW 264.7 macrophages *in vitro* and murine peritoneal exudate cells (PECs) *in vivo* were investigated. As macrophages produce these inflammatory mediators in response to pathogenic infection, parallel cultures of macrophages were studied for phagocytosis and intracellular killing of *Salmonella enterica*. EPA and EP *in vitro* inhibited NO production and TNF-α release in a dose-dependent manner. RAW 264.7 cells treated with EA or EP showed decreased killing over 24 hours although EA enhanced bacterial phagocytosis. Upon bacterial infection, RAW 264.7 cells produce high levels of NO; however, an *Echinacea*-mediated

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decrease in NO production was observed. *Echinacea* alcohol extracts administered orally at 130 mg kg^{-1} d^{-1}× 7d had a weak effect on NO production and phagocytosis by LPS-stimulated PECs. The results indicated that all *Echinacea* species significantly decreased inflammatory mediators in vitro, however, only EA and EP reduced bacterial killing. Oral administration of *Echinacea* alcohol extracts did not adversely affect the development and anti-bacterial function of inflammatory PECs *in vivo*, however, NO production was decreased during bacterial infection of PECs.

Introduction

The genus *Echinacea* is a popular natural immunostimulant. Reports indicate that *Echinacea* increases phagocytosis by neutrophils and macrophages, and stimulates these inflammatory cells to produce proinflammatory cytokines and free radicals (Burger et al. 1997; Goel et al. 2002; Steinmuller et al. 1993; Stimpel et al. 1984), which are responsible for the antimicrobial activity. Contrary to these reports, *Echinacea* is also used as an anti-inflammatory agent. The anti-inflammatory effects have been demonstrated using murine inflammatory models such as carrageenan-induced paw oedema and abraded skin (Raso et al. 2002; Speroni et al. 2002; Tubaro et al. 1987). These seemingly contradictory, but interdependent biological activities of *Echinacea* products are, to a very large extent, related to their immunomodulating properties and may be due to the variability in composition of the herb products utilized in each study (Percival 2000). Several active components have been identified in *Echinacea*, including lipophilic alkamides, polar caffeic acid derivatives (mainly echinacoside and cichoric acid) and high molecular weight polysaccharides (Barnes et al. 2005). In the current herbal market, *Echinacea* products are a mixture of many varieties and may be blended with other herbs in various forms, making it hard to clarify which components play a predominant role in the immunomodulatory activity. The interaction between the components of *Echinacea* crude preparations is also unclear. The effects of *Echinacea* products

might also depend on the host condition. *Echinacea* may enhance immune function in the body with a weakened immune system (Currier & Miller 2000; Bodinet et al. 2002), but suppress it when an inflammatory response predominates. Our previous animal study found that *Echinacea* treatment restored mild stress-induced immune changes to normal levels (Zhai et al. 2007).

The inflammatory response *in vivo* involves infiltration of macrophages that function through secretion of inflammatory mediators, i.e. tumour necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β) and nitric oxide (NO) (Park & Barbul 2004). The inflammatory cytokines TNF-α and IL-1β enhance phagocytosis by macrophages and trigger the release of NO (Nussler & Billiar 1993). NO, a signal of macrophage activation, is central to macrophage cytostatic and cytotoxic activity against various bacteria and tumour cells (Burgner et al. 1999; MacMicking et al. 1997). However, when produced in excessive quantities, NO is detrimental to both pathogenic agents and the host's own tissues (Brune et al. 1997; Chang et al. 2000). Therefore, production of NO and other inflammatory mediators is tightly regulated by a set of complex anti-inflammatory mechanisms which involve IL-4 and IL-10 (Salmon-Her et al. 2000; Sato et al. 1999). Our previous *ex vivo* observations show that orally administered alcohol extracts of *Echinacea* increased the production of IL-4 and IL-10, but inhibited TNF-α and IL-1β secretion by mitogen-stimulated splenocytes (Zhai et al. 2007). Other research groups show that the alkamides from *Echinacea* downregulate NO production in activated macrophages *in vitro* (Chen et al. 2005) and decrease TNF-α release in LPS-stimulated human whole blood cells *ex vivo* (Woelkart et al. 2006). These studies suggest that alcohol extracts of *Echinacea* as well as its alkamide fraction possess anti-inflammatory activity.

In this study, the effects of alcohol extracts of the three commonly used *Echinacea* species, *E. angustifolia* (EA), *E. pallida* (EPA), and *E. purpurea* (EP) were further examined both *in vivo* and *in vitro* by determining the production of inflammatory mediators, NO, TNF-α and IL-1β in activated macrophages**.** Parallel cultures of *ex vivo* macrophages and the

macrophage cell line RAW 264.7 cells were studied for phagocytosis and intracellular killing of *Salmonella* in order to understand if *Echinacea* alcohol extracts compromise the antibacterial function of macrophages through inhibition of inflammatory mediators.

Materials and Methods

Preparation of alcohol extracts

The plants EA, EPA and EP were harvested in the USDA North Central Regional Plant Introduction Station (Ames, IA) with identification numbers PI 631285, PI 631293, and PI 631307, respectively. Alcohol extracts from the dried roots were prepared as previously described (Zhai et al. 2007) followed by evaporation until dry. These dry residues were subsequently dissolved in ethanol and phosphate buffed saline (PBS) to desired concentrations for *in vitro* assays or dissolved in ethanol and Nanopure water for animal studies. Those extracts used for *in vitro* assays were further filter-sterilized using a 0.45 µm microfilter (Costar, Corning, NY). Aliquots of alcohol extracts were stored at -80°C (for *in vitro* assays) or -20°C (for animal studies) and freshly thawed for each experiment. The endotoxin levels were evaluated in aliquots using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD) according to the manufacturer's specifications for a microplate assay and were detected to be below the limit of detection (0.1 EU ml^{-1}). Phytochemical analysis was performed to detect alkamides and caffeic acid derivatives in aliquots using high performance liquid chromatography (HPLC) as described previously (Wu et al. 2004).

Animals

Animal care and experimental procedures were approved by the Iowa State University Committee on Animal Care. Male BALB/c mice at 8 weeks of age were obtained through Iowa State University Laboratory Animal Resources from Harlan Laboratories (Indianapolis, IN) and allowed to acclimatise to the new environment for 2 weeks. The mice were housed

three/cage and provided free access to food and water under a reverse 12 h light/dark regimen (lights off at 9:00 AM).

Echinacea administration in vivo

The mice were randomly assigned to five groups. Groups 1-3 were gavaged with one of the three alcohol extracts. Group 4 was gavaged with an equal volume of 5% ethanol as vehicle control. Group 5 served as a no gavage control. The vehicle control and the no gavage control were established to control for the effects of the vehicle as well as handling stress. The extracts were orally administered to the animals at 130 mg kg^{-1} body weight once daily for 7 consecutive days. This dosage and regimen was chosen based on an extrapolation of the maximum dose recommended for humans and calculated as previously described (Zhai et al. 2007). Three days prior to euthanasia, all mice were injected intraperitoneally with 3% proteose peptone (Difco Laboratories, Detroit, MI) to induce an inflammatory cell response. The animal experiment was repeated three times.

Peritoneal exudate cell collection

Twelve to fifteen hours after the last gavage, the mice were euthanized by CO_2 asphyxiation, and peritoneal exudate cells (PECs) were immediately collected by peritoneal lavage with ice-cold EDTA (0.1%; w/v) in Hank's balanced salt solution (Fortier & Falk 1994). For each group, PECs were pooled and washed twice with RPMI 1640 medium supplemented with 2 mM glutamine, 25 mM Hepes, 50 μg ml^{-1} gentamicin and 10% heated-inactivated foetal bovine serum (complete medium).

Macrophage cell line

The murine peritoneal macrophage cell line RAW 264.7 cells were acquired from American Type Culture Collection (ATCC, Rockville, MD) and passaged in complete medium with heated-inactivated iron-fortified bovine calf serum (JRH Biosciences, Lenexa, KS) at 37°C in a 7% CO_2 incubator. Cells between passage 5 and 20 were used in this study.

Cell viability assay

Mitochondrial reduction of MTS, a tetrazolium compound, to a coloured formazan was determined as an indicator of cell viability. RAW 264.7 cells and PECs were seeded at 4×10^4 cells per well in 96-well tissue culture plates with or without 1µg ml^{-1} lipopolysaccharide (LPS, E. coli 055:B5, L6529; Sigma, St. Louis, MO). For cultures of RAW 264.7 cells, one of alcohol extracts was added at 0-500 µg ml^{-1} at the same time the cells were plated in the absence or presence of LPS. After overnight incubation, MTS (Promega, Madison, WI) was added and cultures then successively incubated for 3 (PECs) or 1 (RAW 264.7 cells) h. The extent of formazan formation was determined photometrically at absorbance 490 nm using a Bio Kinetics Reader (Bio-Tek Instruments, Winooski, VT). The absorbance of cell-free complete medium with or without vehicle or *Echinacea* alcohol extracts was used as a blank and subtracted from the value of the corresponding treatment groups.

Growth and freezing of Salmonella

Salmonella enterica subspecies enterica serovar Typhimurium were obtained from Dr. H. Harris (Iowa State University) and grown overnight at 37°C in Luria-Bertani (LB) broth with shaking to a density of 10^9 cells ml^{-1}. The *Salmonella* were washed once in PBS and frozen at -80°C in LB broth containing 16% sterile glycerol. A freshly thawed aliquot of *Salmonella* was used for each experiment.

Gentamicin protection assays

Engulfment and killing of *Salmonella* were performed using both RAW 264.7 cells and PECs. The protocol for each cell type was identical with the exception that *Echinacea* alcohol extracts were added to cultures of RAW 264.7 cells. PECs were collected from mice which had been gavaged with one of the alcohol extracts *in vivo*. RAW 264.7 cells and PECs were seeded at a concentration of 4×10^5 cells per well (1 ml) in 24-well plates and incubated overnight. After harvest of culture supernates for NO and cytokine assays,

described below, cells were washed three times in gentamicin-free complete medium. *Salmonella* were then added to the adherent RAW 264.7 cells and PECs at a multiplicity of infection (MOI) of 100 bacteria per cell. Following 2 h of infection, cells were washed in warm complete medium containing 50 μg ml^{-1} of gentamicin and maintained in gentamicin-containing complete medium for the remainder of the experiment to kill all extracellular bacteria. At 0, 4 and 24 h post infection, one set of parallel cell cultures were washed with sterile PBS to remove gentamicin and then lysed in distilled water to release the intracellular bacteria. Intracellular bacteria were enumerated by serial dilution and plate count on LB agar. Lysate dilutions on LB agar were incubated overnight at 37°C and counts were expressed as colony forming units (CFU) ml^{-1}. CFU at the 0 h time point reflects the amount of phagocytosis by the cultured RAW 264.7 cells and PECs, while the reduction in CFU at 4 and 24 h time points reflects the killing of intracellular bacteria by the cells. As *Echinacea* treatment induced some changes in eukaryotic cell growth (see Fig 3.1), the intracellular bacteria quantitation was adjusted based on the parallel MTS assay to normalize each treatment group to the same cell count before bacterial infection by using the formula: number of intracellular bacteria = number of viable bacteria counted × (control cells MTS OD) / (treated cells MTS OD).

Nitrite assay

NO in the presence of oxygen is rapidly converted to nitrite. Supernates collected from RAW 264.7 cells and PECs cultured with and without LPS or with *Salmonella* (as described above) were tested for nitrite based on the Griess reaction (Chou et al. 1997). The absorbance at 550 nm was measured using a microplate reader. The cell-free culture medium alone containing a trace amount of nitrite was subtracted from each value obtained with cells. The NO levels were calculated based upon the absorbance of sodium nitrite (0.39-100 μM) as a standard. The NO levels in supernates from RAW 264.7 cells post *Salmonella* infection were adjusted based on the MTS data to normalize for differences in cell number.

In the absence of LPS, very low amounts of NO (< 0.5 μM) were measured in culture supernates from RAW 264.7 cells incubated *in vitro* for 24 h. Thus, we report only NO production of cultures from LPS stimulated cells.

Cytokine assays

Supernates from RAW 264.7 cells and PECs incubated with LPS for 24 h (as described above) were assayed for TNF- α (RAW 264.7 cells) or IL-1β and IL-10 (PECs) by enzyme-linked immunosorbent assay (ELISA). In the absence of LPS *in vitro*, very low (or lower than limit of detection) amounts of the cytokines of interest were measured in culture supernates of both RAW 264.7 cells and PECs. In the presence of LPS, RAW 264.7 cells secrete high levels of TNF-α and PECs secrete high levels of IL-1β and IL-10. ELISAs for these cytokines were conducted according to the manufacturer's protocols (BD Biosciences, San Diego, CA). The cytokine levels were calculated by using purified recombinant mouse cytokines as a standard.

Statistical analysis

Statistix software (version 8.0, Analytical Software, Tallahasee, FL) was used for the statistical analysis. Differences between the vehicle and other groups were tested by two-way analysis of variance (group × experiment). A value $p<0.05$ was considered significant.

Results

Phytochemical analysis

Echinacea alcohol extracts contain several chemical components. Lipophilic alkamides and hydrophilic caffeic acid derivatives are two groups of the most studied metabolites. The concentrations of phytochemicals identified and quantified using HPLC are listed in Table 3.1. Alkamides were the major phytochemicals detected, especially in EA and EP alcohol extracts. Note that amides 7 and 8 appear in the present EPA extracts though they were undetectable in

our previously prepared alcohol extracts (Zhai et al. 2007). Among the caffeic acid derivatives, echinacoside was found in higher quantities in both EA and EPA extracts while cichoric acid was the main form in EP extract.

In vitro experiments

Before measurement of the production of inflammatory mediators by RAW 264.7 cells, we first evaluated the cytotoxicity of *Echinacea* alcohol extracts by using the MTS-based assay. *Echinacea* alcohol extracts displayed different patterns of effects in the presence as opposed to the absence of LPS (Fig 3.1). In the absence of LPS, all three alcohol extracts at 200 and 500 μg ml^{-1} significantly increased cell number when these two groups were jointly compared to the vehicle control, but in the presence of LPS, EPA and EP at 500 μg ml^{-1} significantly decreased cell number when compared to cells treated with vehicle (0.25% ethanol) (*p'* s ≤0.006). Treating RAW 264.7 cells with the vehicle had modest effects on cell number. The vehicle, in comparison to the baseline control, decreased cell number in the absence of LPS, but increased cell number when LPS was present.

To assess the effect of *Echinacea* alcohol extracts on NO production, RAW 264.7 cells were exposed to LPS plus alcohol extracts for 24 h. The alcohol extracts reduced NO production in a dose-dependent manner (Fig 3.2A). In comparison to the vehicle control, EPA at 100 μg ml^{-1} significantly decreased the production of NO ($p = 0.009$). When the herbal concentration was raised to 200 μg ml^{-1}, NO production was significantly inhibited by all three alcohol extracts (p values ≤ 0.042). None of the three alcohol extracts at the concentrations tested interfered with the reaction between nitrite and the Griess reagent (data not shown).

RAW 264.7 cells activated by LPS secrete high levels of TNF-α (reaching up to 9000 pg ml^{-1}). The effect of *Echinacea* alcohol extracts on the TNF-α levels was evaluated in the same culture supernates analyzed for the NO levels. All three extracts had a similar inhibitory effect on TNF-α secretion as on NO production (Fig 3.2B). When compared to the

vehicle control, both EPA and EP at a concentration of 100 µg ml^{-1} significantly decreased TNF-α secretion ($p < 0.02$ and $p < 0.0001$, respectively). EA, as well as EPA and EP at the highest concentration tested of 200 µg ml^{-1} also inhibited TNF-α secretion (p's < 0.04).

After incubation with *Echinacea* alcohol extracts overnight, RAW 264.7 cells were assessed for phagocytosis (CFU at 0 h) and bacterial killing (reduction in CFU at 4 and 24 h) after addition of *Salmonella*. Fig 3.3 shows the survival of bacteria in RAW 264.7 cells pretreated with one of the three alcohol extracts in the absence of LPS. Incubation of RAW 264.7 cells with EA at 100 - 200 µg ml^{-1} enhanced the subsequent phagocytosis ($p's < 0.04$), but inhibited bacterial killing at 4 ($p's < 0.026$) and 24 h ($p's < 0.026$) post-infection. EPA displayed no significant effects on bacterial phagocytosis, but increased bacterial killing over 24 h incubation ($p = 0.002$ at 200 µg ml^{-1}). EP had no effect on bacterial phagocytosis, but decreased later bacterial killing. EP at 100 - 200 µg ml^{-1} significantly inhibited bacterial killing 4 h post-infection ($p's < 0.037$). At 24 h post-infection, EP at 10 µg ml^{-1} also showed an inhibitory effect on bacterial killing ($p = 0.011$).

NO production was also evaluated in the culture of RAW 264.7 cells following exposure to *Salmonella* (Fig 3.4). RAW 264.7 cells pretreated with *Echinacea* alcohol extracts showed a dose-related trend of decreased NO production 24 h after exposure to bacteria. When compared to the vehicle, EA and EPA at 200 µg ml^{-1} significantly inhibited NO production when assayed at 24 h post-infection ($p = 0.036$ and $p = 0.018$, respectively). At 4 h post-infection, EP at 100 µg ml^{-1} significantly decreased NO production ($p = 0.030$), but the decrease in NO was not significant at the 24 hour time point for EP.

Ex vivo experiments

Parallel studies were performed using proteose peptone-elicited PECs. After mice were gavaged with *Echinacea* alcohol extracts for 7 consecutive days, PECs were harvested and incubated overnight in the absence or presence of LPS. There were no significant differences in cell number as determined by the MTS assay (Fig 3.5A) and NO production (Fig 3.5B)

between the vehicle control and any of the *Echinacea* treatment groups. Also, no significant differences in secretion of cytokines IL-1β and IL-10 in culture supernates of PECs in the presence of LPS were associated with oral administration of the extracts as compared to the vehicle control (Fig 3.5C). For all these assays of PECs (cell viability, NO production and cytokines), no significant difference was observed between the two control groups (the no gavage control and the vehicle control).

After incubation of PECs overnight without LPS stimulation, cells were further assessed for phagocytosis and bacterial killing as well as NO production at 0, 4 and 24 h post-infection. In comparison to the no gavage control, the vehicle control displayed significantly decreased phagocytosis ($p < 0.02$) (Fig 3.6A). However, one of the three extracts, EPA, showed a marginal increase in bacterial phagocytosis over the vehicle control ($p = 0.052$). With increasing time after infection, there were no significant differences in bacterial killing between the vehicle control and any of other groups. Measurement of NO production post-infection showed that mice gavaged with the vehicle displayed increased potential in NO production and a significant increase in the vehicle control was found at 4 h after infection as compared to the no gavage mice ($p < 0.05$) (Fig 3.6B). Conversely, three *Echinacea* treatments showed to some extent decreased NO production when compared to the vehicle control and EP exhibited a significant decrease in NO production compared to the vehicle control ($p < 0.03$) at 4 h, but not 24 h post-infection.

Discussion

This study demonstrates that *Echinacea* alcohol extracts *in vitro* inhibit LPS-induced generation of NO and TNF-α in macrophages. Although NO and TNF-α are needed for clearing and containing bacterial infection, excessive NO and TNF-α are implicated in a pathological role in inflammatory responses. Such an inhibitory property may endow *Echinacea* alcohol extracts as an effective anti-inflammatory remedy. In the present study we

found evidence that *Echinacea* can decrease inflammatory mediators *in vitro*, but that the effect *in vivo* was observed only in comparison with the vehicle control group which exhibited an excessive production of NO during *Salmonella* infection. Interestingly enough the excessive NO production and suppressed NO production did not correlate with any biological effects of bacterial killing in the PECs.

Echinacea alcohol extracts consist of both hydrophilic and lipophilic fractions. There is evidence to believe that polar caffeic acid derivatives have anti-inflammatory activities (Speroni et al. 2002). There are several types of caffeic acid derivatives in *Echinacea* (Table 1). Echinacoside was predominately present in EPA and EA, whereas cichoric acid was the principal caffeic acid derivative of EP. In recent years much attention has been paid to the antioxidant activities and free radical scavenging abilities of *Echinacea*-derived caffeic acid derivatives (Dalby-Brown et al. 2005; Facino et al. 1995; Hu & Kitts 2000; Pellati et al. 2004). The alkamide mixture of *Echinacea* alone shows weak or no antioxidant activity, however, the alkamides have been found to significantly increase the antioxidant activity of the caffeic acid derivatives, possibly due to the lipophilic nature of the alkamides (Dalby-Brown et al. 2005). Moreover, individual alkamides and mixtures of alkamides have been reported to have anti-inflammatory effect by inhibiting NO production in LPS-stimulated macrophages *in vitro* (Chen et al. 2005). The observed inhibition of inflammatory mediators by *Echinacea* alcohol extracts *in vitro* in the present study might be due to the additive or synergistic action of hydrophilic fractions (caffeic acid derivatives) and lipophilic fractions (alkamides).

The NO and TNF-α inhibitory potential of the three alcohol extracts *in vitro* is generally EPA>EP>EA. The basis of this difference in the modulation of NO and TNF-α production may rest on the distinct amounts and types of phytochemicals between the three herbal preparations. HPLC data showed that EPA and EP had higher levels of echinacoside or cichoric acid, respectively, and they share high levels of some individual alkamides, such as

alkamide 2 which occurs in relatively small amount in EA. These herbal components may be quantitatively important as modulators of NO and TNF-α production. Further studies are needed to address the respective role of individual phytochemicals and their interaction in the modulation of inflammatory mediators.

NO generated by LPS-activated macrophages is a strong inducer of cell damage and apoptosis (Brune et al. 1997; Chang et al. 2000). The downregulation of NO production by *Echinacea* alcohol extracts possibly protects macrophages from NO damage. Since production of TNF-α and NO by macrophages are precisely controlled by several ubiquitous transcription factors including NF-κB (Baeuerle & Baltimore 1996), *Echinacea*-mediated reduction of both inflammatory mediators likely results from the perturbance of a common upstream signalling pathway.

Interestingly, Sharma et al. (2006) recently reported that an alcohol tincture from EP roots increased the nuclear expression of multiple pro-inflammatory transcription factors (e.g. NF-κB and STATs) in nonactivated human bronchial epithelial cell line BEAS-2B, but inhibited the expression of these transcription factors when the cells were infected with rhinovirus, providing strong mechanistic evidence to explain the observed phenomena in this study; that is, *Echinacea* alcohol extracts have different influences on the nonactivated and activated macrophages and the effects of *Echinacea* on the inflammatory mediators are associated with the modulation of transcription factor expression.

The opposite effects of *Echinacea* alcohol extracts on the nonactivated and activated macrophages were also seen in cell numbers with the use of the MTS assay (Fig 3.1). In comparison to the vehicle, *Echinacea* resulted in decreased cell number in the presence of LPS. While macrophages are in a resting state, *Echinacea* stimulated to some extent an increase in cell number. *Echinacea* alcohol extracts might stimulate the nonactivated macrophages (e.g. increase cell number and/or function), but reduce macrophage proliferation when they are activated.

