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A comparison of both water and ethanol extracts prepared from *Echinacea purpurea* and *Echinacea angustifolia* on the response to Influenza A/PR/8/34 infection in mice

By

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ABSTRACT

Influenza is a contagious respiratory disease causing mild to severe illness. The emergence of a new Influenza virus H1N1 pandemic strain in 2009 has increased the risk of another pandemic. Some concern regarding the potential resistance to neuraminidase inhibitors, along with concerns regarding a readily available vaccine to target emerging viruses, and insufficient evidence to recommend use of antibiotics for upper respiratory tract infection (Aroll B *et al*, 2005), have motivated researchers to look for alternative medicines and other therapies including herbal remedies. Plants species belonging to Genus *Echinacea* are among the most extensively used herbal remedies for “flu- like” symptoms, and are also known to be used traditionally in North Americans native populations for respiratory illness, wounds, digestive problems, and poisoning (Felter *et al.*, 1983, Hobbs, 1994). Recent research on *Echinacea* species has been primarily focused on the immune modulatory properties, particularly in preventing and treating respiratory tract infection (Barnes *et al.*, 2005). Several studies using *Echinacea* extract treatments have reported beneficial effects in preventing and treating respiratory tract infections such as influenza or rhinovirus infections, but the efficacy of *Echinacea* is debatable due to inconsistent findings. In this dissertation, we report the results of investigation into the effect of different extracts prepared from two commonly used *Echinacea* species, *E. angustifolia* and *E. purpurea*. The *in vivo* disease model used to test the efficacy of these extracts is a murine model of influenza infection. Both aqueous and ethanol extracts from *E. angustifolia* and *E. purpurea* were tested in mice subsequently infected with influenza virus. All extracts tested harbored some level of

immune modulatory potential, but showed large variability based on plant species and extraction method used. Aqueous extracts from both species of *Echinacea* demonstrated greater stimulatory effects on immune responses than did ethanol extracts. The most striking effects were improved survival rate and increase in wide range of cytokine/chemokines in the lungs by water extracts. With respect to a species effect, *E. angustifolia* extracts tended to have more potent activity than *E. purpurea* extracts and this held true for both water and ethanol extracts. Modulation of specific cell populations in the lung was also found, but this effect varied by type of extract. In spite of these immunomodulatory changes, there was no reduction in the lung viral load, or any change in weight loss or food intake up to day 8 post-infection.

CHAPTER ONE: GENERAL INTRODUCTION

Literature Review

In recent years, there has been renewed interest in evaluating the pharmacological and physiochemical properties of botanicals, including one of the most commonly used products, *Echinacea*. The immunomodulatory properties of *Echinacea* are the main focus of research, although other potential activities such as anti-viral (Binns *et al.*, 2002; Ghaemi *et al.*, 2008; Birt, 2008), anti-fungal (Morazzoni *et al.*, 2005), and antioxidant properties (Mishima *et al.*, 2004; Dalby-brown *et al.*, 2005) have also been documented. However, there are still many challenges before *Echinacea* can be recommended for medicinal purposes due to inconsistent results. The variability of results regarding the ability of *Echinacea* to modify the outcome of infection can be partly attributed to lack of standardized *Echinacea* extracts, along with the use of different models of infection, and/or different *in vitro* systems used to assess pharmacological properties (Hudson *et al.*, 2005; Wolkert K., 2008; Vimalanathan *et al.*, 2009). In subsequent sections, we will examine literature related to properties of *Echinacea* species and its effectiveness as an immunomodulator particularly in relation to influenza infection.

Phytoactive compounds and their variability

Extracts obtained from *Echinacea* plants are a mixture of compounds that can be broadly characterized as alkamides, caffeic acid derivatives (cichoric, chlorogenic and cafeoyltartaric acid), glycoproteins or polysaccharides (arabinogalactans, fructofuranosides, heteroxylans). Each constituent has shown individual bioactivity and contributes to the

pharmacological activity of a given extract, but the concentration of each constituent is variable depending on the *Echinacea* species, plant organ used for extraction (e.g., roots, stems, aerial portions), growing condition (Sloley *et al.*, 2001; Gray *et al.*, 2003), and method of extraction (Hall *et al.*, 2003; Perry *et al.*, 2001). Only three out of nine known species of *Echinacea* have been studied extensively for their medicinal properties, and few studies directly compare the constituent profile and pharmacological properties of these species. Bioactive compound accumulation in the plant has been shown to be dependent on organ type and age of the plant (Wu *et al.*, 2004; Barnes *et al.*, 2005). In research conducted by Binns *et al.* (2002a), the quantitative phytochemical diversity of all species in genus *Echinacea* was assessed, and the results showed that differences in constituents across species accounted for a range of activities in *Echinacea* extracts. These studies also revealed the presence of the highest amount of cichoric acid in the flower portion of *E. pallida*, whereas *E. purpurea* roots had the highest quantities of alkamides. As an example of varied bioactivity by species, Binns *et al.*, (2002b) demonstrated that 70% ethanol extract of *E. pallida* roots exhibited the most potent inhibition of herpes simplex virus grown on Vero cells, followed by cichoric acid and *E. purpurea* root extract.

In separate studies, Barnes *et al.*, (2005) documented the presence of two structurally different kinds of alkamides in *E. angustifolia* and *E. purpurea*, but an absence of alkamides in *E. pallida*. Other recent studies have demonstrated the presence of alkamides in extracts of *Echinacea* species as the main contributor to anti-inflammatory activity (reduced PGE2 production from LPS stimulated RAW cells) (Lalone *et al.*, 2007). In addition to the bioactivity attributed to alkamides, it has been shown that polyphenolic compounds may also have bioactivity. A study by Pellati *et al.*, (2004) quantified the phenolic composition in

multiple *Echinacea* species using a Lichrospher RP-18 (RP-LC) method and measured radical scavenging activity. Their results suggested that the anti-oxidant effect was greatest in extracts derived from *E. purpurea*, and they concluded that *E. purpurea* has a higher phenolic content than other *Echinacea* species. Another constituent, cynarin, (a phenylpropanoid) has been shown to block CD28 dependent activation of T lymphocytes (Dong *et al.*, 2009), and this constituent has been shown to be present in *E. angustifolia* roots but not in other *Echinacea* species (Sloley *et al.*, 2001). In addition to alkamides and caffeic acid, polysaccharides present in *Echinacea* extracts were also shown to be bioactive (Bauer *et al.*, 1998). Polysaccharide rich *Echinacea* extracts have been shown to activate non-specific innate immune response to antigenic stimuli (Sullivan *et al.*, 2008; Morazzoni *et al.*, 2005; Pillai *et al.*, 2007). Others have concluded that arabinogalactan-containing glycoproteins and high molecular mass polysaccharides present in *Echinacea* extracts are responsible for the varied range of immune-stimulatory activity and again, these components all vary by species (Classen *et al.*, 2006).

The extraction process also plays a major role in determining the concentration of constituents. Alkamides are the main hydrophobic component of plant root extracts and ethanol extraction results in greater concentrations of alkamides, whereas the more hydrophilic polysaccharides are retained in water extracts (Bauer *et al.*, 1998; Hall *et al.*, 2003). Phenolic compounds may be extracted by water-alcohol extraction methods and are also present separately in water and ethanol extracts (Perry *et al.*, 2001). The extraction method and resulting compounds isolated are of major importance with respect to the biological activity. For example, in a recent study, opposite results were found regarding the immunomodulatory activity of *Echinacea* extracts depending upon extraction method and

plant part used. Benson *et al.*, (2010) compared the effects of different extracts of *E. purpurea* on murine dendritic cell function. The results showed that polysaccharide rich root extract increased the expression of cell surface bio-markers (MHC II, CD86, CD54) and production of pro-inflammatory cytokines (IL-6, TNF α) whereas alkamide-rich leaf extracts inhibited the expression of both cell surface biomarkers and cytokine production. These findings illustrate that the immunomodulatory effects of *Echinacea* may be linked to the portion of plant and extraction method used.

Echinacea as immunomodulator

Earlier literature described *Echinacea* as immunostimulatory (Burger *et al.*, 1997) but in view of more recent reports, the term “immunomodulatory” may be a more appropriate term to describe the effect of *Echinacea* on the immune cells. A wide range of immunological parameters (both *in vivo* and *in vitro*) have been measured with respect to the effect of *Echinacea*. However, there is no consensus regarding the exact mechanisms of action by which *Echinacea* alters immune response. The lack of consensus is most likely due to a lack of standardized preparation and variability in the models chosen to study immune response. The mechanisms by which *Echinacea* is effective in modulating the immune response are still elusive, but it is possible to identify general patterns of response based on current literature.

Overall it appears that *Echinacea* has greater immunodulatory effects on innate immunity as compared to adaptive immune responses. However, adaptive immune responses have been less well studied and, therefore, the lack of data showing effects on adaptive immunity may simply be a result of less information rather than no effect. Although

infection models are limited, one study showed that *E. purpurea* polysaccharides reduced the bacterial load in *Listeria monocytogenes*-infected mice, and this activity was attributed to increased macrophage cytokine production. Also, in a herpes simplex infection model, the polysaccharide fraction of *Echinacea* showed anti-viral effects against Herpes Simplex virus, and this effect may have involved increased IFN γ production induced by polysaccharides (Ghaemi *et al.*, 2008). Numerous other studies have shown that the polysaccharide rich fractions of *Echinacea* tend to exhibit the greatest immunostimulatory activity. For example, Sullivan *et al.*, (2008) in an *in vitro* study demonstrated activation of peritoneal macrophages by *E. purpurea* polysaccharides resulting in increased production of inflammatory cytokines (TNF α , IL-1 α , IL-6, and IL-12) and nitric oxide (NO). Similar results (increased IL-1, TNF α , IL-10) were observed in an earlier study on human peripheral blood macrophages using *E. purpurea* extract (Burger *et al.*, 1997) and increased TNF α and NO production by alveolar and spleen macrophages using water-ethanol extract of *E. purpurea* (Goel *et al.*, 2002). Other data suggests that the greatest immunostimulatory activity (defined as increased expression of activation marker CD69 on T cells, NK cells, and B cells) was found in polysaccharide rich fractions of *E. angustifolia* and *E. purpurea*, but almost no activity was found in the fractions containing lipophilic small molecules (echinoacoside, cichoric acid, polyenes) extracted with ethanol. Natural Killer (NK) cells, an important part of anti-viral innate response, are also thought to be modulated by use of *Echinacea* polysaccharides. One study conducted using NK cells present in the human peripheral blood mononuclear cell population demonstrated that a water extract of *Echinacea* increased NK cytotoxicity (Gan *et al.*, 2003). *In vivo* experiments using *Echinacea purpurea* root extract showed an increase in NK cell numbers in the spleen (Currier *et al.*, 2000; Brousseau *et al.*, 2005; Sun *et al.*, 1999;

Gan *et al.*, 2003), and also appeared to increase survival rate of the mice by middle age when *E. purpurea* supplemented diet was fed (13 months) (Brousseau, 2005).

In contrast to the findings on polysaccharide rich extracts, alkalamide rich ethanolic extracts of *Echinacea* have been shown to stimulate anti-inflammatory cytokines (IL-10) and inhibit the secretion of pro-inflammatory (e.g., TNF α) cytokines from murine macrophage cell line (Chen *et al.*, 2005; Chicca *et al.*, 2009; Senchina *et al.*, 2006). Also, data collected by Sharma *et al.*, 2009, in a series of experiments using ethanolic extract of *E. purpurea* showed inhibition of pro-inflammatory (e.g., IL-6, IL-8) cytokine production in a virus-infected human bronchial epithelial cell line. Similar findings showed that the production of inflammatory mediators (IL-1 β , TNF α , NO) by *Salmonella enterica*-infected RAW 264.7 macrophages and peritoneal exudates macrophages was decreased when cultured with ethanol extracts of *E. purpurea* or *E. pallida* (Zhai *et al.*, 2007a). It was also found that ethanol extracts of *Echinacea* exhibited antiviral activity against Herpes Simplex virus grown on Vero cells, but the activity varied with extract type and plant species (Binns *et al.*, 2002). A recent study on mouse macrophage cells stimulated by LPS identified individual alkalamides from *Echinacea* species responsible for inhibition of PGE₂ production, an important inflammatory mediator, using high performance liquid chromatography (Lalone *et al.*, 2007 & 2009). In summary, much of the data published on ethanol extracts and/or alkalamide-rich fractions has revealed an anti-inflammatory effect.

Although most of the work performed to date has used *in vitro* models, there are limited data from *in vivo* studies that have demonstrated immune modulation of both innate and adaptive responses with the use of different *Echinacea* preparations (Rehman *et al.*, 1999; Zhai *et al.*, 2007b). Findings from one *in vivo* study suggested that *Echinacea* extracts

containing varying doses of cichoric acid, polysaccharides, and alkylamides stimulated non-specific immune responses as evidenced by increased alveolar macrophage TNF α and NO release, increased splenocyte IFN γ production, and enhanced phagocytic activity by alveolar macrophages in a dose dependent fashion (Goel *et al* 2002). A similar study compared immunomodulatory effects of ethanol extracts prepared from three different species (*E. angustifolia*, *E. purpurea*, *E. pallida*) and found that splenocytes stimulated with ConA had greater IL-2 and IL-4 production when cultured with *E. angustifolia* extract, greater IL-5 production with *E. pallida* extract, and reduced IL-1 β and TNF α when cultured with LPS plus extracts from all 3 species of *Echinacea* (Zhai *et al.*, 2007b). Enhancement of adaptive immune responses in rats after repeated exposure to keyhole limpet hemocyanin antigen was observed by increased antigen-specific immunoglobulin production in conjunction with *Echinacea* treatment (Rehman *et al.*, 1999). Similar results were observed following dietary administration of *E. purpurea* root extract as evidenced by enhanced proliferation of B cell and T cell in tumor cell immunized mice (Currier *et al.*, 2002), and increased IgM-specific antibody forming cells in mice immunized with sheep red blood cells (Freier *et al.*, 2003). It is also possible that changes in adaptive immune response are mediated via *Echinacea*-induced modulation of innate immunity. Recently, findings by Mishima *et al.* (2004), suggested that *E. purpurea* extract activated macrophage cytokine production, resulting in increased T cell proliferation especially CD4⁺ and CD8⁺ T cell subsets in radiation induced leukopenic mice. Therefore, in future studies, it would be worthwhile to determine the extent to which the T and B cell changes that occur in *Echinacea*-treated animals may be due to innate activation of cytokines that stimulate T and/or B cell function, as opposed to a direct effect on T or B cells.

Influenza Virus and Immune response

Influenza virus, belonging to the *Orthomyxoviridae* family, results in acute infection causing substantial morbidity and mortality in humans worldwide. In the United States, every year, between 5 % - 20 % of the population suffers from respiratory illness caused by influenza virus (<http://www.cdc.gov/flu/about/disease/index.htm>). Influenza viruses also have a zoonotic potential, and are highly contagious for birds, horses, pigs as well as humans. Viral hemagglutinin is required for binding to sialic acid on surface of host cells determining its tropism (Weis *et al.*, 1988). Influenza virus primarily binds to columnar epithelial cells of respiratory tract which also is the site of viral replication (Lamb *et al.*, 1996). Replication leads to cytopathic effects on epithelial cells characterized by down-regulation of host cell protein synthesis (Katze *et al.*, 1986; Sanz-Esquerro *et al.*, 1995) and apoptosis (Wiley *et al.*, 2001) resulting in acute disease onset. In addition, hyperactivity of bronchial system (Utell *et al.*, 1980; Little *et al.*, 1978), small airway obstruction (Hall *et al.*, 1976) and impaired diffusion capacity (Horner *et al.*, 1973) are main contributors to respiratory symptoms. Influenza infection also initiates a cascade of nonspecific and adaptive immune responses which contribute substantially toward clinical signs and symptoms. Respiratory epithelial cells infected by virus respond by producing cytokines/chemokines (IFN α/β , IL-1, IL-6, and TNF α) to attract appropriate immune populations to the site of infection, but the major source of interferon and pro-inflammatory cytokine production are macrophages and dendritic cells which are indirectly stimulated by viral replication (Ronni *et al.*, 1997; Sareneva *et al.*, 1998). These inflammatory cytokines (IL-1, IL-6, IL-12, and TNF α) may further activate NK cells (Nguyen *et al.*, 2004), induce a febrile response (IL-1 β , IL-6 and TNF α), and may contribute to immunopathological lesions (Wareing *et al.*, 2004).

Infected respiratory epithelial cells along with alveolar macrophages and plasmacytoid dendritic cells (pDC) respond by producing type I interferon (Kumagi *et al.*, 2007; Ronni *et al.*, 1997; Jewell *et al.*, 2007). Type I interferon's (IFN α and IFN β) constitute the first line of antiviral response as they induce apoptosis of infected cells and potentiate innate and adaptive immune response through activation of NK cells and effector T cells (Stetson & Medzhitov., 2006). Type I interferons also have direct anti-viral activity (Katze *et al.*, 2002). Further, apoptotic infected epithelial cells release heat labile factors and chemokines such as IL-8 (or KC in the mouse) that promote recruitment of neutrophils and enhance phagocytosis by macrophages and neutrophils (Hashimoto *et al.*, 2007). Along with these cytokines, influenza virus infection results in an upregulated mRNA for MCP-1, MIP-1 α , MIP-1 β , RANTES, IP-10, MIP (neutrophils attractant), and MIP-3 α (immature DC) (Wareing *et al.*, 2004). These chemokines induce an inflammatory infiltrate comprised of mononuclear cells and neutrophils.

Both alveolar macrophages and monocyte-derived inflammatory macrophages are important in phagocytosis of infected cells, antigen presentation, and production of pro-inflammatory cytokines and chemokines. Studies of H1N1 influenza virus infection in mice have indicated a significant increase in recruitment of macrophages and neutrophils to lungs; however, depletion of these cell populations has been shown to increase mortality, suggesting that these cell populations may have both an inflammatory and protective role (Tumpey *et al.*, 2005). In addition to the role of monocytes, macrophages, and dendritic cells in producing inflammatory cytokines, virus infected mononuclear cells are also an important source of IL-12, which is required in early production of IFN γ and induction of cytotoxic T lymphocytes responses (but may not be absolutely necessary for recovery from influenza

infection) (Monterio *et al.*, 1998). Another important aspect of anti-viral innate immunity involves NK cells, which can be detected in pulmonary lymphocyte populations 48 hrs after the initiation of influenza virus infection. NK cells produce IFN γ which may block virus spread by lysis of virus infected cells (Biron *et al.*, 1999). Influenza virus infection of mice lacking NKp46, a NK cell receptor, results in increased mortality demonstrating the importance of NK cells in anti-viral immune response (Gazit *et al.*, 2006).

Mononuclear cells are essential for effective induction of adaptive immune response and preventing secondary bacterial infection (Brion *et al.*, 1999; Legge *et al.*, 2003) but the role of neutrophils in altering the course of infection is not fully understood. Some studies have suggested that neutrophils do not play any role in viral clearance (Wareing *et al.*, 2007) although excessive neutrophil influx may contribute to lung tissue injury (Sakai *et al.*, 2000). A recent study has shown exacerbation of mild disease in the absence of, or with impaired neutrophil influx (Tate *et al.*, 2009). In summary, multiple inflammatory cell populations are recruited to the lungs early during influenza infection that contribute to activation of NK cells and subsequent T cell and B cell responses through cytokine production, but may also contribute to immune-mediated pathology.