Macrophages constitute one of the first lines of host defence against microbial infections based on their abilities to produce NO and reactive oxygen species. NO is believed to represent an important effector molecule in the killing of a variety of pathogens, including *Salmonella* (Alam et al. 2002; Babu et al. 2006; Burgner et al. 1999; MacMicking et al. 1997). Since *Echinacea* preparations, mainly from fresh-pressed juice or the high molecular weight polysaccharide fraction, have been shown to upregulate production and secretion of proinflammatory cytokines and oxygen radicals, consistent with an immune activated antimicrobial effect (Burger et al. 1997; Steinmuller et al. 1993; Stimpel et al. 1984), it is interesting to know the possible consequence upon inhibition of NO production by *Echinacea* alcohol extracts. We attempted to investigate and correlate the phagocytic activity and bacterial killing of macrophages with NO production following exposure to *Echinacea* alcohol extracts. Although EA enhanced phagocytic activity, treatment with EA or EP clearly showed an inhibitory effect on intracellular bacterial killing. This inhibition may be a secondary effect of the downregulation of NO production. Macrophages infected with bacteria produce high levels of NO that can be blocked by alcohol extracts of *Echinacea*, especially at high concentrations. Nonetheless, EPA at 200 $\mu g\ ml^{-1}$ simultaneously increased the potential of bacterial killing but inhibited NO production by macrophages. A reasonable explanation is that macrophages may depend on multiple mediators (i.e. reactive oxygen intermediates (Cherayil & Antos 2001)), not just NO, to provide them with *Salmonella*-killing activity.

RAW 264.7 cells closely resemble the murine peritoneal macrophages in their response to inflammatory stimuli and pathogenic microbes. We expected that *Echinacea* alcohol extracts could exert a similar, although not identical, effect on PECs as they did on the macrophage-like RAW 264.7 cells. Unfortunately, a weak *in vivo* effect on inflammatory PECs was seen. The only notable change in PECs was an EP-mediated reduction in NO production at the 4 h time point after *Salmonella* engulfment. Interestingly, the reduction in

NO production did not result in a significant decrease in bacterial killing. The *in vivo* effect in this regard might be largely influenced by several factors, such as the metabolism of the phytochemicals, the activation state of PECs and the complex *in vivo* environment (e.g. constitutive expression of cytokines, growth factors and hormones (Pruett et al. 2005)). Although inflammatory agents, such as proteose peptone and thioglycollate, are commonly applied to induce an inflammatory response resulting in an influx of strongly activated macrophages into the peritoneum and thus an increased peritoneal macrophage yield, it is unclear as to how and what extent these inflammatory agents affect the functional state of macrophages (Fortier & Falk 1994). On the other hand, lack of an inhibitory effect of orally administered *Echinacea* alcohol extracts on bacterial phagocytosis and killing by PECs may indicate that *Echinacea* will not adversely affect these important innate immune functions.

To summarize, *Echinacea* alcohol extracts have a potential anti-inflammatory activity, but this effect was mainly observed *in vitro*, especially at relatively high concentrations of the extracts. Increased knowledge of the biological properties and the mode of action of the physiologically relevant concentrations of *Echinacea* alcohol extracts are necessary.

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References

Alam MS, Akaike T, Okamoto S, Kubota T, Yoshitake J, Sawa T, Miyamoto Y, Tamura F, Maeda H. 2002. Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. Infect Immun 70:3130-3142.

Babu US, Gaines DW, Lillehoj H, Raybourne RB. 2006. Differential reactive oxygen and nitrogen production and clearance of *Salmonella* serovars by chicken and mouse macrophages. Dev Comp Immunol 30:942-953.

Baeuerle PA, Baltimore D. 1996. NF-kappa B: ten years after. Cell 87:13-20.

Barnes J, Anderson LA, Gibbons S, Phillipson JD. 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt.,*Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. J Pharm Pharmacol 57:929-954.

Bodinet C, Lindequist U, Teuscher E, Freudenstein J. 2002. Effect of an orally applied herbal immunomodulator on cytokine induction and antibody response in normal and immunosuppressed mice. Phytomedicine 9:606-613.

Brune B, Gotz C, Messmer UK, Sandau K, Hirvonen MR, Lapetina EG. 1997. Superoxide formation and macrophage resistance to nitric oxide-mediated apoptosis. J Biol Chem 272:7253-7258.

Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG. 1997. *Echinacea*-induced cytokine production by human macrophages. Int J Immunopharmacol 19:371-379.

Burgner D, Rockett K, Kwiatkowski D. 1999. Nitric oxide and infectious diseases. Arch Dis Child 81:185-188.

Chang CY, Tucci M, Baker RC. 2000. Lipopolysaccharide-stimulated nitric oxide production and inhibition of cell proliferation is antagonized by ethanol in a clonal macrophage cell line. Alcohol 20:37-43.

Chen Y, Fu T, Tao T, Yang J, Chang Y, Wang M, Kim L, Qu L, Cassady J, Scalzo R, Wang X. 2005. Macrophage activating effects of new alkamides from the roots of *Echinacea* species. J Nat Prod 68:773-776.

Cherayil BJ, Antos D. 2001. Inducible nitric oxide synthase and *Salmonella* infection. Microbes Infect 3:771-776.

Chou SH, Kojic LD, Cunnick JE. 1997. Evidence for the involvement of catecholamines in the 2-DG-induced immunomodulatory effects in spleen. Brain Behav Immun 11:79-93.

Currier NL, Miller SC. 2000. Natural killer cells from aging mice treated with extracts from *Echinacea purpurea* are quantitatively and functionally rejuvenated. Exp Gerontol 35:627-639.

Dalby-Brown L, Barsett H, Landbo AK, Meyer AS, Molgaard P. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. J Agric Food Chem 53:9413-9423.

Facino RM, Carini M, Aldini G, Saibene L, Pietta P, Mauri P. 1995. Echinacoside and caffeoyl conjugates protect collagen from free radical-induced degradation: A potential use of *Echinacea* extracts in the prevention of skin photodamage. Planta Med 61:510–514.

Fortier AH, Falk LA. 1994. Isolation of murine macrophages. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strobber W, editors. Current protocols in immunology. New York: John Wiley and Sons, Inc. p 14.1.1-14.1.9.

Goel V, Chang C, Slama JV, Barton R, Bauer R, Gahler R, Basu TK. 2002. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. Int Immunopharmacol 2:381-387.

Hu C, Kitts DD. 2000. Studies on the antioxidant activity of *Echinacea* root extract. J Agric Food Chem 48:1466-1472.

MacMicking J, Xie QW, Nathan C. 1997. Nitric oxide and macrophage function**.** Annu Rev Immunol 15:323-350.

Nussler AK, Billiar TR. 1993. Inflammation, immunoregulation, and inducible nitric oxide synthase. J Leukocyte Biol 54:171-178.

Park JE, Barbul A. 2004. Understanding the role of immune regulation in wound healing. Am J Surg 187:11S-16S.

Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F. 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. J Pharm Biomed Anal 35:289-301.

Percival SS. 2000. Use of *Echinacea* in medicine. Biochem Pharmacol 60: 155-158.

Pruett SB, Fan R, Zheng Q, Schwab C. 2005. Differences in IL-10 and IL-12 production patterns and differences in the effects of acute ethanol treatment on macrophages *in vivo* and *in vitro*. Alcohol 37:1-8.

Raso GM, Pacilio M, Di Carlo G, Esposito E, Pinto L, Meli R. 2002. In-vivo and in-vitro anti-inflammatory effect of *Echinacea purpurea* and *Hypericum perforatum*. J Pharm Pharmacol 54:1379-1383.

Salmon-Her V, Ramont L, Godeau G, Birembaut P, Guenounou M, Bernard P, Maquart FX. 2000. Implication of interleukin-4 in wound healing. Lab Invest 80:1337-1343.

Sato Y, Ohshima T, Kondo T. 1999. Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing. Biochem Biophys Res Commun 265:194-199.

Sharma M, Arnason JT, Hudson JB. 2006. *Echinacea* extracts modulate the production of multiple transcription factors in uninfected cells and rhinovirus-infected cells. Phytother Res 20:1074-1079.

Speroni E, Govoni P, Guizzardi S, Renzulli C, Guerra MC. 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. J Ethnopharmacol 79:265-272.

Steinmuller C, Roesler J, Grottrup E, Franke G, Wagner H, Lohmann-Matthes ML. 1993. Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. Int J Immunopharmacol 15:605-614.

Stimpel M, Proksch A, Wagner H, Lohmann-Matthes ML. 1984. Macrophage activation and induction of macrophage cytotoxicity by purified polysaccharide fractions from the plant *Echinacea purpurea*. Infect Immun 46:845-849.

Tubaro A, Tragni E, Del Negro P, Galli CL, Della Loggia R. 1987. Anti-inflammatory activity of a polysaccharidic fraction of *Echinacea angustifolia*. J Pharm Pharmacol 39:567-569.

Woelkart K, Marth E, Suter A, Schoop R, Raggam RB, Koidl C, Kleinhappl B, Bauer R. 2006. Bioavailability and pharmacokinetics of *Echinacea purpurea* preparations and their interaction with the immune system. Int J Clin Pharmacol Ther 44:401-408.

Wu L, Bae J, Kraus G, Wurtele ES. 2004. Diacetylenic isobutylamides of *Echinacea*: synthesis and natural distribution. Phytochemistry 65:2477-2484.

Zhai Z, Liu Y, Wu L, Senchina DS, Wurtele ES, Murphy PA, Kohut ML, Cunnick JE. 2007. Enhancement of innate and adaptive immune functions by multiple *Echinacea* species. J Med Food 10: 423-434.

Table 3.1. The levels of amides and caffeic acid derivatives in *Echinacea* alcohol extracts

	In vitro study			*In vivo* study		
Phytochemicals	***E. angustifolia***	***E. pallid***	***E. purpurea***	***E. angustifolia***	***E. pallida***	***E. purpurea***
Total alkamides	**17.05**	**10.44**	**26.07**	**74.84**	**12.62**	**55.01**
amide 1	0.42	0.00	2.57	1.35	0.00	4.90
amide 2	0.26	3.72	4.72	0.84	4.50	9.11
amide 3	0.64	0.89	4.25	2.68	1.12	8.80
amide 4	0.08	0.77	2.06	0.33	1.00	4.40
amide 5	0.43	0.38	0.83	1.90	0.49	1.70
amide 7	0.00	0.23	1.11	0.00	0.34	2.49
amide 8	9.35	3.40	6.62	40.88	3.76	14.48
amide 9	1.26	0.46	3.47	5.86	0.65	7.64
amide 10	0.72	0.22	0.25	3.49	0.31	0.64
amide 11	1.77	0.38	0.20	8.28	0.46	0.83
amide 12	0.91	0.00	0.00	3.71	0.00	0.00
amide 13	0.68	0.00	0.00	2.92	0.00	0.00
amide 14	0.54	0.00	0.00	2.58	0.00	0.00
Total caffeic acid derivatives	**7.80**	**15.93**	**5.23**	**10.02**	**18.28**	**6.72**
caftaric acid	0.00	0.58	0.65	0.00	0.70	1.06
chlorogenic acid	0.90	2.13	0.00	0.95	2.22	0.00
cichoric acid	0.00	0.61	4.58	0.00	0.89	5.66
cynarin	2.96	0.00	0.00	3.02	0.00	0.00
echinacoside	3.94	12.61	0.00	6.05	14.46	0.00

* Units are μg metabolite mg^{-1} dried extract.

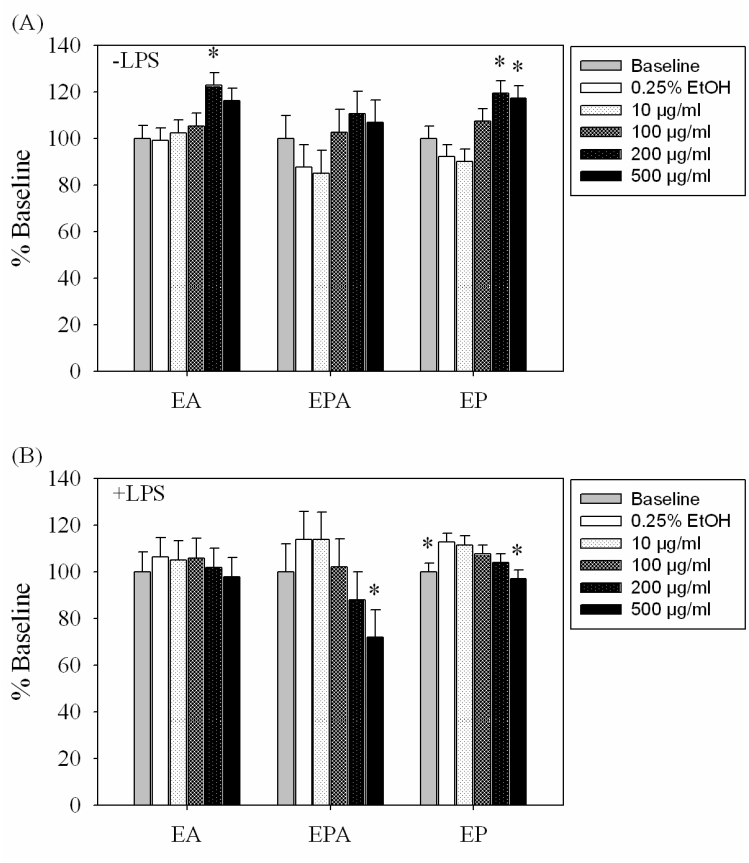

Fig. 3.1. Viability of RAW 264.7 cells exposed to *Echinacea* alcohol extracts. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. Cells were treated with the extracts in the presence (B) or absence (A) of LPS (1 µg ml^{-1}). Cell viability was determined by the MTS assay. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates. Absorbance of baseline LPS treated cells (no vehicle and no alcohol extracts) was expressed as 100%. * $p<0.05$ for the individual group vs the corresponding vehicle control.

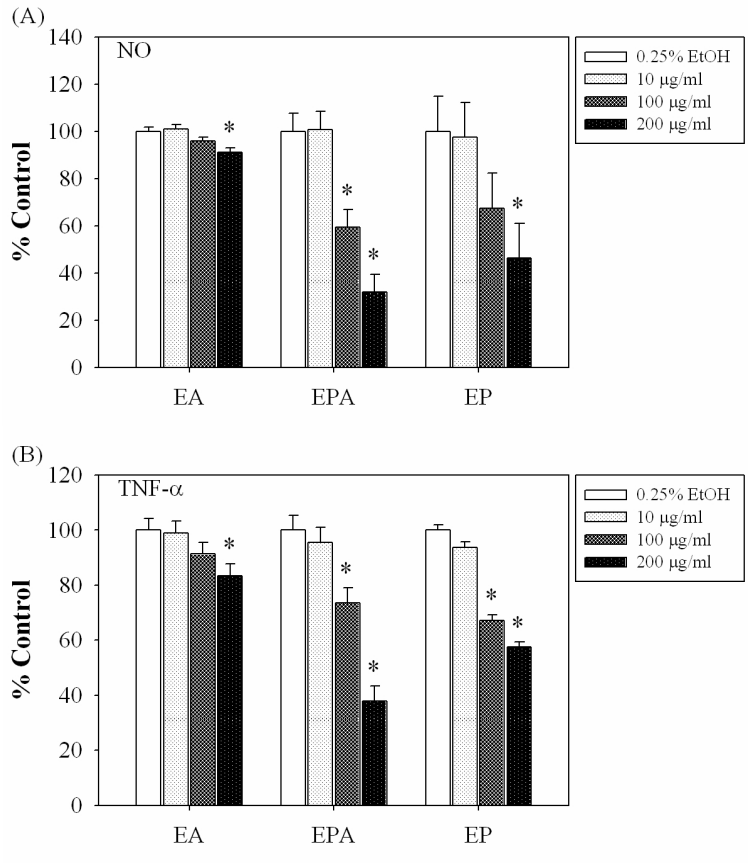

Fig. 3.2. Effect of *Echinacea* alcohol extracts on NO production and TNF-α secretion in LPS-activated RAW 264.7 cells. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. Cells were incubated in the presence of LPS (1 µg ml^{-1}) and alcohol extracts for 24 h. The culture supernates were harvested for nitrite accumulation (A) and TNF-α (B) assays. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates. Values of LPS treated cells cultured with vehicle were referred to as 100%. * $p<0.05$ for the individual group vs the corresponding vehicle control.

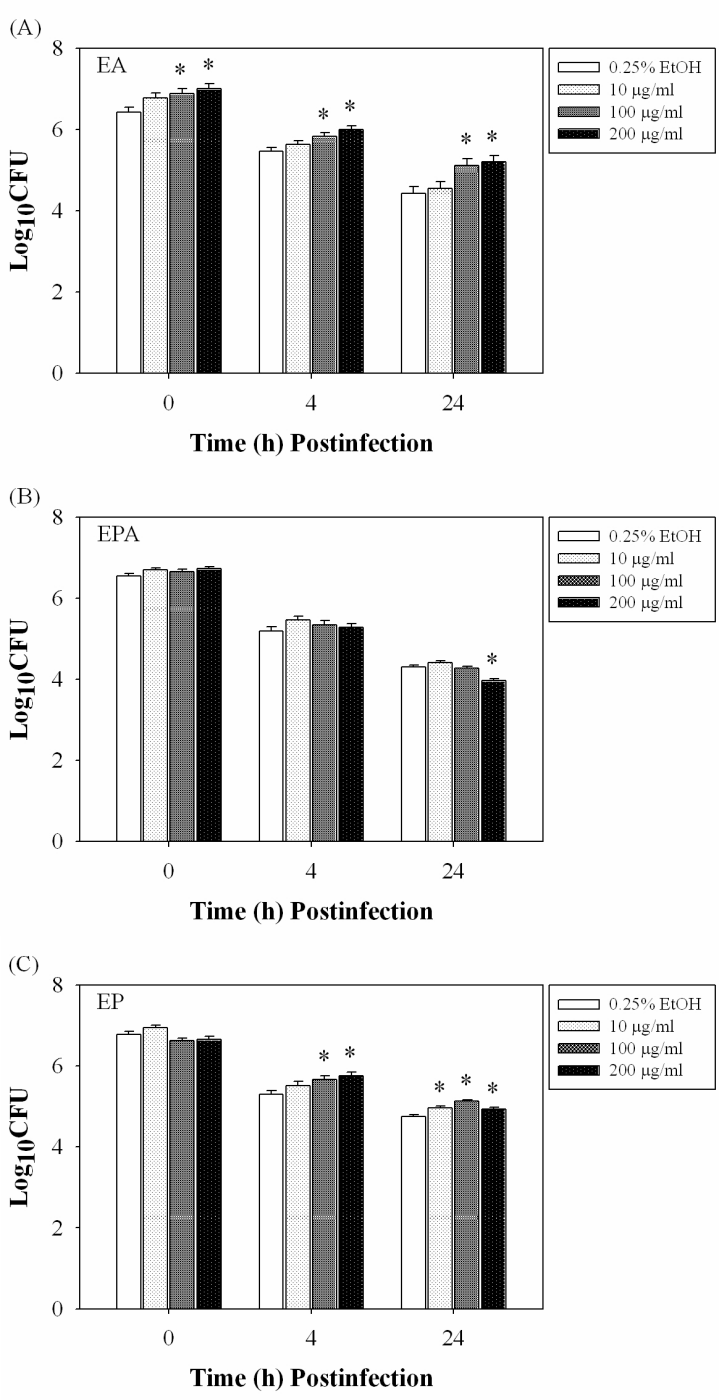

Fig. 3.3. Survival of *Salmonella* within RAW 264.7 cells pretreated with indicated concentrations of *Echinacea* alcohol extracts. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. Cells were infected at an MOI of 100:1 for 2 h. At set time points, internalized bacteria were assessed by gentamicin protection assay. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates. * $p<0.05$ for the individual group vs the vehicle control at the same time point.

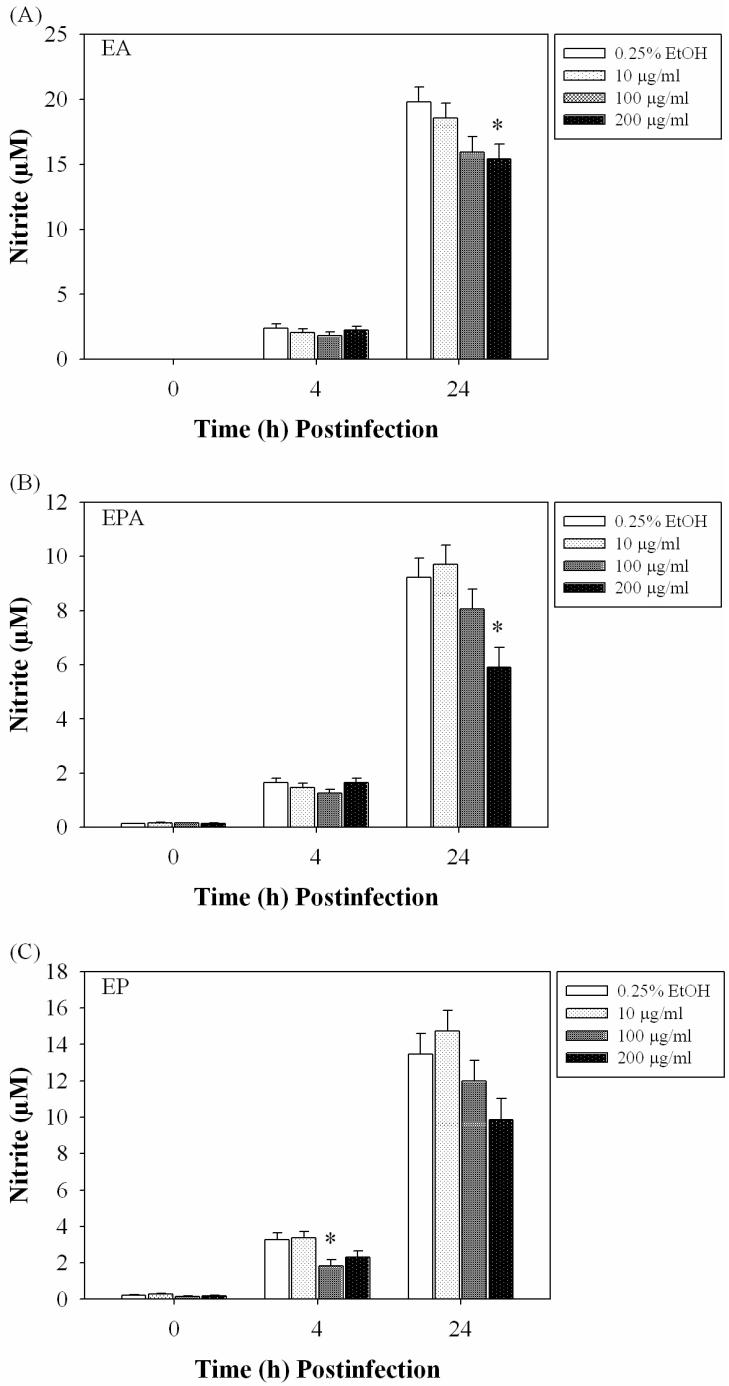

Fig. 3.4. NO production by RAW 264.7 cells infected with *Salmonella* at an MOI of 100:1 for 2h. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. Before infection, cells were treated with indicated concentrations of *Echinacea* alcohol extracts overnight. At set time points after exposure to bacteria, NO production was measured by the Griess reaction. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates.
* $p<0.05$ for the individual group vs the vehicle control at the same time point.

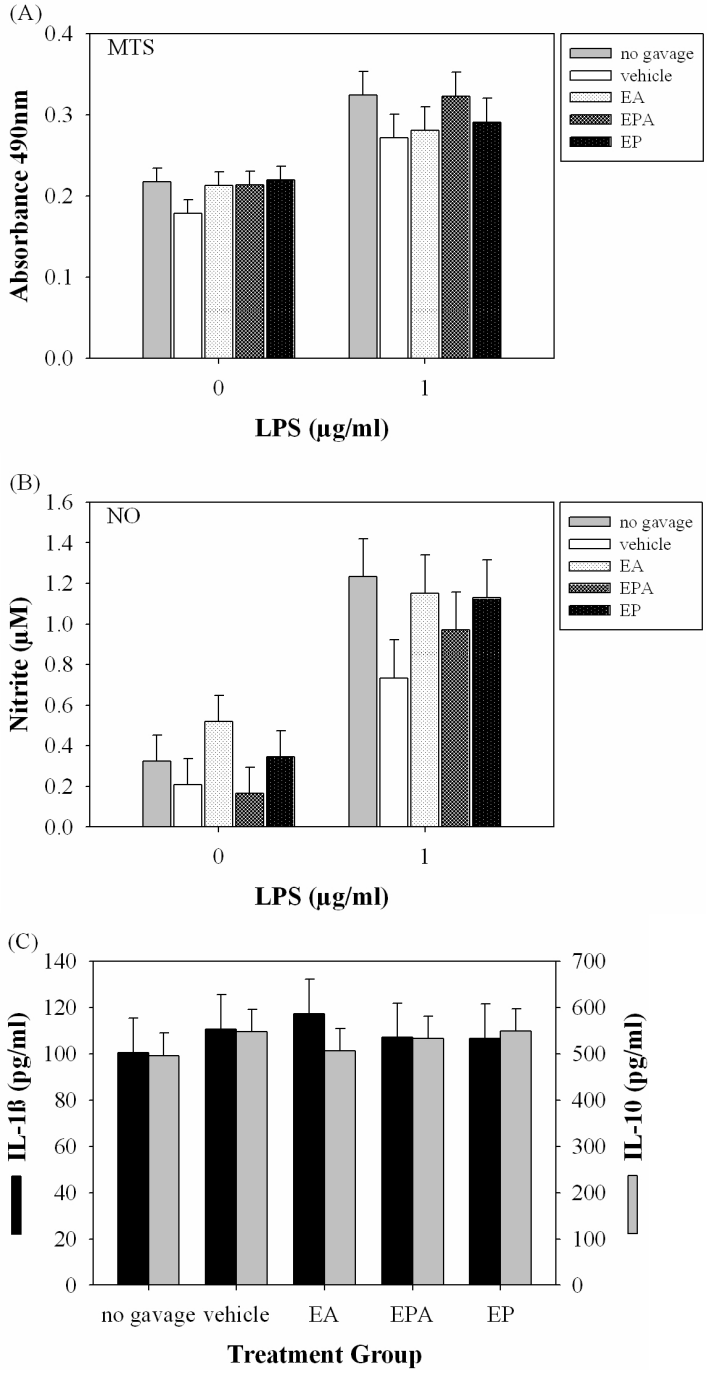

Fig. 3.5. Effect of *Echinacea* alcohol extracts *in vivo* on cell viability of PECs as well as NO and cytokine release by PECs. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. Upon completion of Echinacea administration at 130 mg kg^{-1} d^{-1}× 7d, PECs were harvested and incubated in the absence or presence of LPS (1 µg ml^{-1}) for 24 h. The culture supernates were harvested for nitrite accumulation and cytokine assays. Cells were assessed for viability by the MTS assay. Only cytokine data with LPS stimulation are shown. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates.

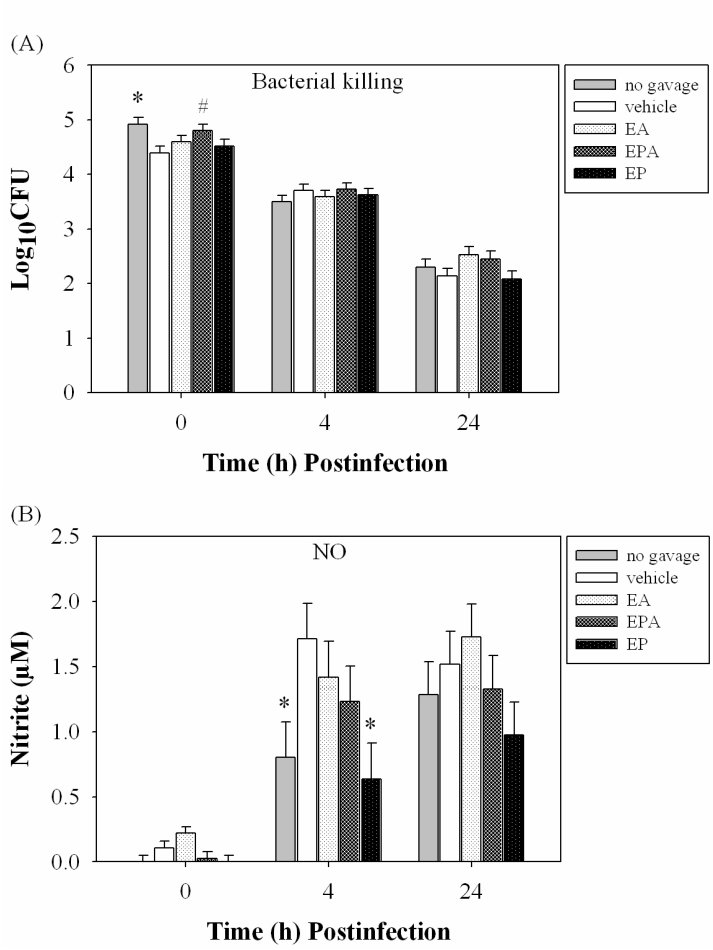

Fig. 3.6. Survival of *Salmonella* within PECs (A) and NO production by PECs (B) harvested from mice treated with *Echinacea* alcohol extracts. EA. *E. angustifolia*; EPA. *E. pallida*; EP. *E. purpurea*. PECs were infected at an MOI of 100:1 for 2 h. At set time points, internalized bacteria were assessed by gentamicin protection assay and nitrite accumulation in the culture assayed by the Griess reaction. Data are expressed as mean ± standard error of the mean of 3 independent experiments in duplicates. * $p<0.05$ and # $p<0.1$ for the individual group vs the vehicle control at the same time point.

CHAPTER 4. *ECHINACEA* INCREASES ARGINASE ACTIVITY AND HAS ANTI-INFLAMMATORY PROPERTIES IN RAW 264.7 MACROPHAGE CELLS INDICATIVE OF ALTERNATIVE MACROPHAGE ACTIVATION

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Abstract

The genus *Echinacea* is currently a popular immunomodulator. Recent reports indicated that *Echinacea* products inhibit nitric oxide (NO) production in activated macrophages. Since decreased NO production is related to the anti-inflammatory nature of the herb, we further determined the inhibitory effects of alcohol extracts and individual fractions of alcohol extracts of *Echinacea* on NO production, and explored the mechanism underlying the pharmacological activity. The results showed that alcohol extracts of three medicinal *Echinacea* species, *E. angustifolia*, *E. pallida* and *E. purpurea*, significantly inhibited NO production by lipopolysaccharide (LPS)-activated macrophage cell line RAW 264.7 cells, among them *E. pallida* was the most active. The *Echinacea*-mediated decrease in NO production was unlikely due to a direct scavenging of NO because it lacked an ability to directly inhibit NO released from an NO donor, sodium nitroprusside. An immunoblotting assay demonstrated that alcohol extract of *E. pallida* inhibited inducible nitric oxide synthase (iNOS) protein expression in LPS treated macrophages. The enzymes iNOS and arginase metabolize a common substrate, L-arginine, but produce distinct biological effects. While iNOS is involved in inflammatory response and host defense, arginase participates actively in anti-inflammatory activation.

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Arginase activity of RAW 264.7 cells stimulated with 8-bromo-cAMP was determined and found to be significantly increased by alcohol extracts of all three *Echinacea* species. Among five fractions prepared from alcohol extracts, the polar fraction containing caffeic acid derivatives was capable of enhancing arginase activity, while the lipophilic fraction containing alkamides exhibited a potential of inhibiting NO production and iNOS expression. These results suggest that the anti-inflammatory activity of *Echinacea* might be due to multiple active metabolites, which work together to switch macrophage activation from classical activation towards alternative activation.

Introduction

Botanical supplements are currently popular in the United States due to their multiple purported health benefits yet low side effects. The genus *Echinacea*, one of the top-selling botanical supplements, in fact, has been widely used for centuries in North America and later in Europe for many therapeutic purposes. There are nine known species of *Echinacea*, three of them, *E. angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP), are of similar and important medicinal values in the modulation of the immune system (Borchers et al., 2000). Three *Echinacea* species are rich in bioactive metabolites in which lipophilic alkamides, water-soluble phenolic compounds (mainly caffeic acid derivatives) and polysaccharides are the most recognized for their immunomodulatory properties (Barnes et al., 2005). Although historically polysaccharides were considered critical for stimulation of the nonspecific immune responses (Borchers et al., 2000; Percival, 2000), recent research interest focuses on two other groups of phytochemicals, namely, alkamides and caffeic acid derivatives. Caffeic acid derivatives are good antioxidants in cell-free free radical generation systems (Hu et al., 2000; Pellati et al., 2004; Dalby-Brown et al., 2005) and echinacoside, the main caffeic acid derivative in EA and EPA, has been functionally linked to anti-inflammatory and wound healing properties of *Echinacea* when applied locally (Speroni et al., 2002; Rousseau et al.,

2006). Pharmacokinetic studies of natural or synthesized caffeic acid derivatives (e.g. caftaric acid, chlorogenic acid and echinacoside) showed that they are quickly absorbed in the rat stomach, but rapidly eliminated from the blood circulation (Jia et al., 2006; Lafay et al., 2006; Vanzo etal., 2007). Matthias et al. (2004) suggested a low bioavailability for caffeic acid derivatives of *Echinacea* based on the observations that they permeate poorly through the Caco-2 monolayers, a model for the intestinal epithelial barrier. These data indicate that there might be a limited pharmacological role for caffeic acid derivatives when consumed orally. In this regard, lipophilc alkamides might play a more important role as they are considered to be orally bioavailable (Matthias et al., 2005; Woelkart et al., 2005).

There is evidence to substantiate that *Echinacea*-derived alkamides have immunomodulatory and anti-inflammatory activity (for review see Woelkart et al., 2007). The alkamides inhibit production of proinflammatory tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) in activated murine macrophage cell line RAW 264.7 cells (Chen et al., 2005; Matthias et al., 2007). Similarly, alkamide-containing alcohol extracts had similar inhibitory activities with low cytotoxicity *in vitro* (Zhai et al., 2007a). When alcohol extracts of *Echinacea* were administered orally to mice, a significantly decreased production of TNF-α and interleukin (IL)-1β by activated splenocytes was seen (Zhai et al., 2007b). Although *Echinacea* extracts containing alkamides exhibit suppressive effects on the tested endpoints of inflammatory response, the underlying cellular mechanism is unclear.

TNF-α and NO are two important components of the inflammatory response produced by macrophages, and their production involves the NF-κB signal pathway (Yamamoto and Gaynor, 2001). When macrophages are in a resting state, the transcription factor NF-κB is stabilized in the cytosol by binding to its inhibitory protein IκB-α. Upon induction by certain inflammatory stimuli, e.g., bacterial lipopolysaccharide (LPS), IκB-α is rapidly phosphorylated and degraded by the 26S proteosome (Katsuyama et al., 2001). As a result, free NF-κB translocates into the nucleus where it regulates the expression of a wide variety

of genes involved in inflammatory responses, e.g., the cytokines TNF-α and IL-1β, and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (McKay and Cidlowski, 1999). The enzyme iNOS catalyzes the conversion of amino acid L-arginine to NO, which is cytotoxic and cytostatic (Meurs et al., 2003). Although NO as an active free radical and inflammatory mediator is part of the host defense, excessive production of NO and its reactive nitrogen intermediates is involved in the pathogenesis of many chronic diseases, such as inflammatory bowel disease, arthritis, atherosclerosis and tumors (Chan et al., 2000; Yamamoto and Gaynor, 2001). Selective inhibition of the iNOS pathway is an important strategy for control of many chronic inflammatory diseases (Chan et al., 2000).

L-arginine metabolism can be redirected toward the synthesis of L-ornithine catalyzed by the enzyme arginase. The subsequent products from L-ornithine, such as polyamines and L-proline, are involved in cell growth, collagen synthesis and wound healing (Meurs et al., 2003). Thus, arginase and iNOS are the two enzymes that catalyze a common substrate but produce divergent biological effects. They are generally recognized to be functionally competitive because the stimulation of one enzyme will negatively regulate the other one due to the limited availability of the same substrate (Wang et al., 1995; Gotoh and Mori, 1999). These two cross-regulated metabolic processes may reflect opposite functional states of macrophages. It is well known that macrophages can be either classically activated or alternatively activated, and are therefore termed as inflammatory or anti-inflammatory cells (Munder et al., 1998; Porcheray et al., 2005). A given macrophage may switch from one activated state to another upon a specific signal (Porcheray et al., 2005). Inflammatory mediators and cytokines induce classical activation involving iNOS expression and, on the contrary, anti-inflammatory cytokines induce alternative activation with upregulated arginase activity (Modolell et al., 1995; Munder et al., 1998; Yamamoto et al., 1998). Animal studies showed that alcohol extracts of *Echinacea* increase production of IL-4 and IL-10, but decrease production of TNF-α and IL-1β in activated spleen cells (Zhai et al., 2007b),

suggesting that *Echinacea* may modulate macrophage function to be anti-inflammatory via a decreased proinflammatory/anti-inflammatory cytokine ratio. This finding led us to hypothesize that *Echinacea* can perturb the iNOS/arginase balance in macrophages in favor of increasing arginase activity for alternative anti-inflammatory activation.

In this study, we examined the effects of *Echinacea* extracts and their fractions on the production of NO in activated macrophage cell line as well as the cellular mode of action underlying the biological activity. We found that alcohol extracts of *Echinacea* had opposite effects on NO production and arginase activity, and these differential effects were induced by different fractions (or chemical constituents), which could work coordinately to drive macrophages to alternative activation.

Materials and Methods

Preparation of alcohol extracts

Dried powdered roots of EA, EPA and EP harvested in 2003 were provided by Iowa State University Botanical Supplements Research Center; and by the USDA North Central Regional Plant Introduction Station in Ames, Iowa, with accession numbers PI 631285, PI 631293, and PI 631307, respectively. Alcohol extracts from the root powders of these plants were prepared as previously described (Zhai et al., 2007b) with 95% aqueous ethanol using Soxhlet apparatus for at least 6 h for exhaustive extraction. The extracts were evaporated to dryness using a rotary evaporator set at 30ºC. The residues were redissolved in a 50/50 mixture of ethanol and sterile phosphate buffered saline (PBS) and then diluted to 20 mg/ml in PBS containing 5% ethanol. Aliquots of the dilutions were stored at -80°C for up to 2.5 years during which no obvious herbal efficacy was reduced.

Fractionation of alcohol extracts

For fractionation, the dried powdered roots of the plants harvested in 2005 (EA) or 2006 (EPA and EP) were used to prepare new alcohol extracts using the Soxhlet extraction. The dry

residues were redissolved in 75% ethanol to obtain ≤ 0.6 g/ml. Five milliliters were injected into a semi-preparative high performance liquid chromatography (HPLC). The starting conditions of the semi-preparative HPLC was 90% Pump A, which was 0.1% acetic acid, and 10% Pump B, which was acetonitrile at a flow rate of 3 ml/min. Eluent collected from time 0 to 31 when Pump B increased from 10% - 30% was fraction 1; eluent collected from time 33 to 42 when Pump B increased from 30% - 40% was fraction 2; eluent collected from time 43-83 when Pump B increased from 40% to 80% was fraction 3; eluent collected from time 84-94 when Pump B increased from 80% to 100% was fraction 4; and eluent collected from time 95-115 when Pump B was running at 100% was fraction 5. The gap of 1-2 minutes between fractions indicated the time of stopping the flow of the mobile phase for the change of the collection flasks. The fractions were then evaporated to remove the mobile phase and lyophilized when needed. After redissolution and dilution to 4 mg/ml in PBS containing 25% ethanol, aliquots were kept at -80°C and used within one year.

Phytochemical analysis

The phytochemical analysis was performed to detect bioactive metabolites of alkamides and caffeic acid derivatives in the crude alcohol extracts with the use of HPLC as described previously (Wu et al., 2004). We did not identify ketones as no internal standards for ketones were used. However, in these alcohol extracts ketones are expected to be present as lipophilic compounds and usually concomitantly appear in alkamide fractions of EPA.

Fractions were identified as described by Liu and Murphy (2007). All of the following parts are from Beckman Coulter (Fullerton, CA, USA): System Gold 126 Pump module; System Gold 508 autosampler; System Gold 168 detector (photodiode array); and 32 Karat Software version 5.0. The columns used were YMC-PACK ODS-AM 250 mm length × 4.6 mm I.D. s-5 μm, 12 nm, reversed-phase C_{18} columns.

The mobile phase used for the analytical HPLC method were, for Pump A, 0.1% acetic acid; while for Pump B, acetonitrile. The flow rate was 1 ml/min. The injection volume was 10

µl. UV scan was collected in the range of 190-600 nm. Alkamides were viewed at 260 nm, while caffeic acid derivatives were viewed at 330 nm.

Peak identification was based on relative retention time, similarity of UV spectra, internal standards, spiking and chromatogram fingerprinting (Wu et al., 2004; Liu and Murphy, 2007). Individual standards were run to check for relative retention times and UV spectra. EP sample with identified peaks was also run to obtain the finger print of the chromatogram, the relative retention times of the alkamides and caffeic acid derivatives, and to obtain the UV spectra of identified compounds.

Determination of endotoxin in herbal preparations

All glassware used in the extraction and fractionation procedure was baked at 185°C overnight prior to use in order to minimize endotoxin contamination. Endotoxin-free Nanopure water was used for preparation and dilution of crude alcohol extracts and fractions. The endotoxin levels were evaluated in aliquots of the herbal preparations using the Limulus Amebocyte Lysate Test (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) according to the manufacturer's specifications for a microplate assay, and were found to be below the limit of detection (< 0.1 EU/ml).

Macrophage cell line

The murine peritoneal macrophage cell line RAW 264.7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and grown in RPMI 1640 medium (GIBCO, Invitrogen Corporation, Grand island, NY, USA) supplemented with 2 mM glutamine, 25 mM Hepes, 50 µg/ml gentamicin and 10% heated-inactivated iron-fortified bovine calf serum (JRH Biosciences, Lenexa, KS, USA) at 37°C in a 7% CO_2 incubator. Cells between passage 5 and 20 (Kiemer et al., 2002) were used in this study.

NO and TNF-α assays

RAW 264.7 cells were seeded in flat bottom 96-well tissue culture plates (Corning Inc., Corning, NY, USA) at a density of 8×10^4 cells per well in the presence or absence of various concentrations of *Echinacea* preparations (as indicated in each graph) and /or LPS (*E. coli* 055:B5; Sigma, St. Louis, MO, USA). Final concentration of ethanol used as a solvent of lipophilic metabolites in each well was 0.25% (v/v). Ethanol (0.25%) control wells were tested in parallel. After 23 h incubation, culture supernates were collected for NO and TNF-α assays, and the attached cells were evaluated for cell viability (see below).

In a second set of experiments, *Echinacea* extracts (100 μg/ml) were either added simultaneously with LPS (1 μg/ml) or up to 6 h after LPS addition (Kiemer et al., 2002). Culture supernates from cells activated with LPS for 22 h were collected for NO assay.

Nitrite, a stable NO metabolite, was determined by the method of Griess reaction (Park et al., 2005). A trace amount of nitrite present in cell-free culture medium was subtracted from each value obtained with cells. Sodium nitrite (0.39-100 μM) was used as a standard. It was confirmed that *Echinacea* extracts at 200 μg/ml did not interact with nitrite and interfere with its detection (data not shown).

TNF-α was assayed by BD OptEIA ELISA set (BD Biosciences, San Diego, CA, USA). The cytokine levels were calculated by using a purified recombinant mouse cytokine as a standard.

Scavenging of NO production from NO donor

Sodium nitroprusside (SNP), an NO donor, in aqueous solution at physiological pH spontaneously liberates NO, which rapidly interacts with oxygen to produce nitrite (Sreejayan et al., 1997; Chan et al., 2000; Kiemer et al., 2002). In order to determine if *Echinacea* metabolites directly interact with NO, SNP (final concentration 2.5 mM; Sigma) was mixed with the Griess reagent (a mixture of equal volumes of 10% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylenediamine) in the presence of 0.25%

ethanol or various concentrations of alcohol extracts, and then incubated at room temperature for a set of time points. NO released from SNP in this reaction system was immediately captured by Griess reagent via nitrite, which was sequentially monitored on a plate reader (Bio-Tek Instruments, Winooski, VT, USA). A series of sodium nitrite standard concentrations instead of SNP was set up and measured at the same time. The reading of the standard curve remained stable throughout the observational period.

Determination of arginase activity

Arginase activity was determined according to a microplate method (Corraliza et al., 1994; Munder et al., 1998) with slight changes. Cells were seeded at 4×10^5 cells/ml in 96-well tissue culture plates in the presence or absence of LPS (1 µg/ml), 8-bromo-cAMP (0.25 mM; Sigma) and/or *Echinacea* preparations. After 23-24 h incubation, supernates were collected for NO assay, the cells rinsed with PBS, and then lysed with a CelLytic M lysis solution (Sigma). Following centrifugation at 2500 g for 5 min at 4°C, 50 µl of the lysate was mixed with 40 µl of 25 mM Tris-HCl (pH 7.4) and 10 µl of 10 mM $MnCl_2$. The arginase was activated by heating for 10 min at 56°C. Aginine hydrolysis to urea was conducted by addition of 100 µl of 0.5 M L-arginine (Sigma), pH 9.7, with incubation at 37°C for 60 min. The reaction was stopped with 800 µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The urea concentration was measured at 550 nm after addition of 40 µl of 9% (w/v) α-isonitrosopropiophenone (Sigma) dissolved in 100% ethanol and heating at 95°C for 90 min. A standard curve was performed with 2-fold dilutions of urea (0.03-4 mM) followed by mixing with the stop reagent and heating. One unit of arginase activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol urea per min at 37°C. Protein concentrations in cell lysate were determined using a micro BCA (Bicinchoninic Acid) protein assay (Pierce Labs, Rockford, IL, USA).