Optimal induction of innate response not only prevents the dissemination of virus but also promotes effective adaptive immune responses. Dendritic cells have shown to be essential in bridging innate and adaptive immune response. There are two major subtypes of DCs: plasmacytoid DC (pDC), which primarily produces IFN α in response to influenza infection whereas the conventional DCs (cDC) subtype undergoes maturation after infection and migrates to draining lymph nodes for antigen presentation (Grayson *et al.*, 2007). In addition to these functions, both subtypes of DCs produce chemokines and cytokines in

successive waves coordinating the recruitment of NK cells and neutrophils in the acute phase and T and B cells in later phase of influenza infection (Piqueras *et al.*, 2006). Subset of DC producing TNF and iNOS have been shown to accumulate in lungs infected with lethal dose of influenza virus and are required in proliferation of influenza specific CD 8⁺ T cells in lungs (Aldridge *et al.*, 2009).

Cytokines such as IL-12 and IFN γ produced by antigen presenting cells (APC's) in the presence of antigen results in differentiation of naïve CD4⁺ cells into T helper 1 (Th1) cells (Abbas *et al.*, 1996). Influenza virus infection predominantly induces a Th1 response, and CD4⁺ Th1 cells primarily secrete IL-2, TNF α , and IFN γ which promotes proliferation of CD8⁺ CTL (Mosmann *et al.*, 1996; Ridge *et al.*, 1998; Riberdy *et al.*, 2000). These cytokines also enhance macrophage function and B cell differentiation to preferentially secrete IgG2a antibody (Ada *et al.*, 1986). Cytotoxic CD8⁺ T cells are essential in influenza virus clearance, and the primary lytic pathways involve perforin release and Fas cytotoxic mechanism (Topham *et al.*, 1997). In localized influenza infection, CD4⁺ cells also play a major role by maintaining cytolytic T cell function and the transition to immune memory (Belz *et al.*, 2002). In summary, the immune response to influenza involves multiple host defense mechanisms, ranging from early innate defenses that may restrict viral spread to enhancement of inflammatory pathways that serve to activate appropriate adaptive immune responses.

Echinacea in Influenza Infection

In the literature, there are reports of several clinical and experimental studies that have been conducted to evaluate the use of *Echinacea* in upper respiratory tract infection.

However, the results are inconclusive, as some studies have found a beneficial effect of *Echinacea* on symptoms and the severity of infection (Berg *et al.*,1998; Brinkeborn *et al.*,1999;Freier *et al.*,2003; Goel *et al.*,2004&2005; Hall *et al.*,2006; Hoheisel *et al.*,1997; Lindenmuth *et al.*,2000; Melchart *et al.*,2000, Saunders *et al.*, 2007) while others show no reduction in severity of symptoms with use of *Echinacea* (Barrett *et al.*,2002;Classen *et al.*,2006; Grimm *et al.*,1999; Taylor *et al.*,2003; Turner *et al.*,2000&2005, O'Neil *et al.*, 2008; Schwarz *et al.*,02; Sperber *et al.*,2004). While much of the previous work has involved cell lines, *ex vivo* studies, or symptomatic evaluation of human subjects, there are only few studies exploring the effects of different *Echinacea* extracts on immune response *in vivo*. A recent study by Fusco *et al.*, (2010) investigated the effect of neutral and weak acidic polysaccharide extract prepared from aerial part of *E. purpurea* on a murine model of influenza A infection. The investigators observed less weight loss in the *Echinacea*-treated mice but no change in viral titer, suggesting a modifying effect of the extract on clinical course of infection (less body weight loss) without any evident anti-viral effect. In this same study, at the early phase of infection (day 3), increased IFN γ , IL-12, KC in both lung and serum and increased IL-10 only in serum was found in *Echinacea* treated mice. By day 7 post-infection, *Echinacea* treatment resulted in reduced IL-10 in serum and lung, as well as decreased serum IFN γ . It is not clear from this study how immunomodulatory changes might relate to the reduction in weight loss, or to specific immunopathology in the lungs. Only one other published study using influenza challenge of mice examined the effect of plant extract mixture which included *Echinacea* as well as *Baptisia tinctoria* and *Thuja occidentalis* (Bodinet *et al.*, 2002). The combination of extracts used in this study resulted in an improved survival rate, and reduced lung lesion score and viral titer. Although there was a

benefit in terms of a reduction in the infection severity, it is difficult to draw any conclusions from the study regarding the specific role of *Echinacea* because the treatment included a mix of herbal remedies, rather than *Echinacea* alone (Bodinet *et al.*, 2002).

The purpose of our present investigation was to verify the specific immunomodulatory effects *Echinacea* species alone (without other herbal constituents) in an animal model of influenza infection. In addition, we sought to determine whether the immunomodulatory effects of two commonly used species of *Echinacea*: *E. purpurea* and *E. angustifolia*, are consistent or variable. Last, we evaluated the effect of water extracts compared to ethanol extracts prepared from *E. purpurea* and *E. angustifolia*. The hypothesis to be tested was that *polysaccharide rich aqueous extract of both Echinacea species would be more effective than ethanolic extract of the same Echinacea species in stimulating an immune response following an influenza virus infection in mice*. We focused on innate immune responses for two reasons. The majority of published data demonstrated potent effects on innate immunity, and secondly, adaptive immune responses are largely dependent upon appropriate activation of innate host defense. This hypothesis was tested by gavaging mice with extract or vehicle 2 days before infection with influenza virus, and continuing gavage with extract or vehicle throughout the course of infection until the time at which mice were euthanized. Experiments evaluate host responses and viral titer at both acute phase and late phases of infection to establish the effect on lung pathology, cytokine/chemokines analysis, illness markers, and viral titer and pulmonary cell populations.

Hypothesis

It is hypothesized that *polysaccharide rich aqueous extract of two Echinacea species; E. purpurea and E. angustifolia, would be more effective than ethanolic extract of the same Echinacea species in stimulating an immune response following an influenza virus infection in mice.* To test this hypothesis, an *in vivo* murine model of influenza A virus infection was used, with immune responses assessed at multiple time points post-infection. We expect that polysaccharide rich water extract will have greater stimulatory effect on immune response as compared to alkalamide rich ethanol extracts. In particular, we expect that cytokine and chemokine production will be increased in the lungs of water extract treated mice as compared to ethanol extract treated mice. We also expect that this increase in lung cytokine/chemokine production will be accompanied by improved survival, decreased lung viral load and reduced symptom severity.

CHAPTER TWO: IMMUNOMODULATORY PROPERTY OF WATER AND ETHANOL EXTRACTS FROM *ECHINACEA* SPECIES.

Abstract

Echinacea species have been used traditionally for their medicinal properties. In today's world, *Echinacea* is one of highest selling herbal medicines consumed primarily for its immune modulator properties, particularly in preventing and treating respiratory tract infection. Multiple *in vitro* studies of *Echinacea* have been conducted and demonstrate immune modulatory effects. However, the results from *in vivo* studies have shown greater disparity, both human clinical trials as well as animal infection models. Many *in vivo* studies fail to simultaneously evaluate immune response, viral load, and symptoms, and therefore it is difficult to determine whether any immune alterations truly have a benefit. In this study, the effects of *Echinacea* extracts from different species (*E. angustifolia* and *E. purpurea*) and extraction method (water and ethanol) were compared for their potential effects on respiratory immune parameters, viral load in the lungs, and clinical symptoms. It was hypothesized that aqueous extracts of *Echinacea* (rich in polysaccharides) would be more effective in stimulating immune responses during influenza infection than ethanol of same species. Mice were gavaged with extracts every 24 hrs starting two days prior to infection and continued during infection until mice were euthanized. Mice were infected with influenza A/PR/8/34 (H1N1) through an intranasal route. Both early and later phases of infection were evaluated. Aqueous extracts, particularly from *E. angustifolia* had the greatest immunostimulatory effect in terms of increased cytokines and chemokines in bronchoalveolar lavage (BAL) fluid and improved survival rate of infected mice. Lung cell

populations were altered, but this effect varied by extract and species. Also, the number of influenza-responsive IFN γ producing CD8⁺ cells present in the BAL fluid was altered by *Echinacea* treatment, but again this effect varied by extract type such that ethanol extracts decreased the number of these cells, whereas water extracts significantly increase the number of these cells. There was no effect of any extract on viral load or symptoms (body weight loss, food intake), although decreased mortality was seen in mice treated with aqueous extract. These results suggest that aqueous extracts of *Echinacea* have more potential than ethanol extracts for modulating the immune response to influenza virus infection, but do not demonstrate direct anti-viral activity.

Introduction

Echinacea had been historically used by Native Americans as an herbal medicine with a wide range of pharmacological properties including antimicrobial, analgesic, snakebite antidote, and the common cold (Felter *et al.*, 1983; Hobbs, 1994). In the present time, *Echinacea* is one of most common herbal medicines primarily used to alleviate common cold symptoms and upper respiratory tract infection especially particularly rhinovirus and influenza virus infections (Caruso *et al.*, 2005). The genus *Echinacea* is composed of 9 species but typically only 3 (*E. angustifolia*, *E. purpurea*, *E. pallida*) are used and studied for potential medicinal properties. Each of these 3 species has shown immunomodulatory potential (Barnes *et al.*, 2005). Each species also has different phytoactive compounds, and the amount of these compounds is dependent upon plant organ used, growing condition of plants, and method of extract preparation (Perry *et al.*, 2001). The efficacy of *Echinacea* as a therapeutic agent during influenza infection has not been clearly established. Although multiple studies have examined the immunomodulatory effects of *Echinacea* with *in vitro* models or *ex vivo* models, the effectiveness of *Echinacea* administration and the potential constituents that may account for bioactivity during influenza infection are not known. Given the challenges of protecting the population against influenza by large scale rapid vaccination efforts, it would be worthwhile to determine if a readily available botanical product may have therapeutic potential.