Western blotting analysis

Cells were seeded in 24-well plates at a density of 5×10^5 cells per well in the presence or absence of LPS (1 µg/ml) and various *Echinacea* preparations. After 24 h incubation, cells were rinsed with ice-cold PBS and then lysed with the CelLytic M lysis solution containing 1% (v/v) protease inhibitor cocktail (Sigma). Cell debris was removed by microcentrifugation (12,000 g×15 min), and the resultant supernates were kept at -80°C till use. The thawed cell lysates were mixed with 4× NuPAGE SDS sample loading buffer (Invitrogen). Equal amounts of cellular protein (25 µg/lane) were separated on 10% Tris-HCl ready gel (Bio-Rad Laboratories, Hercules, CA, USA), followed by electrotransfer onto a PVDF plus membrane. The immunoblot was performed by incubation with a 1:500 dilution of rabbit polyclonal anti-iNOS (sc-8310, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 5% non-fat milk in PBS/0.1% Tween-20 (PBST) overnight at 4°C. Blots were rinsed with PBST, followed by incubation with a 1:2500 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were washed again, and the bands were visualized using an amplified Opti-4CN Substrate kit (Bio-Rad Laboratories). The bands were photographed and quantified with Kodak Image Station 440 CF and Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY, USA). To assess the changes of iNOS expression and to normalize protein loading, membranes run in parallel were incubated with a 1:5000 dilution of mouse monoclonal antibody against β-actin (Santa Cruz Biotechnology Inc.).

Cell viability assay

Mitochondrial reduction of MTS, a tetrazolium compound, to a colored formazan was used as an indicator of cell viability (Cory et al., 1991). Briefly, after removing of 100 µl of culture supernates (i.e. for NO and TNF-α assays), 15 µl of MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) was added to the remaining 100 µl of culture medium incubation continued for 1 h. The extent of formazan

formation was determined photometrically at absorbance 490 nm on a plate reader. The absorbance of cell-free culture medium with or without vehicle or *Echinacea* preparations was used as a blank (self-control) and subtracted from the value of the corresponding treatment groups.

Statistical analysis

Statistix software (version 8.0, Analytical Software, Tallahasee, FL, USA) was used for the statistical analysis. Differences between the vehicle and other groups were tested by two-way analysis of variance (group × experiment). A value $P<0.05$ was considered significant.

Results

Phytochemical analysis

Table 4.1 lists the amounts of total alkamides and several important caffeic acid derivatives in crude alcohol extracts of three *Echinacea* species.

The chemical constituents in the fractions are shown in Table 4.2. Fraction 1 was rich in polar caffeic acid derivatives. Fraction 3 contained lipophilic constituents including alkamides (in all three species) and ketones (in EPA). Other hydrophobic compounds were found in fractions 4 and 5. For fractions 3-5, the later fractions contained compounds which are more hydrophobic than the earlier ones.

Inhibition of TNF-α production by alcohol extracts

As TNF-α production was regulated by *Echinacea in vivo* (Zhai et al., 2007b), we measured this cytokine in *Echinacea*-treated RAW 264.7 cells. In the absence of LPS, very low amounts (or lower than limit of detection, 15 pg/ml) of TNF-α were measured in the supernates from RAW 264.7 cells incubated with or without alcohol extracts of *Echinacea* overnight. In the presence of LPS, the cells secrete high levels of TNF-α. Vehicle (0.25% ethanol) alone had no effect on the cytokine production, however, all three alcohol extracts at a concentration of

100 µg/ml significantly inhibited TNF-α production (*P* values <0.001, Fig 4.1A). The inhibitory effects of *Echinacea* extracts in the presence of 0.1 µg/ml LPS were dose-dependent as indicated by a significant linear effect of dose (*P* values < 0.05) (Fig 4.1B). Alcohol extracts of *Echinacea* at the test concentration range of 10-200 µg/ml had little effect on cell viability based on the MTS assay (Fig 4.1B), indicating that the inhibitory effect of *Echinacea* extracts on TNF-α is unlikely due to cytotoxicity.

Inhibition of NO production by alcohol extracts

In the absence of LPS, less than 0.5 µM of NO was detectable in culture medium of RAW 264.7 cells incubated *in vitro* for 23 h. These low levels of NO are the product of constitutive NOS rather than iNOS. In the presence of increasing concentrations of LPS, cells produce NO, which reached a maximum with LPS at 1 µg/ml (Fig 4.2A). Exposure to vehicle had no effect on NO production, however, the three *Echinacea* extracts at 100 µg/ml reduced NO production. The inhibitory effect of *Echinacea* extracts on NO production was dose-dependent as indicated by a significant linear effect of dose (*P* values < 0.008) (Fig 4.2B). At 100 µg/ml, the inhibitory potential of three species extracts in the presence of 1 µg/ml LPS is generally EPA > EP > EA. All three extracts at tested concentrations had no effect on cell viability (Fig 4.2B).

Direct scavenging of NO generated by alcohol extracts

NO production was estimated by measuring nitrite using the Griess reaction system. NO derived from SNP increased in a linear time-dependent manner (Fig 4.3A). Vehicle alone had no any effect on the NO production, but alcohol extracts of EA and EPA at a final concentration of 50 µg/ml significantly inhibited nitrite accumulation beginning 120 min after initiating of the reaction (Fig 4.3A) (*P* values <0.018 at 120-min time points). However, the inhibitory rates of NO production by these two extracts throughout the 3-h observation period were small (<15%), and were not concentration-dependent (Fig 4.3B). EP extracts at lower

concentrations of 5-25 µg/ml, but not at higher concentrations (50-200 µg/ml), significantly reduced nitrite accumulation (*P* values <0.015).

Inhibition of iNOS induction by alcohol extracts

Effects of alcohol extracts on iNOS induction were measured by an indirect method described by Kiemer et al. (2002). Once macrophages are activated, iNOS gene transcription is initiated about 1 h after activation, whereas its protein expression occurs some 3-4 h later. If the *Echinacea*-mediated decrease in NO production is through interference with the transcriptional induction process, earlier treatment (i.e. 0.5 h after LPS stimulation) with *Echinacea* extracts should have a more profound effect on NO production than adding the extracts later. Therefore, in these experiments, alcohol extracts (100 µg/ml) were either added simultaneously with LPS (1 µg/ml) or up to 6 h after addition of LPS. The results showed that after LPS activation, the earlier addition of alcohol extracts resulted in more inhibition of NO production (Fig 4.4), indicating that *Echinacea* inhibits the transcriptional induction of iNOS. Of three alcohol extracts, EPA extract was the most active, in agreement with its strongest inhibitory potential of TNF-α and NO production.

Opposite regulation of NO production and arginase activity by alcohol extracts

Raw 264.7 cells were incubated with alcohol extracts in the presence or absence of LPS to stimulate iNOS or 8-bromo-cAMP to induce arginase activity (Morris et al., 1998). The induction of arginase activity by 8-bromo-cAMP was further enhanced by the addition of LPS, which itself is a weak stimulant of arginase. In the presence of LPS alone, alcohol extracts of EP and EPA at 100 µg/ml significantly inhibited NO production (*P* values <0.05) (Fig 4.5A), but their influence on arginase activity did not reach statistical significance. Note that 8-bromo-cAMP alone did not induce NO production, but decreased the NO production when added with LPS to RAW 264.7 cells. Moreover, all three alcohol extracts induced additional suppression of NO production in conjunction with 8-bromo-cAMP. In the presence of 8-bromo-cAMP, the

three *Echinacea* extracts at 100 μg/ml significantly increased arginase activity up to 2-fold (*P* values <0.001) (Fig 4.5B). The enhancing effect of *Echinacea* extracts on arginase activity was also seen when both 8-bromo-cAMP and LPS were present in the culture medium (Fig 4.5B). When the concentrations of *Echinacea* extracts were reduced to 10 μg/ml, an enhancing effect was still seen when cells re exposed to *Echinacea* extracts (Fig 4.5C). The test dose range (10-200 μg/ml) of *Echinacea* extracts had no effect on cell viability (Fig 4.5C).

Differential regulation of NO production and arginase activity by fractions

In order to understand if the same fractions that inhibited NO production are responsible for increased arginase activity, five fractions of alcohol extracts were prepared (Table 4.2). Generally, the HPLC fractions of the three *Echinacea* extracts exhibited very similar patterns of effects on NO production and arginase actvity. Note that the opposite effects of *Echinacea* extracts on NO production and arginase activity resulted from different fractions. For all three species of *Echinacea*, fraction 3 containing abundant alkamides had robust inhibition of NO production. For EA, NO was also suppressed by fraction 4 with greater suppression by fraction 5, and for EP, there is some NO suppression by all fractions with the greatest suppression by fractions 2, 3, and 5 (Fig 4.6A). On the other hand, the polar fractions (fractions 1) induced increased arginase activity (Fig 4.6B). However for EPA, the greatest enhancement of arginase activity was seen with fraction 4 which contains unidentified hydrophobic compounds. The HPLC fractions of alcohol extracts showed no cytotoxicity as indicated by the MTS assay (data not shown).

Inhibition of iNOS protein expression

Decreased NO production might be relative to decreased iNOS protein expression as indirectly indicated in Fig 4.4. To that end, we carried out Western blot analysis of lysates of cells exposed to alcohol extracts of three *Echinacea* species at 100 μg/ml or their fractions at 25 μg/ml for 24 h in the presence of LPS. For EA, iNOS expression was significantly inhibited

by fractions 3, 4 and 5 (P values <0.05). For EPA, the unfractionated alcohol extract and its fraction 3 had a robust inhibiting activity (P values <0.05). The unfractionated alcohol extract of EP and its fractions all showed, to some extent, an inhibiting effect, but they did not reach statistical significance except for fraction 5 (P = 0.046).

Discussion

NO and TNF-α are two key mediators in host defence and inflammatory response. They positively regulate each other (Yamamoto and Gaynor, 2001; Wu et al., 2003) and therefore amplify inflammatory signals. However, improper upregulation of these inflammatory players are implicated in a pathological role in inflammatory processes (Yamamoto and Gaynor, 2001; Kiemer et al., 2002). Our results demonstrated the suppressive effects of *Echinacea* extracts and certain individual fractions on production of NO and TNF-α by an activated macrophage cell line, in accordance with previous *in vitro* observations (Chen et al., 2005; Matthias et al., 2007; Zhai et al., 2007a). In order to understand the cellular mechanism as well as the biological implication underlying the herbal anti-inflammatory activity, we carried out additional experiments. We focused our interest on the mechanistic basis for decreased NO production because NO is an important effector molecule in the classical activation of macrophages (MacMicking et al., 1997; Munder et al., 1998).

Macrophages metabolize arginine to produce either NO through iNOS or ornithine through arginase. These two metabolic processes have important yet divergent roles in the cellular function. NO functions primarily with cytotoxic and cytostatic activity while the products of arginase pathway promote cell proliferation (Meurs et al., 2003). The predominance of one pathway over the other may rest on the presence of desirable inducers in the microenvironment. Macrophages express two arginase isoforms with distinct subcellular localizations: cytosolic type I and mitochondrial type II (Louis et al., 1999). LPS co-induces iNOS and arginase II, but the arginase II expression lags behind iNOS expression

(Wang et al., 1995; Morris et al., 1998; Gotoh and Mori, 1999). The delayed expression of arginase II helps control NO production, and prevent NO-induced cell apoptosis (Munder et al., 1998; Gotoh and Mori, 1999). We hypothesized that *Echinacea*-mediated decrease in NO production insinuates an increased activity of arginase in activated macrophages. As expected, *Echinacea* extracts oppositely regulate NO production and arginase activity even though the determinations of these two parameters were performed with different inducers (LPS or 8-bromo-cAMP). This differential regulation of two cross-regulated metabolic processes by *Echinacea* species may have important implications in the anti-inflammatory activity.

We predicted that one fraction responsible for decreased NO production would also be a strong stimulant of arginase. Surprisingly, this speculation was not supported by our observations that the opposite regulatory effects on NO production and arginase activity were due to different fractions. Lipophilic fraction 3 was found to be the most effective in suppressing NO production while the polar fraction containing caffeic acid derivatives was an inducer of arginase activity, though the polar metabolites from EPA only showed an increasing trend in arginase activity. It is not surprising that the lipophilic fractions mediated a decrease in NO production, since an anti-inflammatory effect of alkamides has been reported previously (Chen et al., 2005; Matthias et al., 2007). Rather, it is noteworthy that caffeic acid derivatives (fraction 1) from EA and EPA have no effects on NO production considering that they are well demonstrated antioxidants (Hu et al., 2000; Pellati et al., 2004; Dalby-Brown et al., 2005). Some natural antioxidants (e.g., flavonoids, resveratrol and curcumin) have been found to have strong inhibitory effects on iNOS expression and NO production in activated macrophages (Surh et al., 2001; Yamamoto and Gaynor, 2001).

Although caffeic acid derivatives have been determined to penetrate poorly across Caco-2 monolayers (Matthias et al., 2004), we suggest that *in vitro* they enter into cells to accumulate high enough concentrations and consequently affect arginase activity. Cyclic

AMP-stimulated arginase expression is through a complicated signal pathway involving intracellular activation of protein kinase A, tyrosine kinase phosphorylation, and p38 mitogen-activated protein kinase activation (Chang et al., 2000), and it has been proposed that Th2 cytokine-induced increase in arginase activity is cAMP-dependent (Corraliza et al., 1997; Wei et al., 2000). Although we can not answer based on the present data if caffeic acid derivatives directly mediate the activation of protein kinase A pathway, it is possible that *in vivo* they affect the signal pathway by upregulating Th2 cytokines, such as IL-4 and IL-10. *Echinacea*-mediated increase in Th2 cytokines after oral administration has been reported by us previously (Zhai et al., 2007b).

The lipophilic fraction of EPA includes two classes of phytochemicals, alkamides and ketones. While alkamides are widely investigated, ketones are almost completely neglected. Ketones are, in fact, the main lipophilic constituents in EPA roots, and one of ketones, ketone 23, is recognized as the diagnostic marker for hydrophobic metabolites (Kraus et al., 2005). The biological activity of ketones from EPA is little known. It has been suggested that ketones have antifungal activity (Binns et al., 2000). Obviously, like other bioactive metabolites of *Echinacea* (Awang, 1999), the antifungal activity of ketones might be through modulation of immune cell function rather than through a direct fungal killing. For lipophilic fraction 3 of EPA, it is possible that in addition to alkamides, ketones play in part role in the regulation of NO production.

In an aerobic environment, NO reacts rapidly with oxygen to form nitrogen dioxide, a gas capable of inducing cell damage (Meurs et al., 2003). Since it has been suggested that the polar caffeic acid derivatives of *Echinacea* possess the antioxidant and radical scavenging nature (Hu et al., 2000; Pellati et al., 2004; Dalby-Brown et al., 2005), which could be enhanced by lipophilic alkamides (Dalby-Brown et al., 2005), we investigated whether *Echinacea* extracts (a mixture of caffeic acid derivatives and alkamides) could scavenge or interact with NO and nitrogen dioxide species directly. Addition of high concentration of alcohol extracts (200

μg/ml) to a series of standard solutions of sodium nitrite (0.39-100 μM) did not interfere with the nitrite detection by Griess reaction, indicating that *Echinacea* does not interact with nitrite ions or nitrogen dioxide species directly. Moreover, we demonstrated that *Echinacea* extracts are a weak scavenger of NO because they could not strongly inhibit nitrite accumulation by competing with oxygen to react with NO generated from SNP. Additional studies showed that *Echinacea* extracts could not effectively protect RAW 264.7 cells from SNP-induced cell damage or cytotoxicity via the release of high levels of NO as determined by tritiated thymidine incorporation (data not shown). Based on these collected data, we could conclude that *Echinacea*-mediated decrease in NO production by macrophages is mainly via the modulation of the production yield of NO, but not direct scavenging of NO.

Our results demonstrated that *Echinacea* present at the onset of LPS-induced iNOS expression resulted in a stronger inhibitory effect on NO production, suggesting that *Echinacea* could block the LPS-initiated upstream signaling pathway. Sharma et al. (2006) recently screened the activation of transcription factors in rhinovirus-infected human bronchial epithelial cell line with a protein/DNA array analysis, and found an alcohol tincture of EP roots significantly inhibited the nuclear expression of multiple pro-inflammatory transcription factors including NF-κB and STATs. It has also been demonstrated that in stimulated macrophages, certain individual alkamides could inhibit NF-κB activity (Matthias et al., 2007). Down-regulation of NF-κB expression will lead to decreased iNOS expression and NO production. Our results suggested that the *Echinacea*-mediated decrease in NO production was generally associated with decreased protein expression of iNOS as indicated by Western blotting assay. The control protein β-actin was not affected under the same conditions suggesting that *Echinacea*-mediated inhibition of iNOS expression did not result simply from a broad decrease in protein expression. Note that several *Echinacea* preparations showed relatively weak inhibition of iNOS protein expression when compared with their effects on NO production, this may suggest other mechanisms in the context of decreased

NO production, i.e. a direct inhibition of iNOS enzyme activity. In fact, NO production correlates with iNOS activity and is commonly used as an indirect indicator of iNOS activity (which can be directly assayed by measuring the production of L-citrulline from L-arginine) (Wang et al., 1995; Munder et al., 1998). In addition, decreased NO production might be in part due to a switch of macrophage function to alternative activation, leading to less availability of the substrate for the iNOS pathway.

Like *Echinacea*, some other natural occurring chemicals (e.g., flavonoids, resveratrol, and curcumin) have both immunomodulatory and anti-inflammatory activity including inhibition of NO production and iNOS enzyme expression (Surh et al., 2001; Yamamoto and Gaynor, 2001). Although we can not extrapolate the action mechanism of *Echinacea* based on these different structural natural metabolites, there may be a certain common nature that determines a similar biological activity. It has been suggested that NF-κB activation could be regulated by the intracellular redox state (van den Berg et al., 2001). There is evidence to support that the above mentioned natural chemicals, flavonoids, resveratrol and curcumin, strongly suppress NF-κB activation by way of blocking upstream signaling, i.e. inhibition of phosphorylation of IκB-α (van den Berg et al., 2001; Surh et al., 2001; Lin, 2007). It is likely that *Echinacea*-derived caffeic acid derivatives as potential antioxidants could regulate the NF-κB activation. However, direct evidence is needed to support this speculation because alkamide fractions, but not caffeic acid fractions exerted robust inhibition of NO production by macrophage cell line.

Taken together, *Echinacea* affects macrophage immune function at multiple levels, resulting in inhibition of NO production and increased arginase activity. Observations on fractions of alcohol extracts of *Echinacea* indicate that *Echinacea*-mediated decrease in NO production is due to bioactive alkamides. Such *in vitro* effects may be directly applicable to *in vivo* administration as alkamides are bioavailable. Moreover, the anti-inflammatory activity of *Echinacea* might be dependent on a synergistic action of both alkamides and

caffeic acid derivatives, which act together to drive macrophages to alternative activation. These noteworthy findings will help define the mechanisms behind the success of traditional use of *Echinacea* in phytotherapy for inflammatory diseases.

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References

Awang, D.V.C., 1999. Immune stimulants and antiviral botanicals: *Echinacea* and ginseng. In: J. Janick (Ed.), Perspectives on new crops and new uses, ASHS Press, Alexandria, VA, pp. 450–456.

Barnes, J., Anderson, L.A., Gibbons, S., Phillipson, J.D., 2005. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): a review of their chemistry, pharmacology and clinical properties. The Journal of Pharmacy and Pharmacology 57, 929-954.

van den Berg, R., Haenen, G.R., van den Berg, H., Bast, A., 2001. Transcription factor NF-kappaB as a potential biomarker for oxidative stress. British Journal of Nutrition 86, S121-S127.

Binns, S.E., Purgina, B., Bergeron, C., Smith, M.L., Ball, L., Baum, B.R., Arnason, J.T., 2000. Light-mediated antifungal activity of *Echinacea* extracts. Planta Medica 66, 241-244.

Borchers, A.T., Keen, C.L., Stern, J.S., Gershwin, M.E., 2000. Inflammation and Native American medicine: the role of botanicals. American Journal of Clinical Nutrition 72, 339-347.

Chan, M.M., Mattiacci, J.A., Hwang, H.S., Shah, A., Fong, D., 2000. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. Biochemical Pharmacology 60, 1539-1548.

Chang, C.I., Zoghi, B., Liao, J.C., Kuo, L., 2000. The involvement of tyrosine kinases, cyclic AMP/protein kinase A, and p38 mitogen-activated protein kinase in IL-13-mediated arginase I induction in macrophages: its implications in IL-13-inhibited nitric oxide production. The Journal of Immunology 165, 2134-2141.

Chen, Y., Fu, T., Tao, T., Yang, J., Chang, Y., Wang, M., Kim, L., Qu, L., Cassady, J., Scalzo, R., Wang, X., 2005. Macrophage activating effects of new alkamides from the roots of *Echinacea* species. Journal of Natural Products 68, 773-776.

Corraliza, I.M., Campo, M.L., Soler, G., Modolell, M., 1994. Determination of arginase activity in macrophages: a micromethod. Journal of Immunological Methods 174, 231-235.

Corraliza, I.M., Modolell, M., Ferber, E., Soler, G., 1997. Involvement of protein kinase A in the induction of arginase in murine bone marrow-derived macrophages. Biochimica et Biophysica Acta 1334, 123-128.

Cory, A.H., Owen, T.C., Barltrop, J.A., Cory, J.G., 1991. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Communications 3, 207-212.

Dalby-Brown, L., Barsett, H., Landbo, A.K., Meyer, A.S., Molgaard, P., 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. Journal of Agricultural and Food Chemistry 53, 9413-9423.

Gotoh, T., Mori, M., 1999. Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. The Journal of Cell Biology 144, 427-434.

Hu, C., Kitts, D.D., 2000. Studies on the antioxidant activity of *Echinacea* root extract. Journal of Agricultural and Food Chemistry 48, 1466-1472.

Jia, C., Shi, H., Wu, X., Li, Y., Chen, J., Tu, P., 2006. Determination of echinacoside in rat serum by reversed-phase high-performance liquid chromatography with ultraviolet detection and its application to pharmacokinetics and bioavailability. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 844, 308-313.

Katsuyama, K., Hirata, Y., 2001. A pyrrolidinone derivative inhibits cytokine-induced iNOS expression and NF-kappaB activation by preventing phosphorylation and degradation of IkappaB-alpha. Journal of Biochemistry 129, 585-591.