Influenza virus causes acute contagious respiratory disease by attaching and infecting epithelial cells lining the respiratory tract (Lamb *et al.*, 1996). The initial host defense response involves activation of innate immunity to restrict spread of the virus by producing pro- inflammatory cytokines/chemokines (IFN α/β , IL-1, IL-6, and TNF α) from epithelial

cells, alveolar macrophages and DC (Ronni *et al.*, 1997; Sareneva *et al.*, 1998). Along with these cytokines, influenza virus infection also have been shown to upregulate the mRNA level of MCP-1, MIP-1 α , MIP-1 β , RANTES, IP-10, MIP (neutrophils attractant) and MIP-3 α (immature DC) (Wareing *et al.*, 2004). These inflammatory cytokines and chemokines induce an infiltration of inflammatory cells including monocytes, macrophages, neutrophils to the respiratory tract, along with activation of NK cells (Nguyen *et al.*, 2004) and induction of febrile response (Wareing *et al.*, 2004). Dendritic cells (DC), especially conventional dendritic cells (cDC) detect viral antigen, undergo maturation and migrate to draining lymph nodes for antigen presentation and induction of subsequent adaptive immune response (Grayson *et al.*, 2007). Dendritic cells also produce IL-12 which promotes the development of an effective adaptive immune response by differentiation of naïve CD4 cells into T helper 1 (Th1) cells (Abbas *et al.*, 1996). Th1 cells secrete cytokines that promote maturation and activation of CD8⁺ cytotoxic lymphocytes which are critical in viral clearance. Th1 cytokines promote antibody production, and antibody has an important role in preventing re-infection from the same or related virus strain (Mosmann *et al.*, 1996; Ridge *et al.*, 1998; Ada *et al.*, 1986). Cytotoxic T lymphocytes and virus specific antibody are very important factors in clearance of virus in a primary infection (Miao *et al.*, 2010). Viral specific cytotoxic T lymphocytes attack and destroy infected cells primarily by Fas and perforin mediated mechanisms (Topham *et al.*, 1997). Although direct activation of effector CD8⁺ cells is responsible for viral clearance, it is possible that an indirect effect such as modulation of innate immune parameters ultimately results in enhanced CD8⁺ response. Therefore, agents that modulate either innate or adaptive immune response against influenza may be beneficial for the host.

Echinacea extracts have been shown to activate non specific innate immune responses by stimulating inflammatory cytokine (TNF α , IL-1 α , IL-6, and IL-12) and nitric oxide (NO) production by macrophages (Sullivan *et al.*, 2008). Another study demonstrated that *Echinacea* likely modulates both innate and adaptive immunity by increasing production of multiple cytokines (IL-1 β , IL-4, IL-10, and TNF α) (Zhai *et al.*, 2007). A study by Pillai *et al.*, (2007) reported that aqueous extract of *Echinacea* (rich in polysaccharide) increased CD4⁺ T helper cell activation, and CD4⁺ cells secrete cytokines that play a major role in maintaining cytolytic T cell function and the transition from effector to memory phase. Increased NK cell cytotoxicity and T cell proliferation have also been reported with use of different *Echinacea* extracts. Although there are multiple studies demonstrating that *Echinacea* modulates immunity, very few studies have been designed to determine the efficacy of *Echinacea* during a respiratory infection, or determine whether a specific change of immune response is beneficial with respect to disease outcome. Also, different *Echinacea* species and different types of extracts have not been compared simultaneously in a respiratory infection model. One recent study (Fusco *et al.*, 2010) did assess one type of *Echinacea* extract in an influenza model. In this study there was less weight loss in the *Echinacea*-treated mice but no change in viral titer, suggesting a modifying effect of the extract on clinical course of infection (less body weight loss) without any evident anti-viral effect. In this same study, at the early phase of infection (day 3) a decrease in lung KC, IL-5, and IL-10 was found in *Echinacea* treated mice. By day 7 post-infection, *Echinacea* treatment resulted in reduced IL-10 in serum and lung, as well as decreased serum IFN γ . It is not clear from this study how the immunomodulatory changes might relate to the reduction in weight loss, or to specific immunopathology in the lungs.

To our knowledge, the effect of different *Echinacea* extracts on the immunomodulatory response to influenza has not been studied. The advantage of evaluating different species and/or types of extract preparation is that this approach will likely provide more information with respect to the constituents that may be responsible for bioactivity. The purpose of our investigation was to determine if differences in *Echinacea* species and/or differences in type of extract would result in differential modulation of immune response to influenza viral infection. We hypothesized that the aqueous extracts of *Echinacea* (typically rich in polysaccharides and polyphenols) would be more effective than ethanolic extracts of *Echinacea* in inducing increased immune activation in response to influenza infection.

Material and Methods

Mice: Male BALB/c mice, at 6-8 weeks of age were used in all experiments. The mice were acclimated to the housing conditions in the animal care facility for at least one week before the start of experiments. Harlan 2014 rodent chow by Teklad diets was fed to mice. The Iowa State University committee on animal care has approved the animal procedures. Mice were assigned randomly to different treatment groups containing 10-12 mice per group (number varies with experiment but constant within experiment).

Extracts: *E. angustifolia* (PI631285) and *E. purpurea* (PI649040 & PI631307) were harvested in USDA North central regional plant introduction station at Ames, IA (USA). Alcohol Extracts from the dried roots of these two *Echinacea* species were prepared by a soxhlet extraction method as described by Lalone *et al*, 2007. A Lipophilic metabolite profile of ethanol extracts was performed by Lankun Wu (2008) (Figure 11). Water extracts were prepared by boiling 6 g of dried root in 100 ml of endotoxin free water in an endotoxin free flask, and stirred for 1 hour at room temperature. Extracts were filtered using endotoxin free glass filter paper, centrifuged for 15 min at 10,000 rpm and pellets discarded before freeze drying. Endotoxin analysis of water extracts showed that the *E. angustifolia* extract used in this study had 1172.6 EU/ml whereas *E. purpurea* water extract had 131.6 EU/ml). The ethanol extract of *E. angustifolia* had a non detectable level of endotoxin and the ethanol extract of *E. purpurea* had 49.9 EU/ ml. The biological significance of these levels of endotoxin has not been thoroughly evaluated. Extracts were diluted to 14.67 mg/ml of extract, and the final concentration of the diluted extract was less than 5%. Each mouse was gavaged at a dose corresponding to 110 mg/kg body weight. The vehicle treated group was

gavaged using equal volume of <5% ethanol vehicle or water. The gavage was performed 24 hrs and 48 hrs prior to infection and continued every 24 hrs after infection until euthanization.

Viral infection: Mice were anesthetized with either CO₂ or isoflurane and infected through an intranasal route with 25 µL of influenza A/PR/8/34 virus (sequence similarity to avian influenza virus isolates but less virulent) at a stock concentration of 10^{10.45} EID using dilutions ranging from 1:1 to 1:8 resulting in a low dose infection (~5% mortality) to a high dose infection (~75% mortality). Mice were housed separately after infection so that body weight, food, and water consumption could be measured every 24 hours.

Viral titer quantification: Virus titers were measured by quantitative fluorogenic real-time reverse-transcription polymerase chain reaction (RT-PCR) with TaqMan chemistry. Using sequences deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and the Influenza Sequence Database (<http://www.flu.lanl.gov>), we engineered virus-specific oligonucleotide primers and a fluorescent probe to target a highly conserved region of the swine influenza virus nucleoprotein. The forward primer (SIVRTF:5_-CGGACGAAAAGGCAACGA-3_) and reverse primer (SIVRTR:5_-CTGCATTGTCTCCGAAGAAATAAG-3_) were synthesized by a commercial vendor (Integrated DNA Technologies). A TaqMan MGB probe with a 5_ reporter 6-carboxyfluorescein (FAM) and a 3_ nonfluorescent quencher (SIVRTP: 5_-6- from 50 mL of lung and Bronchiolar Alveolar Lavage Fluid sample, positive control (H1N1 and H3N2 swine influenza viruses) and negative control (elution buffer) using the Ambion MagMAX Viral RNA Isolation FAMCCGATCGTGCCYTC) was synthesized by Applied Biosystems.

To conduct the assay, viral RNA was first extracted Kit (Applied Biosystems) and KingFisher 96 magnetic particle processor (Thermo Scientific). Real-time RT-PCR was then carried out with the QuantiTect Probe RT-PCR Kit (Qiagen) in a 20 ml reaction volume using 4 mL of extracted template. Primers were added at a final concentration of 0.4 mmol/L each, and the probe was added at a final concentration of 0.2 mmol/L. Polymerase chain reaction amplification was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems) with the 384-well format. Cycling conditions were as follows: (1) reverse transcription for 30 min at 50°C, (2) a 15 min activation step at 95 °C, and (3) 40 cycles of 15 sec at 94_°C and 60 sec at 60_ °C. A set of influenza preparations, each with a known virus titer (EID50/mL), were used to generate a standard curve. Samples with threshold cycle values of 35 were considered positive. The amount of influenza in each sample was calculated by converting the threshold cycle value to a virus titer by using the standard curve. A limitation of using PCR for viral titer is that it does not differentiate live replicating virus from other viral particles.

Bronchoalveolar Lavage and tissue collection: Mice were euthanized and lungs were lavaged three times with 1 ml of phosphate buffered saline to collect bronchoalveolar fluid. The bronchoalveolar lavage (BAL) fluid was centrifuged, the supernatant was stored at -80 °C for cytokine/chemokines analysis and viral titer quantification, and the BAL cells were collected for subsequent flow cytometric analysis of cell populations. Lung lobes were also collected after lavage in some experiments to determine viral titer. If lung lesion scoring (histopathology) was performed in the experiment, the whole lung was collected, fixed with 10% formalin and analyzed for lung lesions by a pathologist blinded to treatment group.

Lung pathology: Lung tissue collected at necropsy was fixed in 10% buffered formalin for histopathological examination. After adequate fixation, the tissues were embedded in paraffin wax, sectioned at 5 μ m thickness, stained with hematoxylin-eosin, and examined with light microscopy. The lungs were examined for bronchiolar epithelial changes, including attenuation, proliferation, degeneration, and necrosis. Epithelial damage with influenza infection typically shows a range of degeneration, necrosis, sloughing and regeneration. The amount and severity of peribronchiolar and alveolar inflammation were also evaluated. Sections of lung were given a score from 0 to 3 to reflect an estimate of the percentage of lung tissue containing lesion and the severity of lesions, according to methods described elsewhere [Richt *et al*;2003]. The lung sections were scored according to the following criteria: 0, no significant lesions or minimal epithelial cells change in < 25% of the lung tissue; 1, mild to moderate epithelial cell changes and interstitial pneumonia in 25% to 50% of the tissue; 2, moderate epithelial cell changes and moderate interstitial pneumonia in ~50% to 75% of the tissue; and 3, significant epithelial cell changes and moderate to severe interstitial to bronchio-interstitial pneumonia in ~75% to 100% of the tissue. For each mouse, the number of lung lobes examined and a score for each lobe was recorded. A single pathologist scored all slides and was blinded to the treatment groups.