Kiemer, A.K., Muller, C., Vollmar, A.M., 2002. Inhibition of LPS-induced nitric oxide and TNF-α by α-lipoic acid in rat Kupffer cells and in RAW 264.7 cells. Immunology and Cell Biology 80, 550-557.

Kraus, G.A., Bae, J., Schuster, J., 2005. The first synthesis of a diynone from *Echinacea pallida*. Synthesis 2005, 3502-3504.

Lafay, S., Gil-Izquierdo, A., Manach, C., Morand, C., Besson, C., Scalbert, A., 2006. Chlorogenic acid is absorbed in its intact form in the stomach of rats. The Journal of Nutrition 136, 1192-1197.

Lin, J.K., 2007. Molecular targets of curcumin. Advances in Experimental Medicine and Biology 595, 227-243.

Liu, Y., Murphy, P.A., 2007. Alkamide stability in *Echinacea purpurea* extracts with and without phenolic acids in dry films and in solution. Journal of Agricultural and Food Chemistry 55, 120-126.

Louis, C.A., Mody, V., Henry, W.L. Jr., Reichner, J.S., Albina, J.E., 1999. Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 276, R237-R242.

MacMicking, J., Xie, Q.W., Nathan, C., 1997. Nitric oxide and macrophage function. Annual Review of Immunology 15, 323-350.

Mckay, L.I., Cidlowski, J.A., 1999. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocrine Reviews 20, 435-459.

Matthias, A., Addison, R.S., Penman, K.G., Dickinson, R.G., Bone, K.M., Lehmann, R.P., 2005. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. Life Sciences 77, 2018-2029.

Matthias, A., Banbury, L., Stevenson, L.M., Bone, K.M., Leach, D.N., Lehmann, R.P., 2007. Alkylamides from *Echinacea* modulate induced immune responses in macrophages. Immunological Investigations 36, 117-130.

Matthias, A., Blanchfield, J.T., Penman, K.G., Toth, I., Lang, C.S., De Voss, J.J., Lehmann, R.P., 2004. Permeability studies of alkylamides and caffeic acid conjugates from *Echinacea* using a Caco-2 cell monolayer model. Journal of Clinical Pharmacy and Therapeutics 29, 7-13.

Meurs, H., Maarsingh, H., Zaagsma, J., 2003. Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness. Trends in Pharmacological Sciences 24, 450-455.

Modolell, M., Corraliza, I.M., Link, F., Soler, G., Eichmann, K., 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. European Journal of Immunology 25, 1101-1104.

Morris, S.M. Jr., Kepka-Lenhart, D., Chen, L.C., 1998. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. The American Journal of Physiology 275, E740-E747.

Munder, M., Eichmann, K., Modolell, M., 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. Journal of Immunology 160, 5347-5354.

Park, E., Kum, S., Wang, C., Park, S.Y., Kim, B.S., Schuller-Levis, G., 2005. Anti-inflammatory activity of herbal medicines: inhibition of nitric oxide production and tumor necrosis factor-alpha secretion in an activated macrophage-like cell line. The American Journal of Chinese Medicine 33, 415-424.

Pellati, F., Benvenuti, S., Magro, L., Melegari, M., Soragni, F., 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. Journal of Pharmaceutical and Biomedical Analysis 35, 289-301.

Percival, S.S., 2000. Use of *Echinacea* in medicine. Biochemical Pharmacology 60, 155-158.

Porcheray, F., Viaud, S., Rimaniol, A.C., Léone, C., Samah, B., Dereuddre-Bosquet, N., Dormont, D., Gras, G., 2005. Macrophage activation switching: an asset for the resolution of inflammation. Clinical and Experimental Immunology 142, 481-489.

Rousseau, B., Tateya, I., Lim, X., Munoz-del-Rio, A., Bless, D.M., 2006. Investigation of anti-hyaluronidase treatment on vocal fold wound healing. Journal of Voice 20, 443-451.

Sharma, M., Arnason, J.T., Hudson, J.B., 2006. *Echinacea* extracts modulate the production of multiple transcription factors in uninfected cells and rhinovirus-infected cells. Phytotherapy Research 20, 1074-1079.

Speroni, E., Govoni, P., Guizzardi, S., Renzulli, C., Guerra, M.C., 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. Journal of Ethnopharmacology 79, 265-272.

Sreejayan, Rao, M.N., 1997. Nitric oxide scavenging by curcuminoids. The Journal of Pharmacy and Pharmacology 49, 105-107.

Surh, Y.J., Chun, K.S., Cha, H.H., Han, S.S., Keum, Y.S., Park, K.K., Lee, S.S., 2001. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutation Research 480-481, 243-268.

Vanzo, A., Cecotti, R., Vrhovsek, U., Torres, A.M., Mattivi, F., Passamonti, S., 2007. The fate of trans-caftaric acid administered into the rat stomach. Journal of Agricultural and Food Chemistry 55, 1604-1611.

Wang, W.W., Jenkinson, C.P., Griscavage, J.M., Kern, R.M., Arabolos, N.S., Byrns, R.E., Cederbaum, S.D., Ignarro, L.J., 1995. Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. Biochemical and Biophysical Research Communications 210, 1009-1016.

Wei, L.H., Morris, S.M., Jr., Cederbaum, S.D., Mori, M., Ignarro, L.J., 2000. Induction of arginase II in human Caco-2 tumor cells by cyclic AMP. Archives of Biochemistry and Biophysics 374, 255-260.

Woelkart, K., Bauer, R., 2007. The role of alkamides as an active principle of *Echinacea*. Planta Medica 73, 615-623.

Woelkart, K., Koidl, C., Grisold, A., Gangemi, J.D., Turner, R.B., Marth, E., Bauer, R., 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. Journal of Clinical Pharmacology 45, 683-689.

Wu, C.H., Chen, T.L., Chen, T.G., Ho, W.P., Chiu, W.T., Chen, R.M., 2003. Nitric oxide modulates pro- and anti-inflammatory cytokines in lipopolysaccharide-activated macrophages. The Journal of Trauma 55, 540-545.

Wu, L., Bae, J., Kraus, G., Wurtele, E.S., 2004. Diacetylenic isobutylamides of *Echinacea*: synthesis and natural distribution. Phytochemistry 65, 2477-2484.

Yamamoto, T., Sawada, Y., Katayama, I., Nishioka, K., 1998. Increased production of nitric oxide stimulated by interleukin-1beta in peripheral blood mononuclear cells in patients with systemic sclerosis. British Journal of Rheumatology 37, 1123-1125.

Yamamoto, Y., Gaynor, R.B., 2001. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. The Journal of Clinical Investigation 107, 135-142.

Zhai, Z., Haney, D., Wu, L., Solco, A., Murphy, P.A., Wurtele, E.S., Kohut, M.L., Cunnick, J.E., 2007. Alcohol extracts of *Echinacea* inhibit production of nitric oxide and tumor necrosis factor-alpha by macrophages *in vitro*. Food and Agricultural Immunology 18, 221-236.

Zhai, Z., Liu, Y., Wu, L., Senchina, D.S., Wurtele, E.S., Murphy, P.A., Kohut, M.L., Cunnick, J.E., 2007. Enhancement of innate and adaptive immune functions by multiple *Echinacea* species. Journal of Medicinal Food 10, 423-434.

Table 4.1. Concentration of known metabolites in alcohol extracts

	Species		
Metabolites	***E. angustifolia***	***E. pallid***	***E. purpurea***
Amides	1.71*	1.04	2.61
Caftaric acid	ND	0.06	0.06
Chlorogenic acid	0.09	0.21	ND
Cichloric acid	ND**	0.06	0.46
Echinacoside	0.39	1.26	ND

* % of dried extract
** ND = not detectable

Table 4.2. *Echinacea* fractions and their chemical constituents

	Species		
Fractions	***E. angustifolia***	***E. pallid***	***E. purpurea***
1	Caffeic acid derivatives	Caffeic acid derivatives	Caffeic acid derivatives
2	Unknown	Unknown	Unknown
3	Alkamides	Alkamides and ketones	Alkamides
4	Alkamide 11 and other very hydrophobic compounds	Very Hydrophobic compounds	Very hydrophobic compounds
5	Most hydrophobic compounds	Most hydrophobic compounds	Most hydrophobic compounds

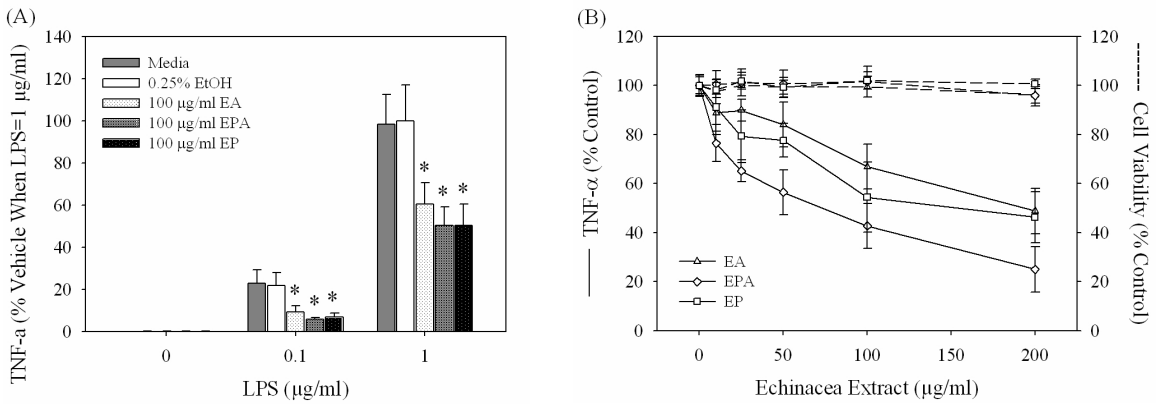

Fig. 4.1. Alcohol extracts of *Echinacea* inhibit production of TNF-α by LPS-stimulated macrophages. RAW 264.7 cells were incubated *in vitro* for 23 h in the presence of indicated *Echinacea* extracts 100 µg/ml plus different concentrations of LPS (A), or incubated with different concentrations of *Echinacea* extracts (0-200 µg/ml) in the presence of 0.1µg/ml LPS (B). Culture supernates were assayed for TNF-α by ELISA (A and B (solid lines)). The attached cells were evaluated for cell viability by MTS assay (B (dashed lines)). The data are expressed as mean ± standard error of the mean of 3 independent experiments performed in triplicate. **P*<0.001 vs the vehicle control at the same concentration of LPS.

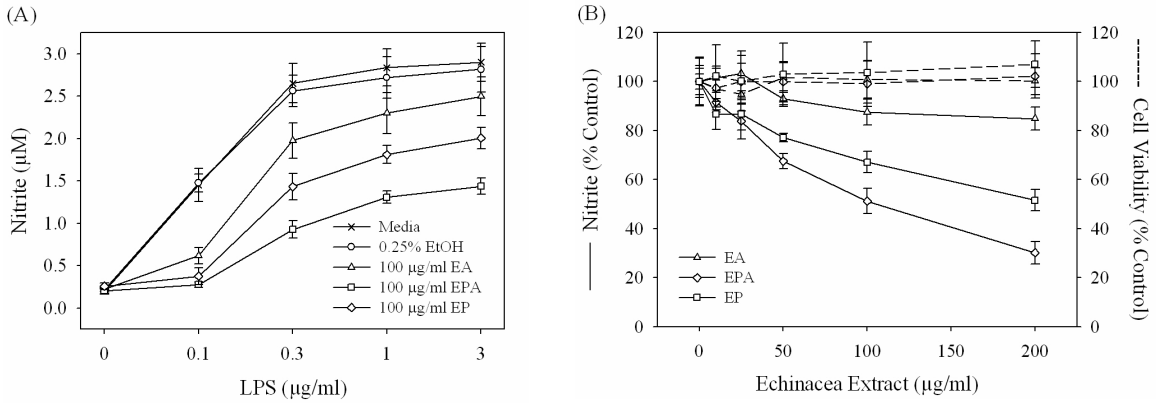

Fig. 4.2. Alcohol extracts of *Echinacea* inhibit production of NO by LPS-stimulated macrophages. Cells were incubated *in vitro* for 23 h in the presence of *Echinacea* extracts 100 μg/ml plus different concentrations of LPS (A), or incubated overnight with different concentrations of indicated *Echinacea* extracts (0-200 μg/ml) in the presence of 1 μg/ml LPS (B). After removing supernates for NO assay (A and B (solid lines)), cell viability was evaluated by addition of MTS (B (dashed lines)). The data are expressed as mean ± standard error of the mean of 3 independent experiments performed in triplicate.

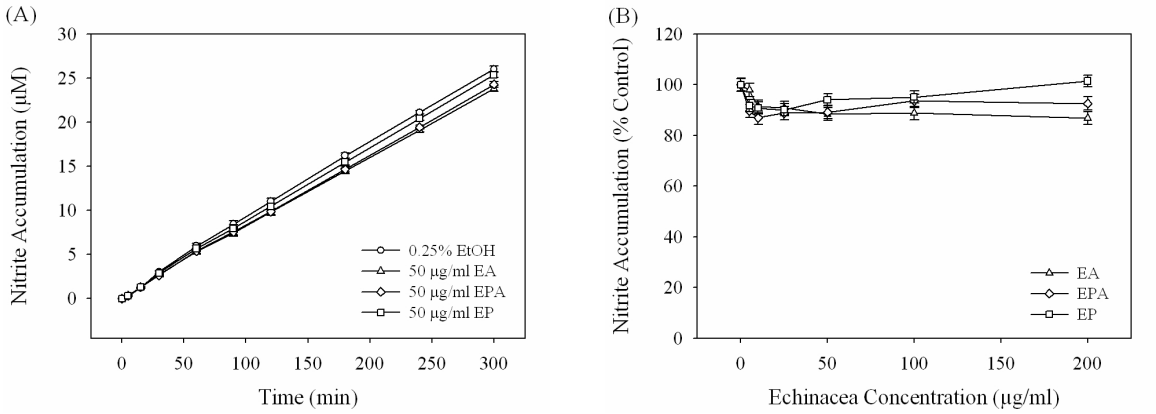

Fig. 4.3. Scavenging of NO derived from SNP by alcohol extracts of *Echinacea*. A. SNP was mixed with Griess reagent in the presence of vehicle (0.25% ethanol) or 50 μg/ml of *Echinacea* extracts and incubated for 6 h. Nitrite accumulation was monitored at 550nm at various time points and calculated based on the absorbance of sodium nitrite standards manipulated in the same way with Griess reagent. B. SNP was mixed with Griess reagents containing various concentrations of *Echinacea* extracts and then incubated for 150 min. The data are expressed as mean ± standard error of the mean of 3 independent experiments performed in triplicate.

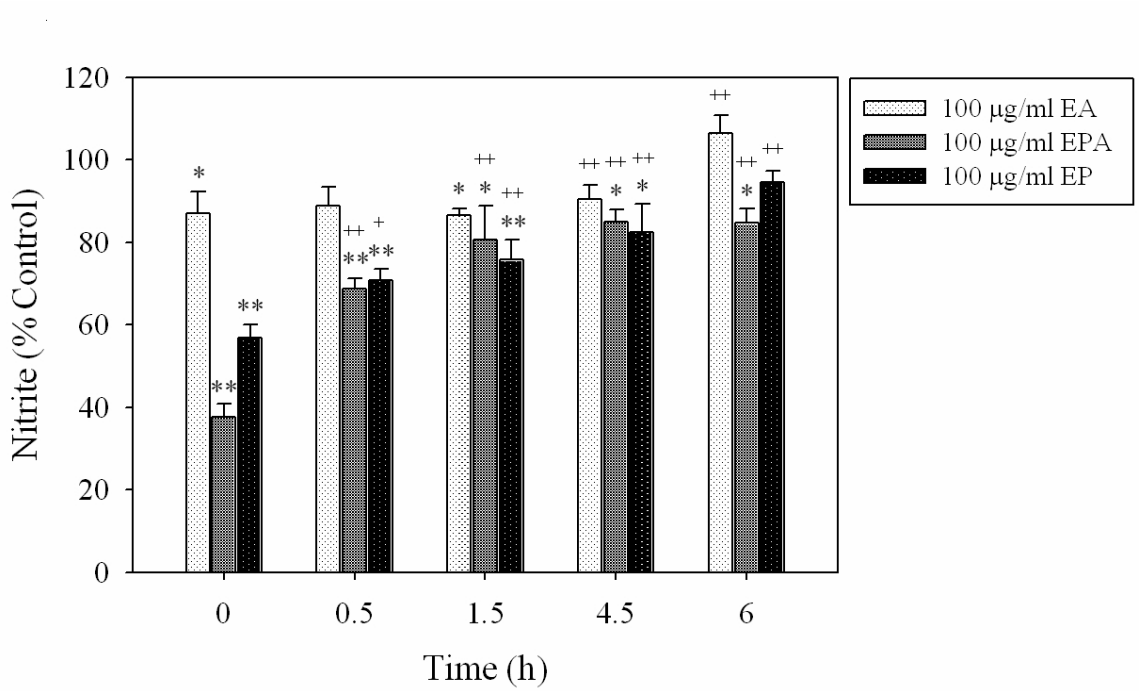

Fig. 4.4. Time course of inhibition of NO production by alcohol extracts of *Echinacea*. *Echinacea* extracts (100 µg/ml) were either added simultaneously with LPS (1 µg/ml) (0 h) or up to 6 h after addition of LPS. The nitrite accumulation by RAW 264.7 cells was determined 22 h after LPS stimulation by the Griess reaction. The data are expressed as mean ± standard error of the mean of 4 experiments performed in triplicate. **P*<0.05 and ***P*<0.001 vs 0.25% ethanol in the presence of LPS (control). +*P*<0.05 and ++*P*<0.001 vs respective group in the presence of LPS at time point 0 h.

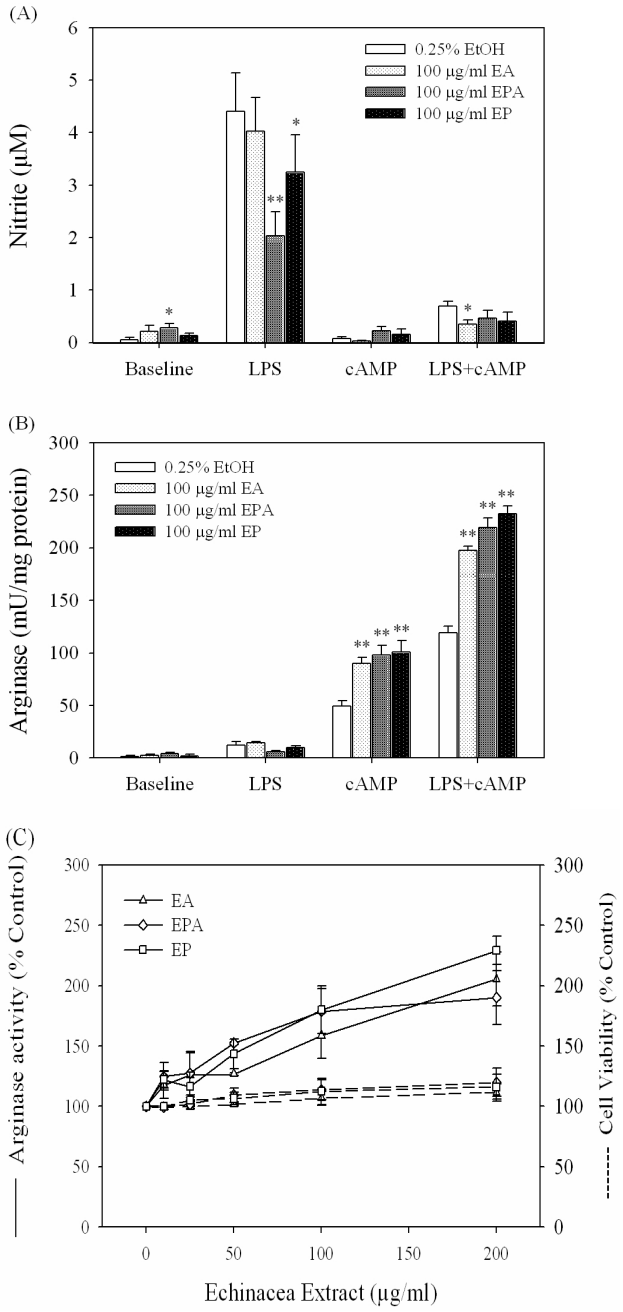

Fig. 4.5. Regulation of NO production and arginase activity of activated RAW 264.7cells by alcohol extracts of *Echinacea*. Cells were incubated in the presence or absence of indicated concentrations of *Echinacea* extracts, LPS (1 μg/ml) and /or 8-bromo-cAMP (abbreviated cAMP, 0.25 mM) for 23-24 h, then supernates were collected for NO assay (A). The cell lysates were assayed for arginase activity (B and C (solid lines)) as described in Materials and Methods. Cell viability was determined by MTS assay (C (dashed lines)). Data are mean ± standard error of the mean of 4-5 experiments performed in triplicate (for NO and cell viability) or duplicate (arginase). **P*<0.05 and ***P*<0.001 vs 0.25% ethanol as control in respective treatment group.

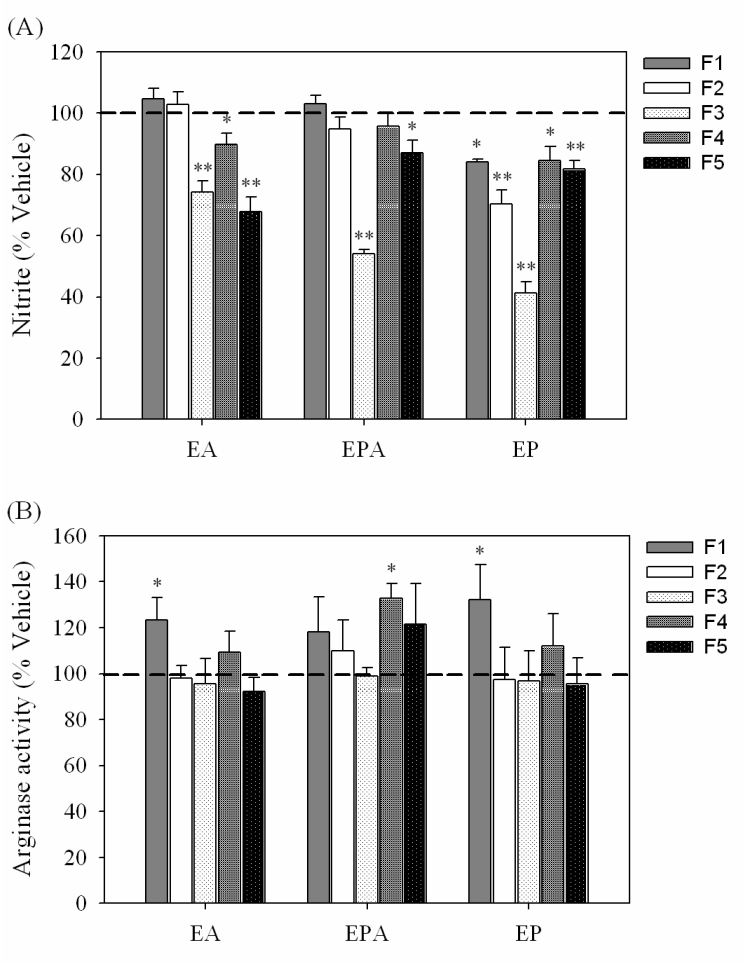

Fig. 4.6. Regulation of NO production and arginase activity of activated RAW 264.7 cells by fractions of alcohol extracts of *Echinacea*. NO production was determined in supernates of cells exposed to LPS (1 µg/ml) plus individual fraction (25 µg/ml) for 24 h (A). Arginase activity was assayed in cells treated with LPS (1 µg/ml), 8-bromo-cAMP (0.25 mM) and individual fraction (25 µg/ml) for 24 h (B). Data are expressed as mean ± standard error of the mean of 4-5 experiments performed in triplicate (NO) or duplicate (arginase). **P*<0.05 and ***P*<0.001 vs 0.25% ethanol as vehicle as in respective treatment group.