Cytokine/Chemokines analysis: BAL supernatants were analyzed for cytokines and chemokines with a Luminex Platform (Bio-plex, Bio-rad) and a 32-plex kit (Millipore). Cytokines and chemokines included interleukin 1 α (IL-1 α), interleukin 1 β (IL-1 β), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin

6 (IL-6), interleukin 7 (IL-7), interleukin 9 (IL-9), interleukin 10 (IL-10), interleukin 12p40 (IL-12p40), interleukin 12p70 (IL-12p70), interleukin 13 (IL-13), interleukin 15 (IL-15), interleukin 17 (IL-17), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-inducible protein 10(IP-10), IFN- γ , keratinocyte-derived chemokine (KC), monocytes chemo attractant protein 1 (MCP 1), leukemia inhibitory factor (LIF), LIX, macrophage inflammatory protein 1 α (MIP 1 α), macrophage inflammatory protein 1 β (MIP-1 β), RANTES (regulated on activation, normal T cell expressed and secreted), and tumor necrosis factor α (TNF α), macrophage colony stimulating factor (M-CSF), monokine-induced by interferon- γ (MIG), macrophage inflammatory protein-2 (MIP-2), vascular endothelial growth factor (VEGF).

Cell population Identification: Cells isolated from the BAL fluid were stained with a combination of the following antibodies, (dependent upon the experiment): allophycocyanin–cyanine 7–conjugated anti-mouse CD8 α (53-6.7) for CD8a cells, fluorescein isothiocyanate–conjugated anti-mouse CD11b and phycoerythrin-conjugated anti-mouse Gr1 in high side scatter population as neutrophils, or CD45⁺ (phycoerythrin- cyanine 7 rat anti-mouse CD45), Gr1-, CD11b- as lymphocytes. Appropriate isotype controls were used and cells were analyzed with BD FACSCanto flow cytometer (BD Biosciences). Also, in some experiments, whole lungs were collected and analyzed for CD8 α ⁺ cells that produced IFN γ in response to influenza virus peptides. Briefly, lungs were homogenized, a single cell suspension was obtained, and cells were incubated at 37 °C for 6 hr with one of the following, medium alone, influenza NP peptide (NP 147-158, TYQRTRALVTG) or influenza HA peptide (HA 533-541, IYSTVASSLVL). Following the incubation, cells were

analyzed by flow cytometry using PE-conjugated anti-CD8 α and intracellular APC-conjugated anti-IFN γ to identify activated CD8 α^+ cells responding specifically to influenza virus. Protein transport inhibitor, Monensin, was used to increase accumulation of cytokine within cell by blocking their intracellular protein transport system

Statistical analysis: A one-way analysis of variance was used to compare viral titer, lung lobe score, cytokine and chemokines levels, and cell populations at each day after infection. SPSS software (version 14.0; SPSS) was used in all analyses, and LSD post hoc analyses were performed as needed. To evaluate changes over time in body weight, food and water intake, a mixed analysis of variance (treatment group by repeated measures) was used.

Results

Survival rate (Ethanol extract and water extract):

E. angustifolia ethanol extract treatment does not significantly improve the survival rate of mice infected with influenza virus compared to infected control mice treated with vehicle, when observed up to 8 days after infection (Fig 1a). On other hand, water extract of *E. angustifolia* from the same accession significantly improves the survival rate of infected mice when compared with the vehicle group (Fig 1b).

BAL viral titer:

Viral titer was measured in all experiments. At day 1, 5, or 7 post-infection there was no significant effect of *Echinacea* treatment on the amount of virus in the BAL fluid or whole lung. This held true regardless of whether *E. angustifolia* or *E. purpurea* was tested, and was also the case with either ethanol extracts or water extracts. No effect on BAL viral titer was found with either extract of both *Echinacea* species tested (Fig 2a & 2b).

Lung cytokine and chemokines (ethanol extracts):

The following cytokines and chemokines were shown to be significantly increased in the BAL fluid upon infection: exotaxin, GCSF, GMCSF, IFN γ , IL-1 α , IL-1 β , IL-2, IL-5, IL-6, IL-10, IL-12p40, IL-15, IP-10, KC, LIF, LIX, MCP-1, MIG, MIP-1 α , MIP-1 β , MIP-2, RANTES and TNF α . The following cytokines or chemokines were not significantly altered by infection at the time points measured in this study: IL-9, IL-13, IL-17; and the following cytokines were not detectable: IL-3, IL-4, and IL-7. At day 5 of infection with influenza virus, *E. angustifolia* treatment induced a modest increase in IL-15 and GM-CSF, with a

trend ($p < 0.10$) towards increased IL-12p40, IL-10, and MIP-2 as compared to infected mice treated with vehicle (Fig 3a, 3b & 3c). In contrast, *E. purpurea*-treated mice did not have significantly different levels of cytokines/chemokines than vehicle-treated mice, but did show a trend towards increased GM-CSF ($p=0.09$) (Fig 4a, 4b & 4c). Given that *E. purpurea* ethanol extract treatment appeared to be less effective in modulating cytokines as compared to *E. angustifolia* ethanol extract, further testing at day 7 post infection was done only with *E. angustifolia* ethanol extract. Similar to the findings at day 5, IL-10 tended to be increased in *E. angustifolia* treated mice at day 7 post-infection; however, the general trend was towards a decrease in multiple cytokines and chemokines (Fig 5a, 5b & 5c). At same time, parameters for illness severity such as weight loss and food intake were similar between the groups, ruling out a change in infection severity between the groups.

Lung cytokine and chemokines (water extracts):

At day 5 of infection with influenza virus, the effect of *E. angustifolia* water extract was more evident, with significantly increased in a total of 13 cytokines or chemokines (IL-1 α , IL-12p40, TNF α , IL-5, GCSF, IL-6, KC, MCP-1, MIP-1 β , MIP-2, IP-10, LIF, MIG) (Fig 6a, 6b & 6c) as compared to vehicle treated mice. The magnitude of water extract-induced changes was greater than ethanol extracts, and the water extracts appeared to affect many more cytokines and chemokines as compared to the ethanol extracts of same species.

BAL lung cell populations:

At day 5 post-infection, treatment with *E. angustifolia* ethanol extract or *E. purpurea* ethanol extract did not alter lung cell populations even though chemokine

differences existed at this time point. However, at day 7 post-infection, *E. angustifolia* ethanol treatments resulted in significantly fewer neutrophils (Fig 7a) but total lymphocyte number was not different. With respect to water extract, at day 5 post-infection, there were also significantly fewer neutrophils (Fig 7b). Therefore, the increase in chemokines did not appear to result in greater cell recruitment, but rather a reduction in neutrophils. There were no changes in total CD8 α^+ cells in any experiments; however, at day 8 post-infection, differences were found in the percentage of influenza-specific IFN γ -producing CD8 $^+$ cells. *E. angustifolia* ethanol extract treated mice had significantly fewer CD8 α^+ IFN γ -producing cells in response to NP peptide than vehicle ($p < 0.05$) (Fig 8a). A similar pattern of response was found with respect to HA peptide. In contrast, treatment with water extract of *E. angustifolia* significantly ($p < 0.05$) increased CD8 α^+ IFN γ -producing cells in response to both NP and HA peptides. The treatment with water extracts of *E. purpurea* resulted in a similar type of response as the *E. angustifolia* water extract (a trend toward increased CD8 $^+$ IFN γ -producing cells in response to NP peptide, Fig 8b)

Illness severity (body weight, food intake):

There was no significant difference in body weight loss between mice treated with *E. angustifolia* ethanol extract, *E. purpurea* ethanol extract or vehicle when mice infected with influenza virus were weighed up to day 8 post infection (Fig 9a). Similarly, there was no difference in body weight loss in mice treated with *E. angustifolia* water extracts, *E. purpurea* water extract and vehicle group infected with influenza up to day 7 post infection (Fig 9b). The food intake results paralleled the body weight results in that no significant effects of any of the *Echinacea* treatments were found.

Pathology:

At day 8 post-infection lung lesions scores were not different between *E. angustifolia* ethanol extract treated mice, *E. purpurea* ethanol extract treated mice, and vehicle treated mice (Fig 10).

Discussion

Although many studies have investigated the effect of *Echinacea* extracts on specific immune cell populations (macrophages, monocytes, NK cells, T cells in cell cultures), data on the impact of *Echinacea* in a relevant *in vivo* disease model are limited. To our knowledge, this is the first study to evaluate the effect of different *Echinacea* species and different types of extract in the same experimental influenza infection model. The one similar study published to date used only aqueous extract from the aerial part of *E. purpurea* in a mouse model of influenza infection (Fusco *et al.*, 2010). A separate study examined the effect of *Echinacea* during influenza infection, but *Echinacea* was just one of a multi-component preparation, and therefore it was difficult to draw any specific conclusions about the role of *Echinacea* alone. Again, neither of these published studies compared different types of extracts from different species to better understand the potential bioactive constituents.

The findings from the present experiments suggested that water extract both from *E. angustifolia* and *E. purpurea* have a more pronounced effect on the immunomodulation of cytokines than ethanol extracts from the same species without affecting viral titer and course of infection during the early phase of infection. In general, it appeared that water extracts have a non-specific stimulatory effect in that the majority of the cytokines and chemokines measured were found to increase (IL-1 α , IL-5, IL-10, IL-12p40, TNF α , KC, MIP-1 α , MIP-1 β , IP-10, GCSF, LIF, MIG, IFN γ), rather than a specific group of cytokines/chemokines. The extraction process plays an important role in isolating different phytochemical from the plant by the use of different type of solvents. Water extracts primarily retain hydrophilic

constituents rich in polysaccharides and some polyphenols whereas ethanol solvent used for alcoholic extracts results in retention of alkaloids and some polyphenols. The polyphenols group in *Echinacea* can be extracted by ethanol-water solvent (Hall, 2003) but it can also be detected in only water and only ethanol extract. Although a phytochemical analysis of water extract used in our study was not available, the literature suggests that high molecular mass polysaccharides are the main component of water extracts from root of *Echinacea* plant.

Polyphenols and alkaloids constitute the ethanolic extract of *Echinacea* root. According to Matthias *et al.* (2004), alkaloids, but not polyphenols are capable of crossing the intestinal barrier and thus only alkaloids are considered bioavailable. The ethanol extracts of *Echinacea* used in our experiments were analyzed for their lipophilic metabolite profile by our center (Lankun Wu, Dissertation p28). This analysis showed that *E. angustifolia* ethanol extract had a relatively high abundance of Amide 8, whereas *E. purpurea* extract had amide 2, amide 3, amide 8, and amide 10 in equal abundance. Therefore, it is possible that the bioactivity observed in the water extracts was due primarily to polysaccharides whereas the activity in ethanol extracts may have been due to the alkaloids, particularly alkaloid 8, although further studies will be required to confirm this.

The effect of ethanol extracts from both plant species with respect to enhancement of cytokines or chemokines was more modest than the effect of water extracts in the early days of infection. At day 5 post-infection, there was an increase in IL-15, GM-CSF, and IL-12p40, but at day 7 post-infection, a decrease in IL-1 β , IL-6, KC, and eotaxin was found. At both time points, there was a tendency towards increased IL-10. Therefore, it is possible that ethanol extracts of *E. angustifolia* act to modestly enhance cytokine/chemokine expression in the early phase of infection, but then exhibit anti-inflammatory effects at the later phase of

infection. In contrast to *E. angustifolia* ethanol extract, the ethanol extracts from *E. purpurea* had no significant effects in the influenza model of infection.

Nearly all extracts of *Echinacea* tested resulted in increased KC expression in the early phase of infection (except *E. purpurea* ethanol extract). KC is considered an important neutrophil attractant. However, findings from another study suggest that macrophage/monocyte recruitment and the chemokines involved in recruitment of these cells (MCP-1, MIP1 α) may have a greater role in overall inflammation and survival than neutrophils (Dawson *et al.*, 2000). Therefore, a change in neutrophil number as induced by *Echinacea* may not have a large impact on overall disease outcome. Water extracts of *E. angustifolia* and *E. purpurea* stimulated a wide range of pro-inflammatory cytokines (IL-1 α , IL-5, IL-6, IL-12p40, and TNF α) and chemokines (KC, GCSF, MCP-1, MIP-1 α , MIP-2, IP-10). Pro-inflammatory cytokines, particularly IL-6 and TNF α are secreted by activated alveolar macrophages, and the finding that IL-6 and TNF α are increased in the BAL corresponds with other research on *Echinacea* in which water extracts have been shown to stimulate macrophage cytokine production *in vitro* (Burger *et al.*, 1997; Sullivan *et al.*, 2008, Goel *et al.*, 2002). Although activated macrophages may have some anti-viral effects, studies have shown that inflammatory monocytes and macrophages and the cytokines produced by these cells at the site of infection are major contributors to influenza-associated immunopathology (Lin *et al.*, 2008). In spite of the *Echinacea*-associated increase in multiple inflammatory cytokines, mortality did not increase, suggesting that the enhancement of multiple cytokines is not detrimental. Therefore, it is possible that *E. purpurea* or *E. angustifolia* water extracts alter in a beneficial manner the careful balance between

promoting a productive immune response through activation of inflammatory responses, yet limiting inflammatory-induced cell damage. It is important to note that although water extracts of *Echinacea* altered cytokines and chemokine response, there was no change in viral load in the lungs, or an improvement in symptoms (body weight loss, food intake) up to day 5 post-infection. However, *E. angustifolia* water extract treatment reduced mortality when assessed up to day 8 post-infection. Therefore, it remains possible that in the later stages of infection (day 7-12) viral clearance is improved and/or symptoms of illness are attenuated by *Echinacea* water extract treatment.

In contrast to wide range of cytokines and chemokines increased with water extracts, the results showed that after influenza infection, during the transition from innate to adaptive host defense (~day 5 post-infection), only IL-15, IL-12, and IL-10 tended to be greater in BAL of mice treated with ethanol extract of *E. angustifolia*. IL-15 may promote NK cell activation and memory T cell survival (Nogusa *et al.*, 2009), and therefore an increase in IL-15 may enhance viral clearance by NK cells. Other studies have shown stimulation of NK cells by *Echinacea* extract (Currier *et al.*, 2000; Brousseau *et al.*, 2005) and it is possible that the mechanism of NK activation is IL-15 dependent. However, it is important to note that by day 5 post-infection, viral clearances was not improved in ethanol extract-treated mice. Therefore, if NK cells have greater activation as a result of *Echinacea* treatment, it either occurs at a later time point or does not translate to more effective viral clearance. With respect to cytotoxic T cell function, the results showed that influenza-stimulated production of IFN γ in CD8⁺ cells was not enhanced by *Echinacea* ethanol extract. Memory T cell survival was not assessed, and therefore in future studies it will be important to determine

whether the enhancement of IL-15 production has an effect on memory T cells. Finally, a trend toward increased production of IL-10 at day 5 and day 7 post-infection in *E.*

angustifolia ethanol extract treated mice suggested there may be an earlier activation of regulatory mechanisms to limit inflammation to prevent further damage of lung tissue. It is also noteworthy that *E. purpurea* ethanol extract did not show any activity in modulation of cytokine in mice infected with influenza virus.

Activation of certain cytokines and chemokines in the early phase of infection as a result of treatment with *E. angustifolia* ethanol extracts might be expected to result in influx of large number of immune cells to local tissue (lung). However, no increase in total number of leukocytes in the BAL was found by day 7 post-infection, and treatment with *E. angustifolia* ethanol extract decreased neutrophil influx to lungs. A similar effect was observed with respect to influenza-stimulated IFN γ producing CD8⁺ cells in the lungs of influenza infected mice treated with *E. angustifolia* ethanol extract. There was a significant decrease in the influx of CD8⁺IFN producing cells to lungs with *Echinacea* ethanol treatment but no effect on IFN γ production on a per cell basis (MFI). Taken together, these findings suggest that ethanol extracts of *E. angustifolia* may exhibit anti-inflammatory activity in the later stages of infection.

The reduction in BAL neutrophil number was observed with both water and ethanol extracts of *E. angustifolia*. The apparent inconsistency between early phase infection increases in neutrophil chemokines (KC) and later phase reductions in neutrophils number may be attributed to either a failure of cytokines to recruit cells (integrins or attachment failure) or may be a compensatory immune mechanism to prevent further lung

immunopathology. The contribution of neutrophils to lung injury has been demonstrated, but neutrophils have also been shown to limit the extent of influenza virus replication and disease progression in lungs (Tate *et al.*, 2009). A decrease in neutrophils can result in enhanced viral replication, high mortality and severe illness, although our findings show no effect of extracts on viral titer, illness markers, or pathological lesion score between any of the extract treatments and vehicle control groups across multiple time points. It is possible that the neutrophil effect is more important during the earliest phase of infection (days 1-4), and by day 5-7, increase in CD8a⁺ cells and initiation of adaptive immune responses become more important in clearing virus, and therefore the *Echinacea*-induced decline in BAL neutrophil number at day 5-7 may not have a negative impact on infection outcome.

In contrast to ethanol extracts, *E. angustifolia* water extract increases the number of influenza specific IFN γ producing CD8⁺ cells in the lungs at day 7 post- infection. Similarly, water extracts of *E. purpurea* tended to increase IFN γ producing CD8⁺ cells. There was no effect on IFN γ production on a per cell basis (MFI) with use of either extract, rather an effect on number of cells. These findings are consistent with wide range stimulation of pro-inflammatory cytokines by treatment with water extracts of *Echinacea*. An increase in influenza-induced IFN γ -producing CD8⁺ cells may be expected to result in a more rapid viral clearance. It is possible that viral clearance was improved at a later time point (8-10 days post-infection), but viral load was not assessed at these later time points in our experiments.

Overall, the findings from these experiments show that water extracts and ethanol extracts prepared from the same species of *Echinacea* have very different effects *in vivo*. This is likely due to the differing constituents that are expected to be present in water extracts

(polysaccharides and polyphenols) as compared to ethanol extracts (alkamides and some polyphenols). The *in vivo* effect of ethanol extracts is consistent with other *in vitro* results from our research group in that anti-inflammatory effects were observed (although these were confined to the later stages of infection). Alkamides have been shown to be present in the ethanol extracts used in these experiments and may account for anti-inflammatory properties. The constituents present in water extracts used in these studies are not yet available, but based on research from other groups, it is likely that water extracts contain polysaccharides (and polyphenols) which appear to exhibit immunostimulatory effects. Finally, it is important to note that although immunomodulatory effects were observed, there was no reduction in viral load in the lungs up to day 5 post-infection (with peak viral titers expected between day 2-4 post-infection). It remains possible that benefits would be observed in the resolution phase of infection (day 10-14), and those experiments are the focus of future research. It is also possible that the active fractions in each extract interact in such a way with each other or constituents of diet as to nullify the effects of each other, resulting in no change in symptom severity. Again, further work will seek to identify the role of specific bioactive components.