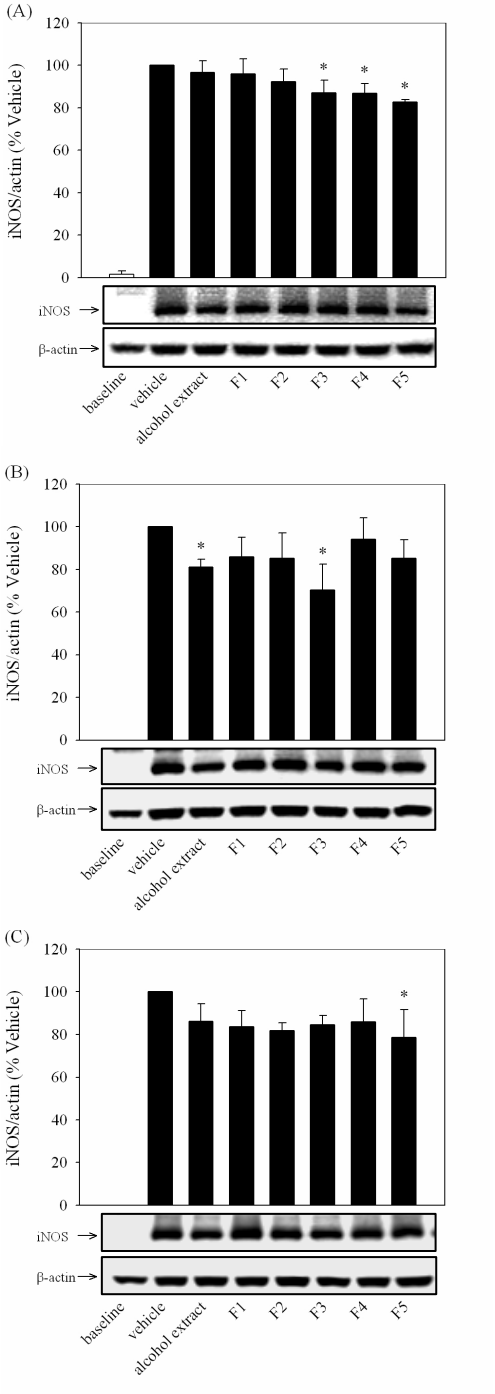

Fig. 4.7. Regulation of iNOS expession by alcohol extracts and fractions of *Echinacea*. Cells were incubated with or without LPS (1 μg/ml) plus *Echinacea* extract (100 μg/ml) or individual fraction (25 μg/ml) for 24 h. The iNOS expression was analyzed by Western blotting and digitally quantitated using image analysis software. The data represent the mean of three independent experiments with different cell preparations. Representative bands are shown. The net intensity of iNOS bands was compared to that of corresponding β-actin with the same treatment, and the ratio was expressed as a percent of the ratio for the vehicle- and LPS-treated cells. A. EA; B. EPA; C. EP. **P*<0.05 vs vehicle plus LPS.

CHAPTER 5. ALCOHOL EXTRACT OF *ECHINACEA PALLIDA* REVERSES STRESS-DELAYED WOUND HEALING IN MICE

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Abstract

Open skin wounds heal gradually beginning with an inflammatory response. Factors that affect this immune event may lead to a variable course of wound recovery. Restraint stress has been well documented to delay wound closure, partially via glucocorticoid (GC)-mediated immunosuppression. *Echinacea*, a popular herbal immunomodulator, is purported to be beneficial for wound healing due to its ability to modulate inflammatory cell function. To test the hypothesis, an alcohol extract of *E. pallida* was administrated orally to male SKH-1 mice at 130 mg/kg per day for 3 days prior to, and 4 days post wounding with a 3.5 mm biopsy punch on the dorsum. At the same time, mice were exposed to 3 cycles of daily 12-h restraint stress prior to, and 4 cycles post wounding. Wound healing was recorded daily by photography. The results showed that *Echinacea* accelerated wound closure in the stressed mice, but had no apparent wound healing effect for the non-stressed mice when compared to their respective controls. To test if the positive healing effect is through modulation of GC release, plasma corticosterone concentrations were measured in unwounded mice that had experienced 4 consecutive cycles of daily restraint stress and were simultaneously treated with the herbal extract for 4 doses, one dose per day. The results showed that *Echinacea* induced an increase, rather than a decrease in the plasma

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corticosterone concentrations when compared to the vehicle control. That data suggest that the improved effect of *Echinacea* in stressed mice is not mediated through modulation of GC signaling.

1. Introduction

The wound repair process is adaptive and progressive; inflammation, cell proliferation and tissue regeneration are three orderly but temporally overlaid stages (Hubner et al., 1996; Park and Barbul, 2004). The inflammatory response that mounts immediately after physical injury holds the key to the efficacy of the subsequent stages in wound healing. Inflammation is a double-edged sword; it has important physiological significance in the normal healing process, but excessive inflammation is detrimental to wound repair (Midwood et al., 2004). Homeostatic regulation of the early inflammatory event is beneficial for faster wound healing.

The genus *Echinacea*, one of the currently popular herbal medicines, is a promising anti-inflammatory agent. Historically, three *Echinacea* species, *E. angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP), have been empirically used in phytotherapy for wound healing, pain relief and alleviation of cold symptoms (Borchers et al., 2000). Recently laboratory studies indicated that alkamides or alkamide-rich alcohol extracts of *Echinacea* inhibit production of inflammatory mediators, i.e. nitric oxide (NO) and prostaglandin E2 in lipopolysaccharide (LPS)-activated mouse macrophage cell line *in vitro* (Lalone et al., 2007; Zhai et al., 2007a). These inhibitory effects may be partially related to blockade of transcriptional factor NF-κB (Stevenson et al., 2005; Matthias et al., 2007) whose activation is mainly responsible for the inflammatory response and production of inflammatory mediators.

Macrophages are important players in the inflammatory response and at the early stage of wound healing. They can undergo classical activation by LPS and tumor necrosis factor-

alpha (TNF-α) to activate inducible nitric oxide synthase (iNOS) and produce NO that causes inflammation, or undergo alternative activation by interleukin (IL)-4 and IL-10 to activate arginase leading to production of L-ornithine (Modolell et al., 1995), an important precursor for cell growth and collagen synthesis in wound recovery (Meurs et al., 2003). We recently investigated the differential effects of *Echinacea* on these two competitive metabolic pathways; alcohol extracts of *Echinacea* reduced TNF-α production, but increased IL-4 and IL-10 production in an *in vivo* gavage model (Zhai et al., 2007b). Moreover, *Echinacea* inhibited NO production, but stimulated arginase activity in activated macrophage cell line (Zhai et al., unpublished). These data further indicate that *Echinacea* presents a clear anti-inflammatory activity that may promote wound tissue recovery.

Nevertheless, there is not sufficient evidence to support *in vivo* wound healing efficacy of *Echinacea*. Alcohol extracts of *Echinacea* are typically composed of two classes of natural chemicals, lipophilic alkamides and water-soluble caffeic acid derivatives. Caffeic acid derivatives are effective antioxidants in free radical generation systems (Dalby-Brown et al., 2005), and have an antihyaluronidase activity (Facino et al., 1993). This later enzyme inhibiting nature has been functionally linked to the healing effect of topical application of a standardized *Echinacea* on vocal fold wounds in a pig animal model (Rousseau et al., 2006). Hyaluronidase catalyzes the hydrolysis of cellular hyaluronan. A suppression of hyaluronidase will allow accumulation of enough hyaluronan in the extracellular matrix for scarless wound repair (Rousseau et al., 2006). In an excision wound model in rats, it was seen that in comparison to the untreated wounds, wound tissues treated topically with EPA extract or its constituent echinacoside, a caffeic acid derivative, for 72 h showed a positive healing process characterized by less inflammatory response and higher hyaluronan contents (Speroni et al., 2002). However, until now no information about the wound healing properties of alkamides from *Echinacea* has been reported, nor a favorable wound healing effect of *Echinacea* via oral administration.

Another advantage not to be ignored in treating wounds with *Echinacea* may reside in the herb's multiple immunomodulatory properties. *Echinacea* is known for strengthening the immune system against pathogenic infections partially through the activation of neutrophils and macrophages to produce inflammatory mediators (Awang, 1999; Goel et al., 2002). The actual action of *Echinacea*, either inflammatory or anti-inflammatory, is largely determined by the host immune cell functional state (Zhai et al., 2007a). *Echinacea* seems to have an ability to help the body's immune system maintain homeostasis and work more efficiently. Although chronic inflammation can disrupt normal wound repair, acute inflammation occurring at the very early stages of an open wound healing process has a critical physiological implication, i.e. removal of cellular debris and pathogens from wound tissue (Kondo and Ohshima, 1996; Weller, 2003; Midwood et al., 2004). Any extrinsic factors that disturb this physiological inflammatory process might adversely affect wound healing. One of the notable examples is a stressor. In a murine model of cutaneous punch biopsy wound healing, a decreased inflammatory response induced by repeated restraint stress resulting in increased corticosterone has been well demonstrated to slow wound healing (Padgett et al., 1998; Rojas et al., 2002). It is likely that the dual biological activities (immunomodulation and alternative macrophage activation) of *Echinacea* are beneficial to wound healing even under an unfavorable condition like chronic stressor, however, direct evidence is needed.

Precisely because of the immunomodulatory, arginase enhancing and anti-inflammatory properties of *Echinacea*, we preliminarily evaluated for the first time the wound healing effect of oral administration of EPA extract on the punch biopsy wound healing process in mice exposed to repeated restraint stress and non-retrained controls. We chose EPA as the *Echinacea* treatment as it decreased NO production to the greatest degree and enhanced arginase activity equivalently to other medicinally used *Echinacea* species, EA and EP (Zhai et al., unpublished).

2. Materials and Methods

2.1. Preparation of EPA extract

The plant EPA was harvested in 2006 in the USDA North Central Regional Plant Introduction Station (Ames, IA) with identification number PI 631293. Alcohol extract from the dried roots was prepared by Soxhlet extraction followed by evaporation to dryness (Zhai et al., 2007b). The dry residue was subsequently dissolved and diluted to 16 mg/ml in 5% ethanol. Aliquots of this dilution were stored at -20°C and thawed for gavage with each aliquot used once. The phytochemicals in the dilution were analyzed using high performance liquid chromatography (HPLC) as described previously (Wu et al., 2004; Lalone et al., 2007). *N*-isobutylundeca-2-ene-8,10-diynamide and 3,5-dimethoxy-4-hydroxy-cinnamic acid were used as internal standards for quantification of lipophilic compounds and water soluble compounds, respectively. To exclude bacterial or endotoxin contamination, the endotoxin levels were evaluated in aliquots using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD, USA) according to the manufacturer's specifications for a microplate assay, and were found to be below the limit of detection (< 0.1 EU/ml).

2.2. Animals

Male SKH-1 mice at 5 weeks of age were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA), and allowed to acclimate to the new environment for 2 weeks. This outbred strain of mice was used to evaluate wound healing, because they are hairless which permits better viewing of the wounds (Padgett et al., 1998), yet immune competent. The mice were housed 2-4/cage under a reverse 12-h light/dark regimen with lights off at 8:30 AM. Unless indicated, the mice had access to water and Harlan Teklad standard rodent chow (#2018; 18% protein rodent diet) *ad lib*. All experimental manipulations were approved by the Iowa State University Committee on Animal Care.

2.3. Animal study 1: Wound healing effect of EPA

This study consisted of two independent experiments (experiment 1 and 2) with an identical study design (total n = 6-7 mice/group).

2.3.1. Restraint stress treatment

Mice were stressed in a manner described previously (Padgett et al., 1998). After the mice were randomly assigned to groups, the restraint stressed groups of mice were placed individually into 50-ml conical centrifuge tubes drilled with holes of 0.4 cm diameter for air flow, heat exchange, and some urine and fecal drainage. The restraint was administrated for 12 h per day (one cycle) during the light phase, with a total of seven cycles applied: three cycles prior to and four cycles post wounding. The first stress cycle after wounding began 30-32 h after wounding. When the stressed mice were subjected to the restraint process, the non-stressed animals were simultaneously food and water deprived (FWD), but remained in their home cages. To determine the potential effect of FWD on wound healing, an extra group was tested with continued free access to food and water (Food *ad lib*) throughout the experimental procedure.

2.3.2. Echinacea administration

An alcohol extract of EPA was orally administered by gavage to the animals at 130 mg/kg body weight once daily for a total of seven doses; three doses were given immediately after each stress cycle prior to wounding, and four doses were given before each stress cycle post wounding. This dosage and regimen was generally selected based on an extrapolation of the dose recommended for humans and calculated as previously described (Zhai et al., 2007b).

2.3.3. Wound biopsy

Mice were anesthetized with an intraperitoneal injection of a mixture of 20 mg/ml rompum and 100 mg/ml ketamine (provided by laboratory Animal Resources of Iowa State University) at a dose of 8.8 µl/g body weight. After the dorsal area was disinfected with 70%

ethanol, a sterile 3.5 mm biopsy punch (Miltex, Inc., York, PA, USA) was used to make two full-thickness wounds on the back (Padgett et al., 1998). In order to prevent dehydration and hypothermia, the mice were subsequently injected intraperitoneally with 1 ml of sterile 0.9% saline solution and placed under a warm lamp until they recovered consciousness.

2.3.4. Wound healing evaluation

Wounds were photographed immediately after wounding and then photographed daily until the end of the experiments. In order to obtain higher quality pictures, beginning from the day after wounding, the mice were temporarily anesthetized using isoflourane (Abbott Laboratories, North Chicago, IL, USA) inhalation. Each individual wound and a standard dot placed beside the wound were analyzed by measuring the horizontal and vertical length using Canvas X software (ACD Systems of America, Miami, FL, USA). The area of an ellipse ($\pi \times$ vertical length $\times$ horizontal length divided by 4) was used to estimate the wound size and the corresponding standard dot size. Beginning 7 days after wounding, healing was also assessed by the absence of an enzymatic reaction during application of 3% hydrogen peroxide (Padgett et al., 1998). Temporary use of this hydrogen peroxide solution has no negative influence on wound healing (Drosou et al., 2003).

2.4. Animal study 2: Immune and corticosterone modulation of EPA

This study extended study 1 and reused the animals from animal study 1. To rule out the potential effect of wounding history on the observational parameters, the animals were given 30-45 days to recover after experimental manipulation in study 1. Animals from experiment 1 were sampled for corticosterone assay and corticosterone was found to return to the baseline levels. The data reported here were collected from the mice used in experiment 2 of study 1. This study 2 includes 7 groups, 3-4 mice per group. Group treatments were randomly reassigned to *Echinacea* and restraint treatments without wounding. Mice were stressed as described above but with a total of four consecutive stress cycles, and were gavaged with

Echinacea extract at 130 mg/kg body weight once daily before each stress cycle. Body weight was recorded daily during the experimental procedure.

2.4.1. Sample collection

Two hours after the last stress cycle, the stressed mice along with the non-restraint stressed mice were euthanized by CO_2 asphyxiation. Body weight and spleen weight were recorded. Blood was collected by heart puncture using a heparinized syringe and then centrifuged at 12,000 g for 10 min. Plasma was collected and frozen at -80°C until assays. The spleens were dissociated into a single cell suspension, and the splenocytes were enumerated using a Hemavet 850 Hematology Analyzer (Drew Scientific, Inc., Oxford, CT, USA) and diluted to 5×10^6 cells/ml in RPMI 1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 25 mM Hepes buffer, 50 µg/ml gentamicin and 10% heated-inactivated fetal bovine serum.

2.4.2. Corticosterone sensitivity assay

Corticosterone suppresses splenocyte proliferation *in vitro* (Avitsur et al., 2001). To test the sensitivity of splenocytes to inhibition by corticostreone, cell viability was evaluated by MTS assay. Cells were seeded at 5×10^5 cells per well in 96-well tissue culture plates with or without 1 µg/ml of LPS (*E. coli* 055:B5, L6529; Sigma, St. Louis, MO, USA) and 0.1-1 µM corticosterone (Sigma) diluted in ethanol with a final concentration of 0.1% (v/v) in cell culture. Following 45 h of incubation, 15 µl of a tetrazolium compound MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Corporation, Madison, WI, USA) was added to 100 µl of cultures with continued incubation for 3 h. The extent of formazan formation from MTS was determined photometrically at absorbance 490 nm using a plate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.4.3. Plasma corticosterone assay

Corticosterone was determined using an enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's protocol. Before assay, plasma samples were appropriately diluted with the assay buffer included with the kit so that samples could be read directly from the standard curve.

2.4.4. Plasma cytokine assay

Plasma IL-6 was determined by an OptEIA ELISA mouse kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol with the exception that the plate was read at 655 nm after addition of the substrate solution for 30 min.

2.5. Statistical analysis

Statistix software (version 8.0, Analytical Software, Tallahasee, FL, USA) was used for the statistical analysis. For analysis of study 1, differences between the treatment groups were tested by two-way analysis of variance (stress × gavage type) with subsequent a priori contrasts. For analysis of study 2, differences between treatment groups were tested by one-way analysis of variance with subsequent a priori contrasts. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Phytochemical analysis

The HPLC profiles of EPA extract are shown in Fig. 5.1 and the amounts of phytochemicals identified and quantified are shown in Table 5.1. The alcohol extract of EPA contains two basic groups of natural chemicals, lipophilic alkamides and ketones and water-soluble caffeic acid derivatives. Alkamide 2 and 8 are the main forms of alkamides, while Echinacoside is the main caffeic acid derivative, followed by cichoric acid. One novel aspect of the EPA extract is that it contains ketones (mainly ketone 20).

3.2. Study 1. Wound healing effect of EPA

3.2.1. Effect of stress and EPA on body weight gain

Restraint stress can cause many physical and psychological changes. Decreased body weight gain and increased plasma conticosterone levels are two typical indicators of chronic stress (Munhoz et al., 2006). The restraint stress paradigm used in this study resulted in a remarkable loss of body weight relative to the non-restraint stressed animals whose body weight increased gradually (Fig. 5.2). Upon discontinuation of stress exposure, the body weight of the stressed mice returned rapidly, but was still lower than that of the non-stressed groups in body weight gain at the end of the experiments ($P < 0.001$). EPA, (FWD+EPA) and (RST+EPA), has no effect on body weight gain when compared to its respective controls in the non-stressed and the stressed mice [(FWD + vehicle) or (RST + vehicle) respectively].

3.2.2. Effect of stress and EPA on wound closure

Fig. 5.3 shows daily changes in wound area compared to the original wound size. The results of the two-way ANOVA of wound size differences between the treatment groups for each day are shown below Fig. 5.3. Among the non-stressed animal groups (Fig. 5.3A), there was no difference in wound closure between the Food *ad lib* and FWD control groups. Thus, these no gavage, no restraint groups were combined during further analysis. Wound healing in the FWD + vehicle group was significantly delayed compared to the combined Food *ad lib* and FWD control groups on days 1-2 and 5-6 post biopsy (P values ≤ 0.031). Interestingly, the FWD + EPA group was not different from the combined Food *ad lib* and FWD control groups except for day 6 ($P = 0.026$. Indicating that gavage was stressful and that EPA administration could partially compensate for delay in wound healing.

Fig. 5.3B displays the data for wound closure for the RST stressed mice along with the FWD control. The RST and the (RST + vehicle) stress groups were not significantly different from each other on any day of the analysis. Thus, these two stressed groups were combined

in during further analysis. The combined stress groups (RST and the RST + vehicle) are significantly delayed in wound healing from day 1 through day 12 compared to the Food *ad lib* and FWD control groups (the Food *ad lib* group is not shown on this graph in order to simplify the graph) (P values < 0.05). More interestingly, the wound closure for the RST + EPA group was equivalent to the Food *ad lib* and FWD controls for days 1-5 post wounding and significantly different from the RST and RST + vehicle groups on these days (P values < 0.05). On day 6 post biospy the positive effect of EPA is no longer seen and the RST + EPA group demonstrated wound healing that is significantly behind than the Food *ad lib* and FWD controls (P = 0.01), and not significantly different from the RST and RST + vehicle groups.

3.2.3. Effect of stress and EPA on healing time

Wound healing was also assessed by determining the total time to complete wound healing (Fig. 5.4). Complete healing is indicated by a lack of enzymatic reaction of hydrogen peroxide applied on the wound. Compared to the Food *ad lib* and FWD groups only the RST and RST + vehicle treatments demonstrated a significant delay in complete re-epithelization (P values < 0.001). The time to complete wound healing for the FWD + EPA and RST + EPA groups was not significantly different from the Food *ad lib* and FWD control groups. The RST + EPA group shortened healing time compared to the RST and RST + vehicle groups (P < 0.001).

3.3. Study 2. Immune and corticosterone modulation of EPA

3.3.1. Effect of stress and EPA on spleen/body weight ratio

Restraint stress induced a gradually decreased body weight gain when compared to the non-stressed mice (data not shown). In order to determine if stress reduces spleen mass, spleen/body weight ratio was calculated. Restraint stress showed a trend to reduce spleen/body weight ratio, but there was no significant difference between the stressed and the non-stressed mice. EPA did not significantly affect the spleen/body weight ratio (Fig. 5.5A).

3.3.2. Effect of stress and EPA on corticosterone sensitivity

The effect of oral EPA on cell viability of splenocytes cultured *ex vivo* was assessed using an MTS assay. Cell number was increased in the presence of LPS, but there were no differences among the seven groups. Addition of corticosterone decreased LPS-stimulated cell proliferation. To better evaluate the corticosterone sensitivity of splenocytes, a "corticosterone resistance" index (Avitsur et al., 2001) was calculated, which represents the percentage of cell viability of each treatment group treated with LPS plus corticosterone compared to that of the same group treated with LPS alone. Splenocytes of the stressed mice lose the sensitivity to inhibition by corticosterone, when compared to the non-stressed mice (Fig. 5.5B). When compared to the RST + vehicle group, the RST +EPA group showed increased MTS incorporation and corticosterone resistance, indicating the RST + EPA group might have higher corticosterone levels *in vivo*.