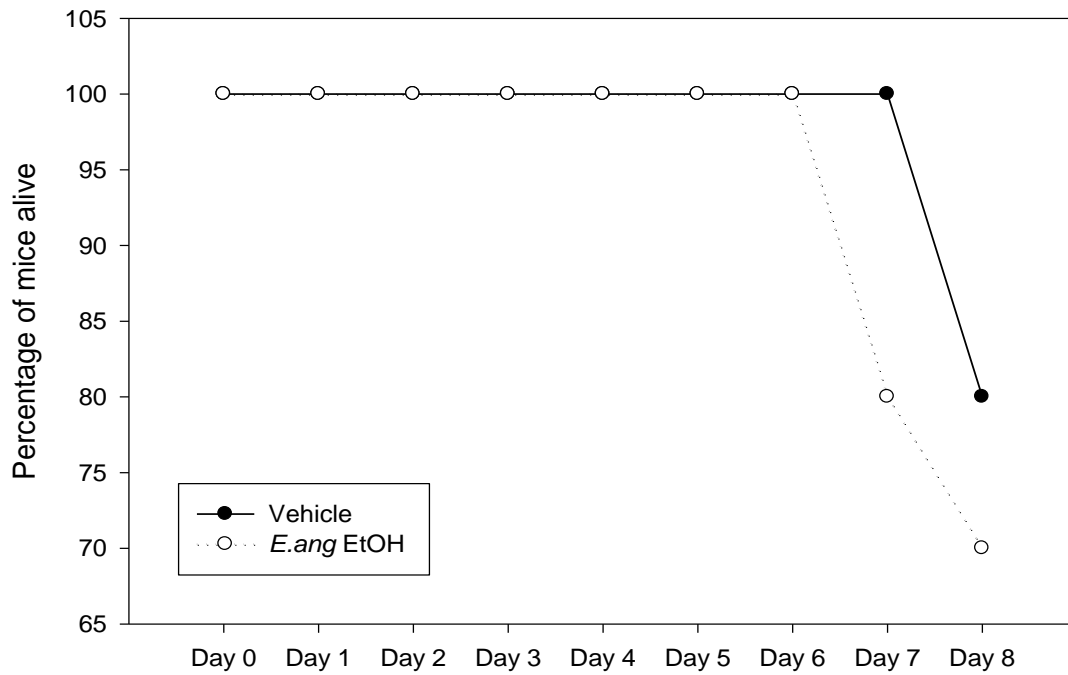
Figures

Figure 1a: Survival rate of mice treated with ethanol extract of *Echinacea angustifolia* (*E.ang* EtOH) at day 8 after infection. 10 mice per group were used and were infected with influenza virus. 5% ethanol was used for vehicle group and *E. angustifolia* ethanol extract was gavaged in the *E.ang* EtOH group.

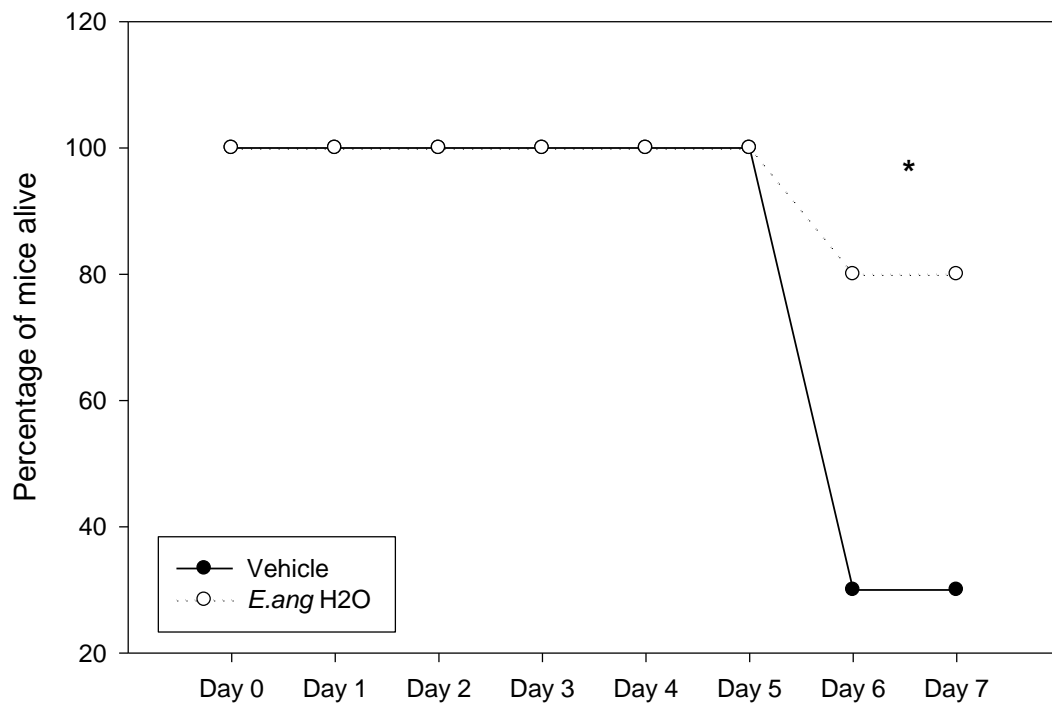


Figure 1b: Survival rate of mice treated with water extract of *Echinacea angustifolia* (*E. ang* H2O) at day 7 after infection. 10 mice per group were used and were infected with influenza virus. Water was used for vehicle group and *E. ang* H2O- *E. angustifolia* water extract was gavaged. Survival rate in the *E. ang* EtOH groups was significantly higher than the vehicle treated group ($p < 0.05$).

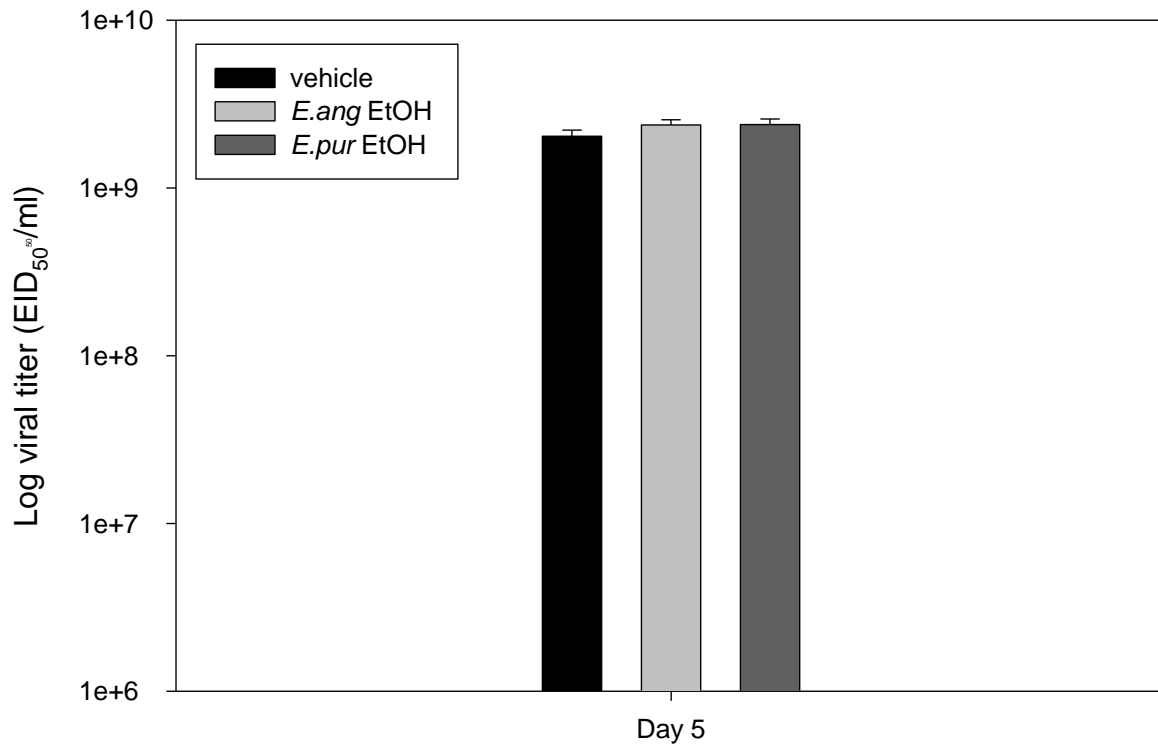


Figure 2a: Viral titer at day 5 post infection using ethanol extract of *Echinacea angustifolia* and *Echinacea purpurea*. 10-12 mice per group were used and lungs were collected to run RT-PCR for quantification of virus. 5% ethanol used as vehicle. *E.ang* EtOH- *E. angustifolia* ethanol extract, *E. pur* EtOH- *E. purpurea* ethanol extract. There were no significant differences between treatment groups.