3.3.3. Effect of stress and EPA on plasma corticosterone and IL-6

As expected, restraint stress significantly increased plasma corticosterone (the RST group *vs* the FWD group, $P = 0.007$) (Fig. 5.5C). For the stressed mice, gavage with vehicle (RST + vehicle), but not EPA (RST + EPA) significantly decreased the stress-induced corticosterone levels ($P = 0.013$).

Restraint stress was also found to increase plasma IL-6 levels (the RST group *vs* the FWD group, $P = 0.002$) (Fig. 5.5D). The RST + vehicle group, rather than the RST + EPA group significantly decreased the IL-6 levels when compared to the RST control ($P = 0.031$).

4. Discussion

Wound healing is a multifactorial process that involves neutrophils, macrophages, fibroblasts and wound remodeling (Diegelmann and Evans, 2004; Park and Barbul, 2004). Neutrophils and macrophages are two important immune cell types responsible for the inflammatory stage of wound healing that takes place from the time of injury to about 5-6

days after injury. Although the inflammatory response reflects host immune defense, it must be tightly regulated in order for the later healing stages to be initiated. Since our previous studies showed that alcohol extracts of *Echinacea* species inhibited NO and TNF-α production by LPS-stimulated macrophage cell line *in vitro* and suppressed TNF-α and IL-1β production by LPS-activated splenocytes *ex vivo* (Zhai et al., 2007a, 2007b), we hypothesized that early treatment with *Echinacea* can improve wound healing through the modulation of macrophage function. We expected a one-week treatment course with EPA extract would help the immune system including macrophages function more efficiently for enhanced and complete wound repair.

Stress was introduced in this wound healing model in order to test the multiple modulating effects of *Echinacea* on wound healing in a different host internal milieu. The restraint stress used in this study is considered a chronic stress. Unlike acute stress which may stimulate the immune system and help the body fight diseases, chronic stress is mostly immunosuppressive in the periphery and detrimental to the body (i.e. worsen existing diseases) (Padgett and Glaser, 2003; Munhoz et al., 2006). It is well documented in a cutaneous wound healing model that chronic restraint stress significantly impaired wound healing (Padgett et al., 1998; Rojas et al., 2002; Sheridan et al., 2004; Head et al., 2006). This study confirmed the previous observations as the combined RST and RST + vehicle groups showed slower wound closure and longer healing time in comparison to the non-stressed control groups. Interestingly, stress-induced negative effects on wound healing could be prevented by EPA extract in the early phases of wound healing, the initial 5-6 days when neutrophil and macrophage function are most important.

There was no improvement of wound healing in the non-stressed mice given EPA (FWD + EPA). Interestingly, this group did not exhibit differences in rates of wound closure as found with the FWD + vehicle group. The alcohol extract of EPA contains high levels of echinacoside (Table 1) which has been shown to benefit wound healing with its

antihyaluronidase activity in animal models when topically applied (Speroni et al., 2002; Rousseau et al., 2006). However, the improved healing effect may not be the result of oral administration of echinacoside because pharmacokinetic data do not support absorption of caffeic acid derivatives in the gastrointestinal tract (Matthias et al., 2004, 2005a, 2005b). In this regard, alkamides are relatively orally bioavailable (Matthias et al., 2004, 2005a, 2005b; Woelkart et al., 2005a). In *vitro*, alkamides have been demonstrated to strongly inhibit LPS-induced end points (i.e. NO and TNF-α) of macrophage cell line and human whole blood (Woelkart and Bauer, 2007). It was, therefore, speculated that the alkamides should behave in a similar manner *in vitro* and *in vivo*. However, it is unclear as to what extent these *in vitro* effects can readily translate into *in vivo* with oral administration. The *in vitro* effects may not be a mirror of *in vivo* effectiveness. We previously compared the effects of *Echinacea* extracts on LPS-activated macrophage cell line *in vitro* and on LPS-activated mouse peritoneal macrophages derived from mice orally gavaged with EPA, and found that *Echinacea* showed a potential anti-inflammatory effect *in vitro*, but not *in vivo* (Zhai et al., 2007a).

Chronic restraint stress caused decreased body weight and increased plasma glucocorticoid (GC) levels. They both appear to be relative to stress-induced activation of corticotrophin-releasing factor receptors (Harris et al., 2002), which is involved in the modulation of circulating GC levels and food intake. Higher levels of GCs released by hypothalamic-pituitary-adrenal (HPA) axis have been directly correlated with stress-impaired wound healing. Upon binding of GCs to their cytosolic receptors, the newly formed ligand-receptor complexes strongly inhibit inflammatory NF-κB function through both direct and indirect pathways (Leung and Bloom, 2003). The subsequent weakened inflammatory response decreases cellular recruitment and proliferation at the wound site and therefore slows wound healing (Rojas et al., 2002; Sheridan et al., 2004).

It remains unclear how EPA reverses restraint stress-delayed wound healing. Since restraint stress displays an anti-inflammatory nature through GC-dependent mechanism (Head et al., 2006), we proposed that *Echinacea* might have anti-GC activity resulting in enhancement of the immune system. Immunostimulatory effects of *Echinacea* have been reported. Goel et al. (2002) found oral administration of alkamides mixed with caffeic acid derivatives and polysaccharides stimulated alveolar macrophages and spleen cells to produce inflammatory mediators in healthy rats. We found in healthy mice alcohol extracts of *Echinacea* increased lymphocyte function (i.e. NK cell cytotoxicity and T-cell cytokine release), but inhibited TNF-α and IL-1β production by spleen cells (Zhai et al., 2007b). Nevertheless, all these effects were seen in a healthy non-stressed host, but not in disease models. It should be noted that both caffeic acid derivatives and polysaccharides are poorly absorbed or uncertain (Bone, 1997; Matthias et al., 2004, 2005a, 2005b). Therefore, the beneficial healing effect of EPA extract is possibly attributed to alkamides. In recent years, alkamides are suggested to be involved in activation of the endocannabinoid system (Gertsch et al., 2004; Woelkart et al., 2005b). This system is composed of endogenous ligands and two cannabinoid (CB) receptors, CB1 and CB2. CB1 receptors are predominantly, but not exclusively found in the brain, while CB2 receptors occur mainly in the immune system (Klein et al., 2003). Due to its distribution and function in the immune system, CB2 receptors have received specific attention in the study of the action mechanism of alkamides. There is evidence to indicate that the *in vitro* immunomodulatory effects of alkamides can be largely ascribed to their binding to CB2 receptors (Woelkart and Bauer, 2007). However, the *in vivo* relevance of the CB2 receptor-involved molecular mechanism is still not clear.

Given that stress-delayed wound healing is associated with elevated plasma GCs, we hypothesized that *Echinacea* can modulate GC release or suppresses activation of the HPA axis, thus reducing GC-induced negative effects. This hypothesis is supported by the findings that the endocannabinoid system modulates the HPA axis function (Rademacher and Hillard,

2007). Activation of CB1 receptors inhibits restraint stress-induced HPA axis activation, whereas blockade of CB1 receptors increases the plasma GC levels (Patel et al., 2004). Alkamides from *Echinacea* have shown some affinity to the CB1 receptors though the binding is much weaker compared to the CB2 receptors *in vitro* (Raduner et al., 2006). Alkamides are highly lipophilic and, therefore, may readily pass through the blood-brain barrier and the plasma membrane of many types of cells in the central nervous system, where they bind to and activate CB1 receptors. We determined both plasma corticosterone levels and splenocyte sensitivity to inhibition by corticosterone. Our speculation was not supported as the alcohol extract of EPA showed no inhibitory effects on the HPA axis activation. On the contrary, the stressed mice treated with EPA showed higher levels of plasma corticosterone and increased splenocyte resistance to corticosterone inhibition when compared to the RST + vehicle control. Because elevated plasma IL-6 is also an indicator of stress (Nukina et al., 2001), we measured plasma IL-6 and found the RST + EPA group showed a non-significant increase in the IL-6 level compared to the RST + vehicle group. Surprisingly, the stressed mice gavaged with vehicle exhibited decreased plasma IL-6 and corticosterone levels. Gavage with the small volume of 5% ethanol may induce some immune responses, however, its role in modulation of IL-6 is unknown.

Since blockade of GC receptors with an antagonist RU486 could only partially reverse stress-delayed wound healing (Padgett et al., 1998), suggesting that in addition to activation of the HPA axis to release GCs, stress may impair wound healing through other mechanisms, i.e. activation of the sympathetic-adrenal mendullary (SAM) axis (Padgett and Glaser, 2003). Stress stimulates the SAM axis to produce catecholamines, which has immune modulating activity through adrenergic receptors expressed on immunocytes, including lymphocytes and macrophages (Padgett and Glaser, 2003). It was demonstrated that phentolamine, an α-adrenergic receptor antagonist, to some extent improves stress-impaired wound healing (Eijkelkamp et al., 2007). The effect of *Echinacea* on the SAM axis needs to be investigated.

In addition to alkamides and caffeic acid derivatives, ketones are also found in alcohol extract of EPA. Like alkamides, ketones are lipophilic and easily absorbed by the intestines (Chicca et al., 2008). Because of their usually concomitant appearance, the interaction between ketones and alkamides is unknown. Ketones have been suggested to have antifungal activity (Binns et al., 2000) and direct cytotoxicity on cancer cells (Pellati et al., 2006; Chicca et al., 2008). The biological activities of ketones need to be further identified.

Taken together, we preliminarily evaluated the modulating effect of EPA extract in a cutaneous wound healing model, and found that this herbal preparation exhibited a beneficial healing effect when the animals were exposed to a chronic restraint stressor. However, the mechanism underlying this result as well as the immunological, biochemical and physiological modulation closely related to the wound healing remain completely unknown. The positive effect of oral administration of *Echinacea* may be attributable to its readily bioavailable alkamides. Because EP and EA contain higher amounts of alkamides than EPA, it is necessary to investigate the healing effect of EP and EA in this wound healing model. On the other hand, topical application of *Echinacea* might be a choice of administration routes. This can avoid the bioavailability problem, and also concentrate high levels of bioactive compounds at the wound site.

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References

Avitsur, R., Stark, J.L., Sheridan, J.F., 2001. Social stress induces glucocorticoid resistance in subordinate animals. Horm. Behav. 39, 247-257.

Awang, D.V.C., 1999. Immune stimulants and antiviral botanicals: *Echinacea* and *ginseng*. In: J. Janick (Ed.), Perspectives on new crops and new uses, ASHS Press, Alexandria, VA, pp. 450–456.

Binns, S.E., Purgina, B., Bergeron, C., Smith, M.L., Ball, L., Baum, B.R., Arnason, J.T., 2000. Light-mediated antifungal activity of *Echinacea* extracts. Planta Med. 66, 241-244.

Bone, K., 1997. *Echinacea*: what makes it work? Alt. Med. Rev. 2, 87-93.

Borchers, A.T., Keen, C.L., Stern, J.S., Gershwin, M.E., 2000. Inflammation and Native American medicine: the role of botanicals. Am. J. Clin. Nutr. 72, 339-347.

Chicca, A., Pellati, F., Adinolfi, B., Matthias, A., Massarelli, I., Benvenuti, S., Martinotti, E., Bianucci, A.M., Bone, K., Lehmann, R., Nieri, P., 2008. Cytotoxic activity of polyacetylenes and polyenes isolated from roots of *Echinacea pallida*. Br. J. Pharmacol. 153, 879-85.

Dalby-Brown, L., Barsett, H., Landbo, A.K., Meyer, A.S., Molgaard, P., 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. J. Agric. Food Chem. 53, 9413-9423.

Diegelmann, R.F., Evans, M.C., 2004. Wound healing: an overview of acute, fibrotic and delayed healing. Front. Biosci. 9, 283-289.

Drosou, A., Falabella, A., Kirsner, R.S., 2003. Antiseptics on wounds: an area of controversy. Wounds 15, 149-166.

Eijkelkamp, N., Engeland, C.G., Gajendrareddy, P.K., Marucha, P.T., 2007. Restraint stress impairs early wound healing in mice via alpha-adrenergic but not beta-adrenergic receptors. Brain Behav. Immun. 21, 409-412.

Facino, R.M., Carini, M., Aldini, G., Marinello, C., Arlandini, E., Franzoi, L., Colombo, M., Pietta, P., Mauri, P., 1993. Direct characterization of caffeoyl esters with antihyaluronidase activity in crude extracts from *Echinacea angustifolia* roots by fast atom bombardment tandem mass spectrometry. Farmaco 48, 1447-1461.

Gertsch, J., Schoop, R., Kuenzle, U., Suter, A., 2004. *Echinacea* alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS Lett. 577, 563-569.

Goel, V., Chang, C., Slama, J.V., Barton, R., Bauer, R., Gahler, R., Basu, T.K., 2002. Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. Int. Immunopharmacol. 2, 381-387.

Harris, R.B., Zhou, J., Mitchell, T., Hebert, S., Ryan, D.H., 2002. Rats fed only during the light period are resistant to stress-induced weight loss. Physiol. Behav. 76, 543-550.

Head, C.C., Farrow, M.J., Sheridan, J.F., Padgett, D.A., 2006. Androstenediol reduces the anti-inflammatory effects of restraint stress during wound healing. Brain Behav. Immun. 20, 590-596.

Hubner, G., Brauchle, M., Smola, H., Madlener, M., Fassler, R., Werner, S., 1996. Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. Cytokine 8, 548-556.

Klein, T.W., Newton, C., Larsen, K., Lu, L., Perkins, I., Nong, L., Friedman, H., 2003. The cannabinoid system and immune modulation. J. Leukoc. Biol. 74, 486-496.

Kondo, T., Ohshima, T., 1996. The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. Int. J. Legal. Med. 108, 231-236.

Lalone, C.A., Hammer, K.D., Wu, L., Bae, J., Leyva, N., Liu, Y., Solco, A.K., Kraus, G.A., Murphy, P.A., Wurtele, E.S., Kim, O.K., Seo, K.I., Widrlechner, M.P., Birt, D.F., 2007. *Echinacea* species and alkamides inhibit prostaglandin E(2) production in RAW264.7 mouse macrophage cells. J. Agric. Food Chem. 55, 7314-7322.

Leung, D.Y., Bloom, J.W., 2003. Update on glucocorticoid action and resistance. J. Allergy Clin. Immunol. 111, 3-22.

Matthias, A., Addison, R.S., Penman, K.G., Dickinson, R.G., Bone, K.M., Lehmann, R.P., 2005a. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. Life Sci. 77, 2018-29.

Matthias, A., Banbury, L., Stevenson, L.M., Bone, K.M., Leach, D.N., Lehmann, R.P., 2007. Alkylamides from *Echinacea* modulate induced immune responses in macrophages. Immunol. Invest. 36, 117-130.

Matthias, A., Blanchfield, J.T., Penman, K.G., Toth, I., Lang, C.S., De Voss, J.J., Lehmann, R.P., 2004. Permeability studies of alkylamides and caffeic acid conjugates from *Echinacea* using a Caco-2 cell monolayer model. J. Clin. Pharm. Ther. 29, 7-13.

Matthias, A., Penman, K.G., Matovic, N.J., Bone, K.M., De Voss, J.J., Lehmann, R.P., 2005b. Bioavailability of *Echinacea* constituents: Caco-2 monolayers and pharmacokinetics of the alkylamides and caffeic acid conjugates. Molecules 10, 1242-1251.

Meurs, H., Maarsingh, H., Zaagsma, J., 2003. Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness. Trends Pharmacol. Sci. 24, 450-455.

Midwood, K.S., Williams, L.V., Schwarzbauer, J.E., 2004. Tissue repair and the dynamics of the extracellular matrix. Int. J. Biochem. Cell Biol. 36, 1031-1037.

Modolell, M., Corraliza, I.M., Link, F., Soler, G., Eichmann, K., 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by TH1 and TH2 cytokines. Eur. J. Immunol. 25, 1101-1104.

Munhoz, C.D., Lepsch, L.B., Kawamoto, E.M., Malta, M.B., Lima Lde, S., Avellar, M.C., Sapolsky, R.M., Scavone, C., 2006. Chronic unpredictable stress exacerbates lipopolysaccharide-induced activation of nuclear factor-kappaB in the frontal cortex and hippocampus via glucocorticoid secretion. J. Neurosci. 26, 3813-3820.

Nukina, H., Sudo, N., Aiba, Y., Oyama, N., Koga, Y., Kubo, C., 2001. Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. J. Neuroimmunol. 115, 46-52.

Padgett, D.A., Glaser, R., 2003. How stress influences the immune response. Trends Immunol. 24, 444-448.

Padgett, D.A., Marucha, P.T., Sheridan, J.F., 1998. Restraint stress slows cutaneous wound healing in mice. Brain Behav. Immun. 12, 64-73.

Park, J.E., Barbul, A., 2004. Understanding the role of immune regulation in wound healing. Am. J. Surg. 187, 11S-16S.

Patel, S., Roelke, C.T., Rademacher, D.J., Cullinan, W.E., Hillard, C.J., 2004. Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. Endocrinology 145, 5431-5438.

Pellati, F., Calò, S., Benvenuti, S., Adinolfi, B., Nieri, P., Melegari, M., 2006. Isolation and structure elucidation of cytotoxic polyacetylenes and polyenes from *Echinacea pallida*. Phytochemistry 67, 1359-1364.

Rademacher, D.J., Hillard, C.J., 2007. Interactions between endocannabinoids and stress-induced decreased sensitivity to natural reward. Prog. Neuropsychopharmacol. Biol. Psychiatry 31, 633-641.

Raduner, S., Majewska, A., Chen, J.Z., Xie, X.Q., Hamon, J., Faller, B., Altmann, K.H., Gertsch, J., 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics. Cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. J. Biol. Chem. 281, 14192-14206.

Rojas, I.G., Padgett, D.A., Sheridan, J.F., Marucha, P.T., 2002. Stress-induced susceptibility to bacterial infection during cutaneous wound healing. Brain Behav. Immun. 16, 74-84.

Rousseau, B., Tateya, I., Lim, X., Munoz-del-Rio, A., Bless, D.M., 2006. Investigation of anti-hyaluronidase treatment on vocal fold wound healing. J. Voice 20, 443-451.

Sheridan, J.F., Padgett, D.A., Avitsur, R., Marucha, P.T., 2004. Experimental models of stress and wound healing. World J. Surg. 28, 327-330.

Speroni, E., Govoni, P., Guizzardi, S., Renzulli, C., Guerra, M.C., 2002. Anti-inflammatory and cicatrizing activity of *Echinacea pallida* Nutt. root extract. J. Ethnopharmacol. 79, 265-272.

Stevenson, L.M., Matthias, A., Banbury, L., Penman, K.G., Bone, K.M., Leach, D.L., Lehmann, R.P., 2005. Modulation of macrophage immune responses by *Echinacea*. Molecules 10, 1279-1285.

Weller, R., 2003. Nitric oxide: a key mediator in cutaneous physiology. Clin. Exp. Dermatol. 28, 511-514.

Woelkart, K., Bauer, R., 2007. The role of alkamides as an active principle of *Echinacea*. Planta Med. 73, 615-623.

Woelkart, K., Koidl, C., Grisold, A., Gangemi, J.D., Turner, R.B., Marth, E., Bauer, R., 2005a. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. J. Clin. Pharmacol. 45, 683-689.

Woelkart, K., Xu, W., Pei, Y., Makriyannis, A., Picone, R.P., Bauer, R., 2005b. The endocannabinoid system as a target for alkamides from *Echinacea angustifolia* roots. Planta Med. 71, 701-705.

Zhai, Z., Haney, D., Wu, L., Solco, A., Murphy, P.A., Wurtele, E.S., Kohut, M.L., Cunnick, J.E., 2007a. Alcohol extracts of *Echinacea* inhibit production of nitric oxide and tumour necrosis factor-alpha by macrophages *in vitro*. Food Agric. Immunol. 18, 221-236.

Zhai, Z., Liu, Y., Wu, L., Senchina, D.S., Wurtele, E.S., Murphy, P.A., Kohut, M.L., Cunnick, J.E., 2007b. Enhancement of innate and adaptive immune functions by multiple *Echinacea* species. J. Med. Food 10:423-434.

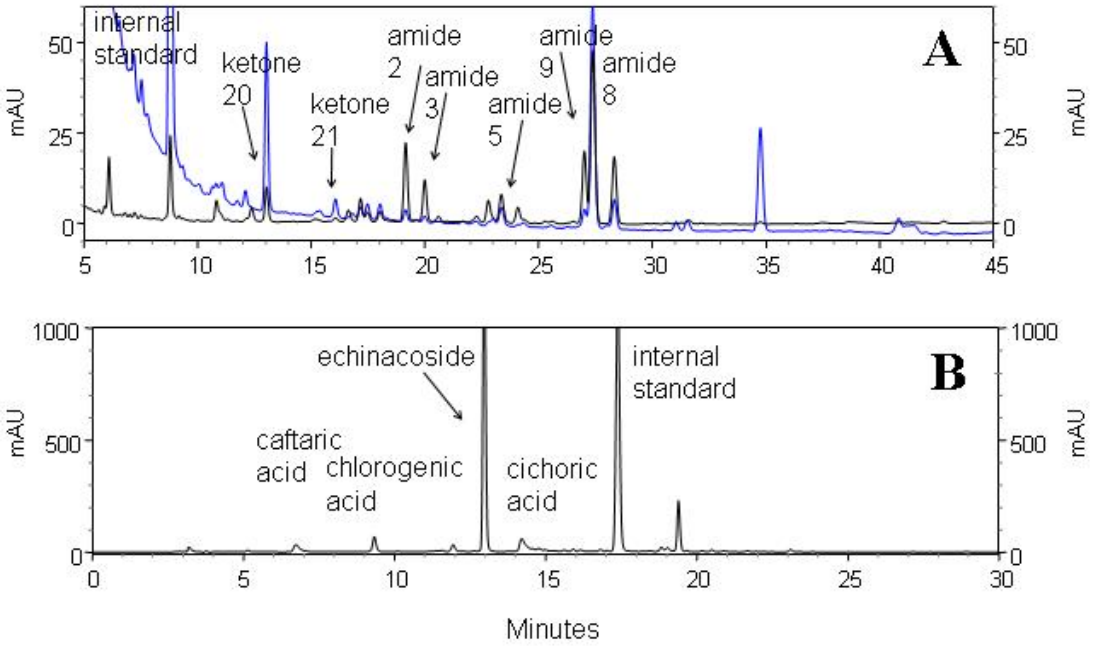


Fig. 5.1. HPLC chromatograms of alcohol extract of EPA: (A) lipophilic alkamides and ketones detected with UV absorbance at 210 nm (upper line) and 260 nm (lower line), and (B) water soluble caffeic acid derivatives detected with UV absorbance at 330 nm. HPLC analysis was performed as described previously by Wu et al. (2004) and Lalone et al. (2007).

Table 5.1. The amounts of alkamides, ketones and caffeic acid derivatives in EPA preparation as determined using HPLC

Group	Metabolite	Concentration*
Alkamide	amide 2	1.01
	amide 3	0.58
	amide 5	0.45
	amide 8	2.35
	amide 9	0.94
Ketone	ketone 20	1.34
	ketone 21	0.17
Caffeic acid derivative	caftaric acid	0.61
	chlorogenic acid	0.67
	cichoric acid	1.07
	echinacoside	7.78

* Values are µg metabolite/mg dried extract

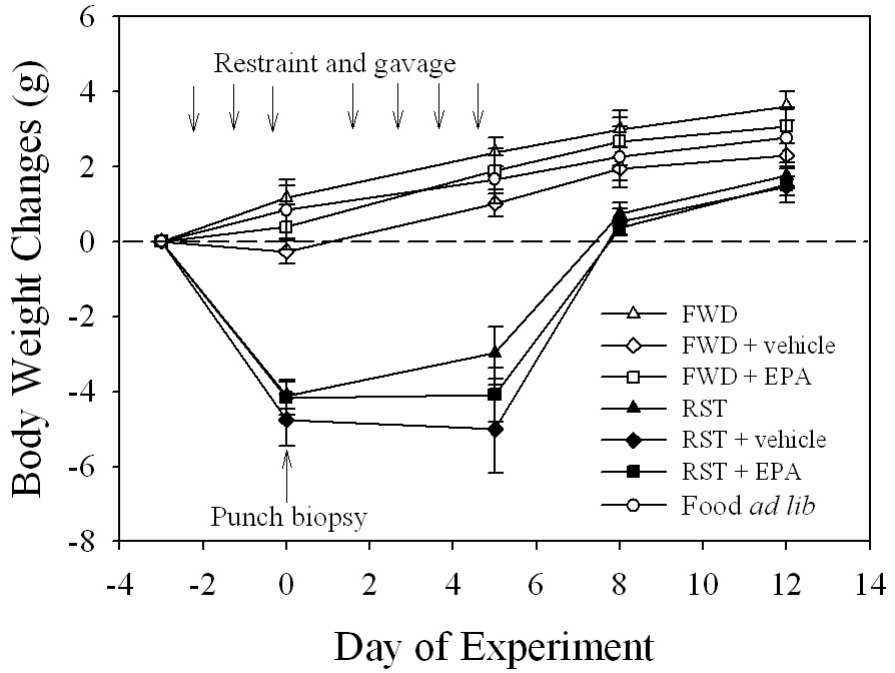
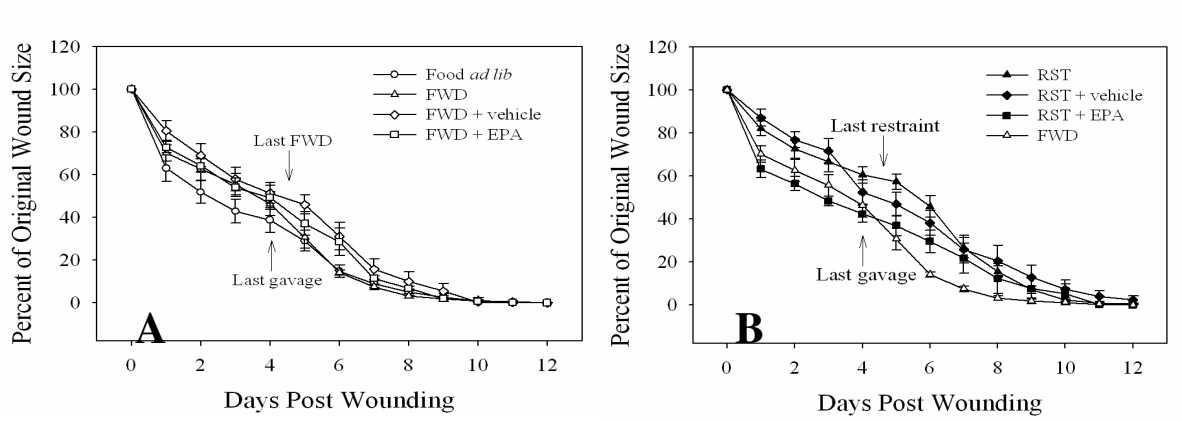


Fig. 5.2. Effects of RST and EPA on body weight loss. A sterile 3.5 mm punch biopsy was used to make two identical wounds on the dorsum on day 0. The stressed animals were administrated three cycles of 12-h interval restraint (RST) stress prior to wounding and 4 cycles post wounding. The non-stressed mice were deprived of water and food (FWD) when the stressed mice were under restraint cycles except the food *ad lib* group, which had free access to food and water throughout the experimental procedure. The mice were orally gavaged three doses of alcohol extract of EPA at 130 mg/kg prior to and four doses post wounding, one dose per day. Body weight was recorded on days as indicated and then was adjusted by the weight at the start of the experiment to reflect change in body weight. Data represent the mean ± SE of each treatment group (n= 6-7 mice/group).



The *P* values of the ANOVA analysis of wound size

	Days post wounding									
ANOVA contrasts	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10
Food ad lib vs FWD										
[Food ad lib + FWD] vs (FWD + vehicle)	0.009	0.031			0.005	0.009				
[Food ad lib + FWD] vs (FWD + EPA)						0.026				
RST vs (RST + vehicle)										
[Food ad lib + FWD] vs [RST + (RST + vehicle)]	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.001	0.009	0.037
[Food ad lib + FWD] vs (RST + EPA)						0.010	0.012			
(RST + EPA) vs [RST + (RST + vehicle)]	0.000	0.001	0.000	0.014	0.015					

Fig. 5.3. Effects of RST and EPA on wound closure of (A) the non-restraint stressed groups and (B) the stressed groups with the non-stressed FWD group. The animals were treated with RST and EPA as described in Fig. 5.1. Wounds were photographed immediately after wounding and then daily until healing. Individual wound size was calculated as a ratio in comparison to a standard-sized dot placed next to the wound and then expressed as the percentage of the original size. Data represent the mean ± SE of each treatment group (n= 6-7 mice/group).

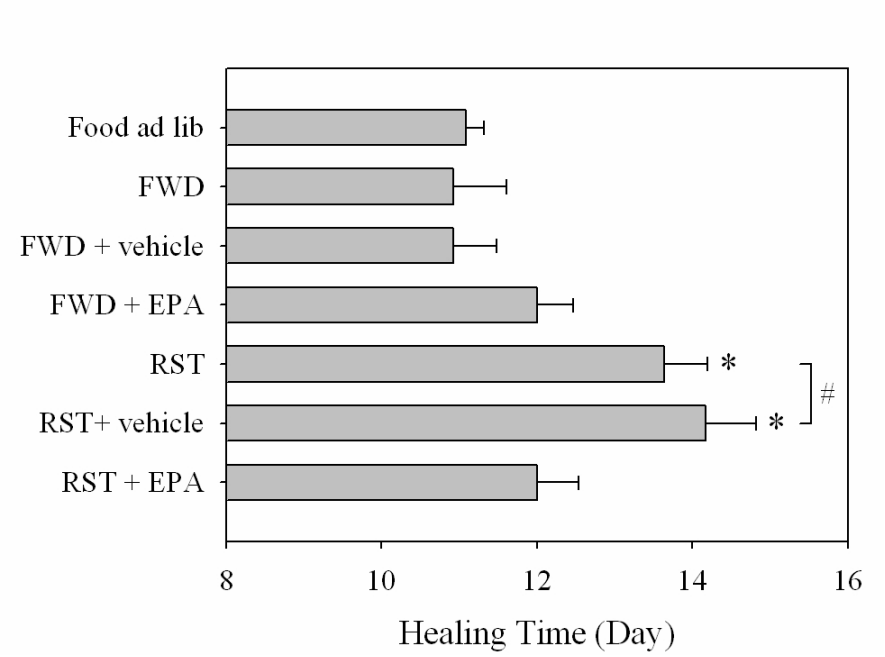


Fig. 5.4. Effects of RST and EPA on healing time. Healing was assessed daily by application of 3% hydrogen peroxide beginning 7 days after wounding and defined as the absence of enzymatic reaction. Data represents the mean ± SE of each treatment group (n=6-7 mice/group). The healing time of each individual animal is the average of its two wounds. *$P < 0.001$ *vs* the Food *ad lib* and FWD groups; # $P < 0.001$ *vs* the RST + EPA group.

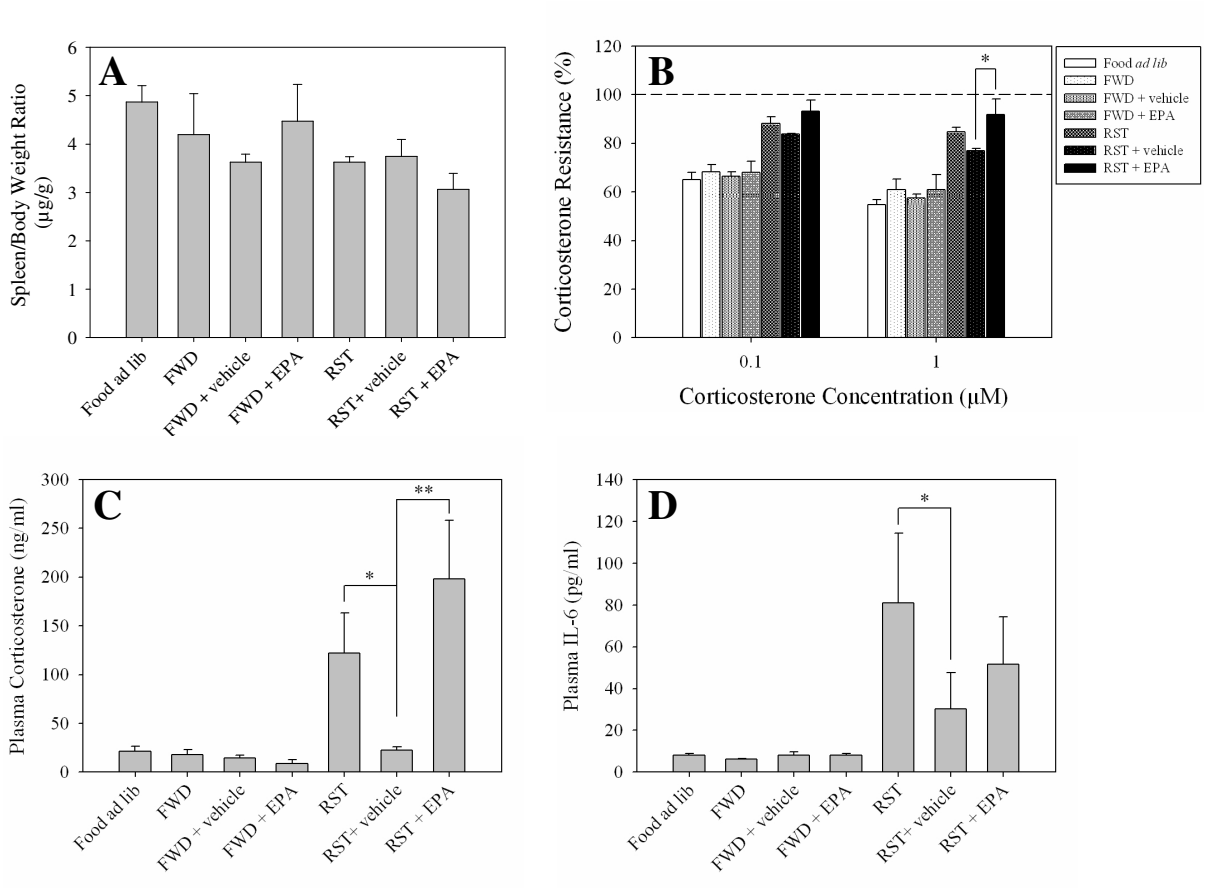


Fig. 5.5. Effects of RST and EPA on (A) spleen/body weight ratio, (B) viability of splenocytes treated with LPS (1 μg/ml) and corticosterone, and plasma concentrations of (C) corticosterone and (D) IL-6. Animals were administrated four consecutive 12-h interval restraint stress cycles (during light phase) and at the same time treated with an alcohol extract of EPA at 130 mg/kg body weight once daily for 4 doses. Two hours after the last stress cycle, animals were euthanized and body weight and spleen weight were recorded. Corticosterone resistance, plasma corticosterone and IL-6 were measured as described in the Material and methods. Dashed line in B indicates cell viability of splenocytes of each treatment group stimulated with LPS alone and referred to 100%. Data represent mean ± standard error of the mean of 3-4 mice. *$P < 0.05$; ** $P < 0.001$.

CHAPTER 6. GENERAL CONCLUSIONS

General Discussion

Chapter 2-5 are original papers in which we demonstrated the immunomodulatory, anti-inflammatory and wound healing properties of *Echinacea* alcohol extracts in *in vitro* or *in vivo* gavage models. We chose the preparation form of alcohol extracts because they consist of two main classes of active metabolites – alkamides and caffeic acid derivatives. Alkamides are identified to be bioavailable (Jager et al, 2002; Matthias et al, 2005a; Woelkart et al, 2005a).

Three medicinally used *Echinacea* species were investigated in parallel in chapters 2-4, thus providing the possibility to compare their efficacy at the same dose (at crude extract level). Generally, the three species harbor similar biological activities. However, they demonstrated quantitative but not qualitative differences in immunomodulatory potentials, which are relevant to their distinct chemical profiles.

Echinacea possesses an ability to modulate both innate immunity and adaptive immunity. This was demonstrated in a healthy animal model in which alcohol extracts were gavaged for 7 consecutive days (chapter 2). Alcohol extracts of *Echinacea* significantly enhanced both B-cell antibody production and T-cell cytokine production. These enhancing effects were more robust when compared to the placebo group, but not the no-gavage group, suggesting that *Echinacea* has a potential to maintain host immune homeostasis. As a dietary supplement, such an immunomodualtory nature may be of more health significance than just a unidirectional immunomodulation.

In vitro, alcohol extracts of *Echinacea* exhibited anti-inflammatory properties by inhibiting production of inflammatory mediators, NO and TNF-α by macrophage cell line RAW 264.7 cells (chapter 3). *Echinacea*-mediated decrease in NO production was not due to a direct scavenging of NO given that caffeic acid derivatives are good antioxidants and free radical scavengers (Hu et al, 2000; Pellati et al, 2004; Dalby-Brown et al, 2005). We suggested

that decreased NO production is at least partially relevant to inhibition of iNOS enzyme expression as determined by Western blot (chapter 4).

NO is generated from L-arginine which can also be metabolized to produce L-ornithine via arginase. These two competitive metabolic processes preferentially occur in different types of activated macrophages. Macrophages undergo classical activation by TNF-α and IL-1β to activate iNOS and produce NO that involves an inflammatory response. Macrophages can also undergo alternative activation by IL-4 and IL-10 to activate arginase and produce L-ornithine which is a precursor of tissue regeneration. We proposed that *Echinacea* alcohol extracts can switch macrophages to alternative activation. They increase production of IL-4 and IL-10 in animal model (chapter 2), while inhibit TNF-α production in RAW 264.7 cell model (chapter 3). Moreover, alcohol extracts of *Echinacea* enhance arginase activity in RAW 264.7 cells (chapter 4).

Alkamides and caffeic acid derivatives are two active principles with clearly different biological natures. The alkamide fraction inhibits NO production and iNOS enzyme expression while caffeic acid and its derivatives increase arginase activities (chapter 4). For arginine metabolism, alkamides and caffeic acid derivatives work in coordination to direct macrophages to produce L-ornithine.

NO and TNF-α are two of main effector molecules of macrophages in pathogenic infection. We were interested in knowing if the *Echinacea*-mediated decrease in NO and TNF-α production could compromise macrophage antimicrobial activities. We found three alcohol extracts had mixed effects on phagocytosis and bacterial killing by macrophages *in vitro*. Especially, EPA had the strongest effect on NO production, but simultaneously increased the potential of bacterial killing (chapter 3), suggesting that *Echinacea* may not adversely affect their important immune functions of macrophages.

However, the *in vitro* anti-inflammatory activities of *Echinacea* were only partially substantiated by evaluating PECs for NO production before and after bacterial infection. The

only statistically significant change in PECs was an EP-mediated decrease of NO production upon infection (chapter 3). Furthermore, the immunomodualtory activities of EPA alcohol extract was evaluated in a skin wound healing model by monitoring the progressive changes in wound size and healing time (chapter 5). No difference was found between the herb and the vehicle group (for no restraint stressed animals). The *in vivo* effect in this regard might be easily influenced by multiple factors, such as bioavailability of active phytochemicals, interplay of different immune cell types and even the physiological complex in actual organism. The *in vitro* effects may not be a mirror of *in vivo* effectiveness.

Interestingly, when the external factor of chronic restraint stress was introduced into the wound healing model, the healing-enhancing effect of EPA alcohol extract was significant. Chronic restraint stress was immunosuppressive via up-regulation of corticosterone release. The wound healing effect of EPA might not be associated with modulation of corticosterone level but through other unknown mechanisms.

Taken together, this dissertation provides new evidence, though limited, that the herb *Echinacea* has immunomodulatory properties. This research work will help understand mechanistically the traditional use of *Echinacea* in phytotherapy for upper respiratory tract infections and inflammatory diseases.

Recommendations for Future Research

As a preliminary basis and applied study of the biological activities of the popular herbal medicine *Echinacea*, this dissertation provides the first step towards a more systematic and in-depth exploration of the herb.

Based on our experimental data, we do not know exactly how *Echinacea* modulates the immune system for a better health, but we propose that *Echinacea* is immunologically active on both innate immune cells and adaptive immune cells. B lymphocytes and T lymphocytes comprise adaptive immunity branch and also involve in host response against infection. It is

necessary to determine whether the *in vivo* influence of *Echinacea* on these cell types is due to a direct enhancing effect or is secondary to activation of other cell types, such as macrophages and neutrophils. Moreover, it is worthy of exploring how *Echinacea* works on these different types of immune cells. If they share a common action mechanism, what it is?

In vitro Echinacea inhibits LPS-induced endpoint products (i.e. NO and TNF-α) in RAW 264.7 macrophages. Although *in vitro* analytical results sometimes can not be translated into *in vivo* effects due to bypassing of ingestion and absorption, *in vitro* studies are relatively easily manipulated and can be considered as a supplementary means towards understanding of *in vivo* assays. In this regard, immune cell lines, like RAW 264.7 cells, might be used to understand the immunological basis for *Echinacea* at a molecular level because LPS-mediated signal pathways are relatively clear. At present, the effects of *Echinacea* on the upstream signaling molecules that activate and regulate NF-κB is completely unknown.

We also observed the wound healing effect of EPA, however, we were unable to identify any potential effects of EPA on the biochemical, physiological and immunological events that occur in wound tissues. Wound tissues might be a good material used for investigation of the biological properties of *Echinacea* by use of various techniques such as immunostaining and histological assays. In addition, our preliminary results suggested the healing effect of EPA in the stressed mice might be not associated with regulation of glucocorticoid release. This needs to be further confirmed. In addition to the glucocorticoid signal, restraint stress might negatively influence early wound healing through other pathways, i.e. α-adrenergic receptors (Eijkelkamp et al, 2007). Stress can induce elevated levels of norepinephrine and epinephrine, which activate α- and β-adrenergic receptors (Cunnick et al, 1990; Padgett & Glaser 2003). The effect of *Echinacea* on the norepinehrine and epinephrine signaling pathways needs to be determined.

We did not obtain a positive effect of EPA in the nonrestraint stressed mice. Two experimental issues should be considered in the future studies. On the one hand, EP instead of

EPA or both EP and EPA could be (comparably) used since EP contains higher levels of alkamides which can survive in the gastrointestinal environment. On the other hand, topical application of the herb might be a choice of administration routes. This can not only avoid the bioavailability problem, but also concentrate high levels of bioactive natural compounds at the wound site, therefore, resulting in the maximum efficacy.

Echinacea is currently used as botanical supplement, in other words, it is being used as mixtures, crude preparations or plants. To our current knowledge, the crude preparations are more efficacious and even safer as opposed to fractions or individual chemicals (Bodinet et al, 2002; Currier et al, 2003; Randolph et al, 2003). Therefore, it is of significance for health practice to study the market used herbal forms and the interactive action of individual compounds within crude preparation. However, the structure-activity relationship of *Echinacea* active principles including alkamides, ketones and caffeic acid derivatives is worthy of special exploration in the future because this may lead to clearly understanding of the interaction between individual components, and also help develop new drugs.

References

Bodinet C, Lindequist U, Teuscher E, Freudenstein J. 2002. Effect of an orally applied herbal immunomodulator on cytokine induction and antibody response in normal and immunosuppressed mice. Phytomedicine 9:606-613.

Cunnick JE, Lysle DT, Kucinski BJ, Rabin BS. 1990. Evidence that shock-induced immune suppression is mediated by adrenal hormones and peripheral beta-adrenergic receptors. Pharmacol Biochem Behav 36:645-651.

Currier NL, Lejtenyi D, Miller SC. 2003. Effect over time of in-vivo administration of the polysaccharide arabinogalactan on immune and hemopoietic cell lineages in murine spleen and bone marrow. Phytomedicine 10:145-153.

Dalby-Brown L, Barsett H, Landbo AK, Meyer AS, Molgaard P. 2005. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions

from *Echinacea purpurea* on *in vitro* oxidation of human low-density lipoproteins. J Agric Food Chem 53:9413-9423.

Eijkelkamp N, Engeland CG, Gajendrareddy PK, Marucha PT. 2007. Restraint stress impairs early wound healing in mice via alpha-adrenergic but not beta-adrenergic receptors. Brain Behav Immun 21:409-412.

Hu C, Kitts DD. 2000. Studies on the antioxidant activity of *Echinacea* root extract. J Agric Food Chem 48:1466-1472.

Jager H, Meinel L, Dietz B, Lapke C, Bauer R, Merkle HP, Heilmann J. 2002. Transport of alkamides from *Echinacea* species through Caco-2 monolayers. Planta Med 68:469-471.

Matthias A, Addison RS, Penman KG, Dickinson RG, Bone KM, Lehmann RP. 2005. *Echinacea* alkamide disposition and pharmacokinetics in humans after tablet ingestion. Life Sci 77:2018-2029.

Padgett DA, Glaser R. 2003. How stress influences the immune response. Trends Immunol 24:444-448.

Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F. 2004. Analysis of phenolic compounds and radical scavenging activity of *Echinacea spp*. J Pharm Biomed Anal 35:289-301.

Randolph RK, Gellenbeck K, Stonebrook K, Brovelli E, Qian Y, Bankaitis-Davis D, Cheronis J. 2003. Regulation of human immune gene expression as influenced by a commercial blended *Echinacea* product: preliminary studies. Exp Biol Med (Maywood) 228:1051-1056.

Woelkart K, Koidl C, Grisold A, Gangemi JD, Turner RB, Marth E, Bauer R. 2005. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. J Clin Pharmacol 45:683-689.

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