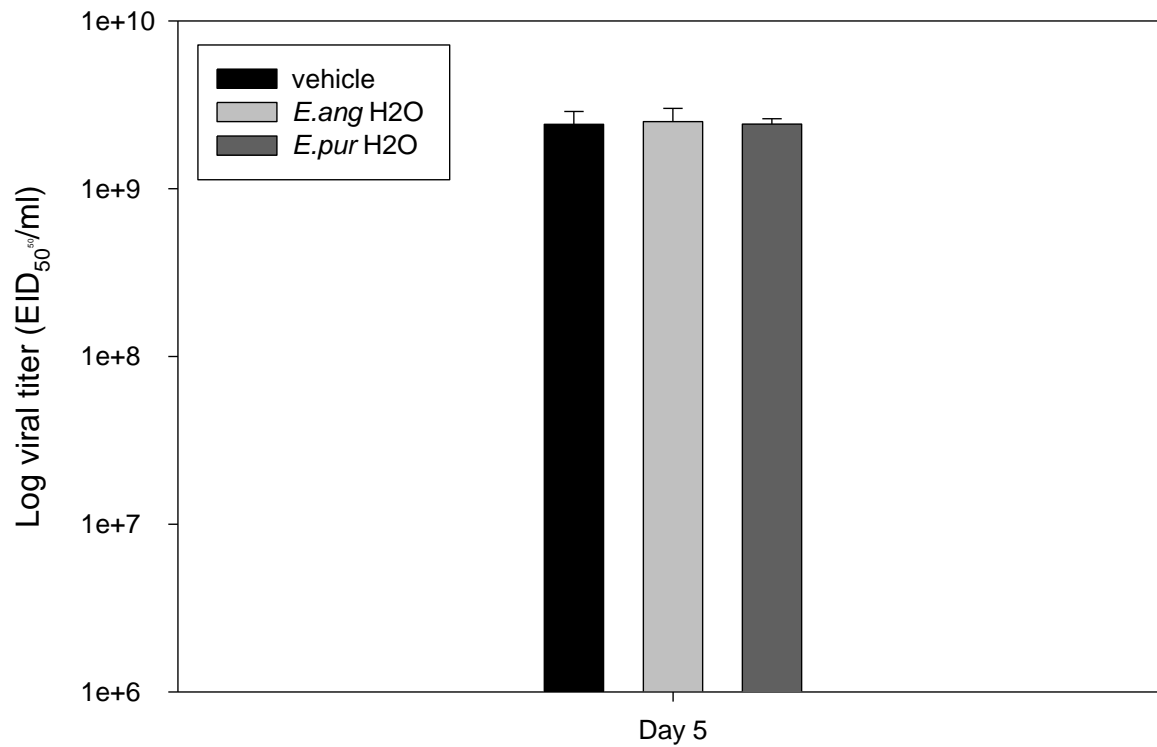


Figure 2b: Viral titer at day 5 post infection using water extract of *Echinacea angustifolia* and *Echinacea purpurea*. 10-12 mice per group were used and lungs were collected to run RT-PCR for quantification of virus. 5% ethanol used as vehicle. *E.ang* H2O= *E. angustifolia* water extract, *E. pur* H2O= *E. purpurea* water extract. There were no significant differences between treatment groups.

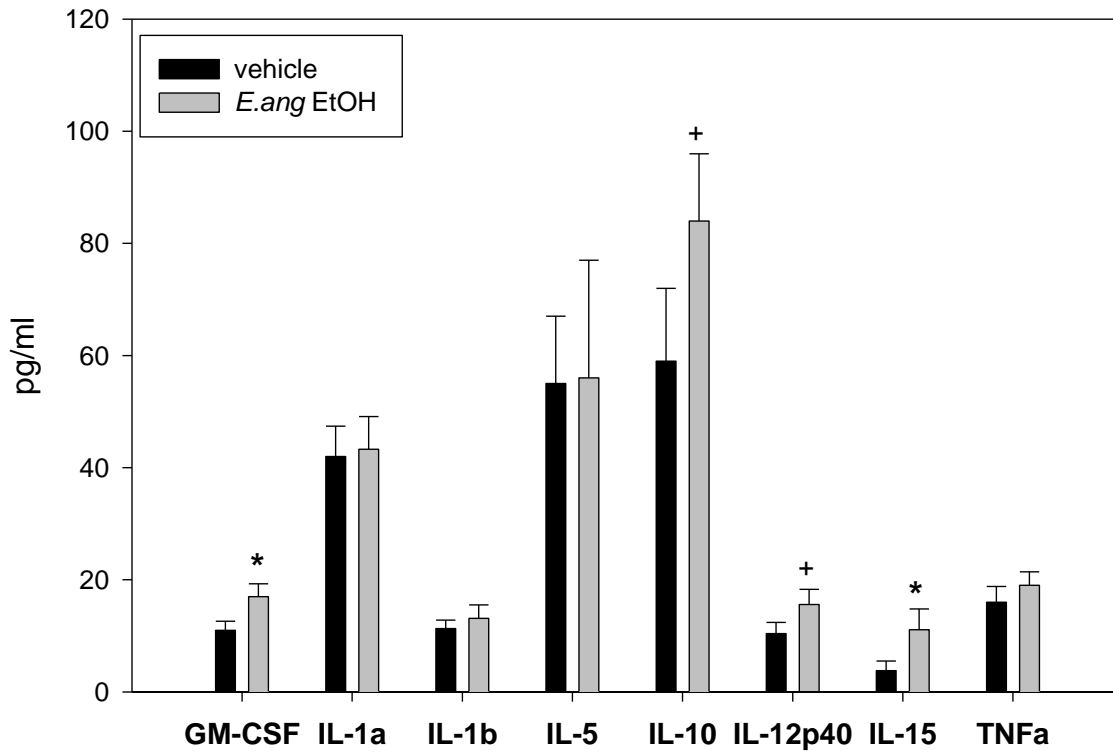


Figure 3a: Cytokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E.ang* EtOH= *E. angustifolia* ethanol extract. (*- $p < 0.05$; +- $p < 0.1$)

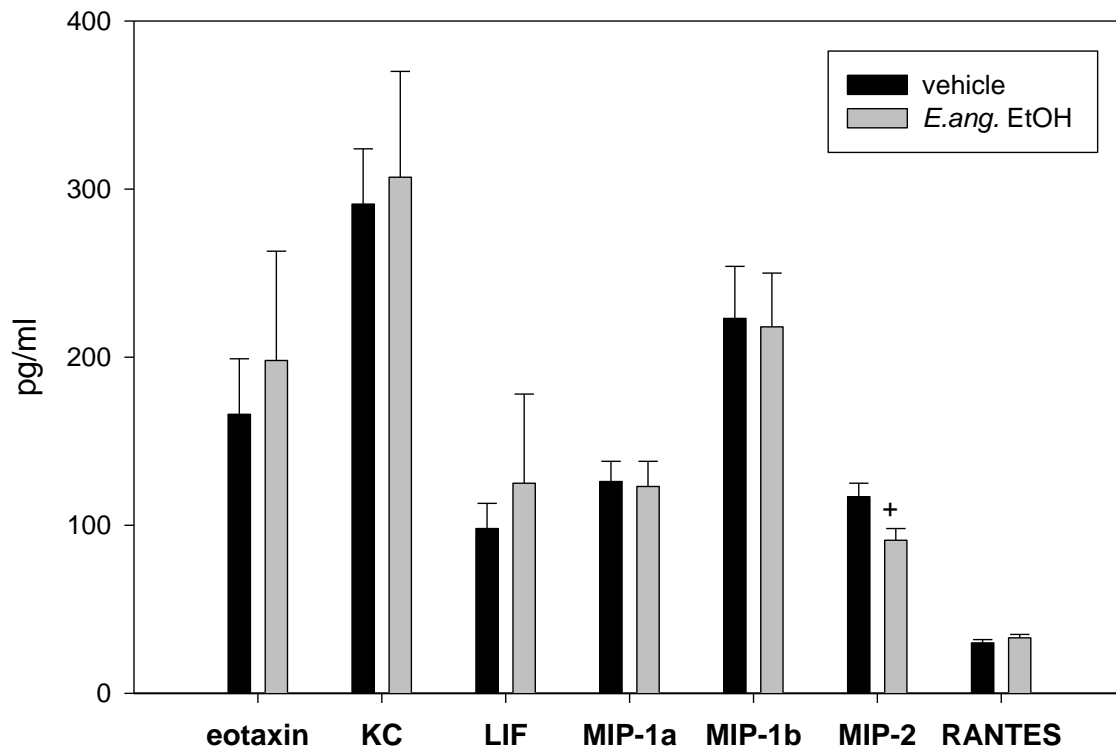


Figure 3b: Cytokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed 32-plex kit. *E.ang* EtOH= *E. angustifolia* ethanol extract. (+-p<0.1)

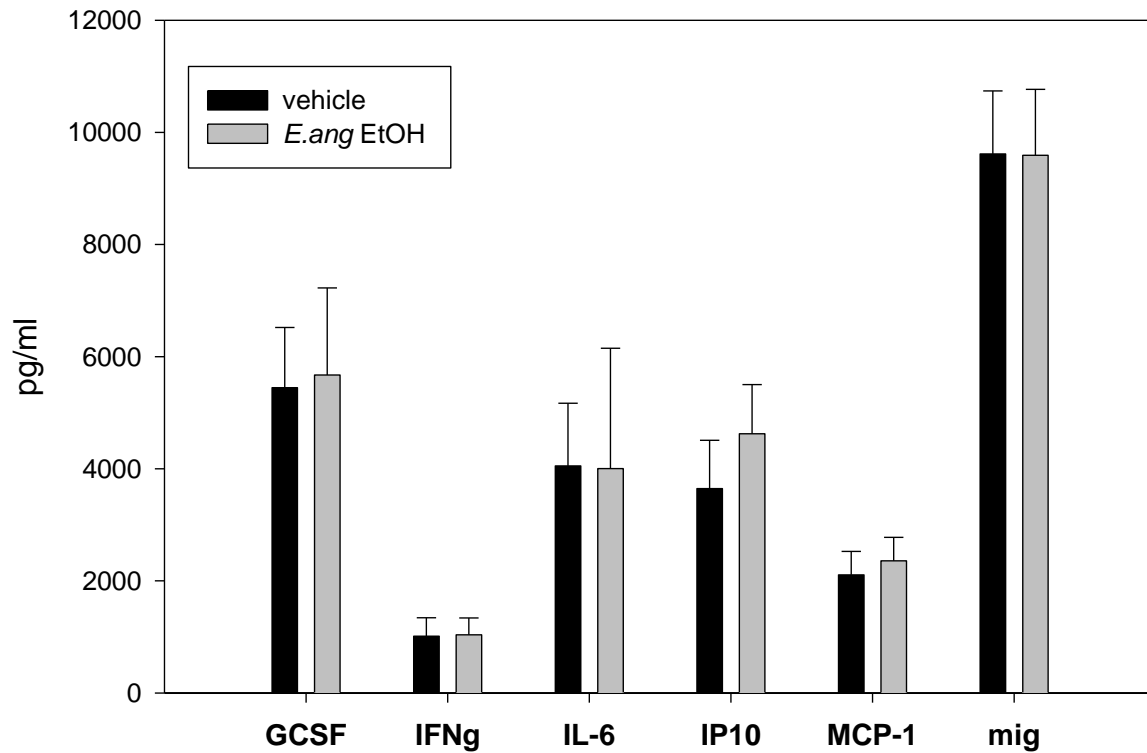


Figure 3c: Cytokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E.ang* EtOH= *E. angustifolia* ethanol extract.

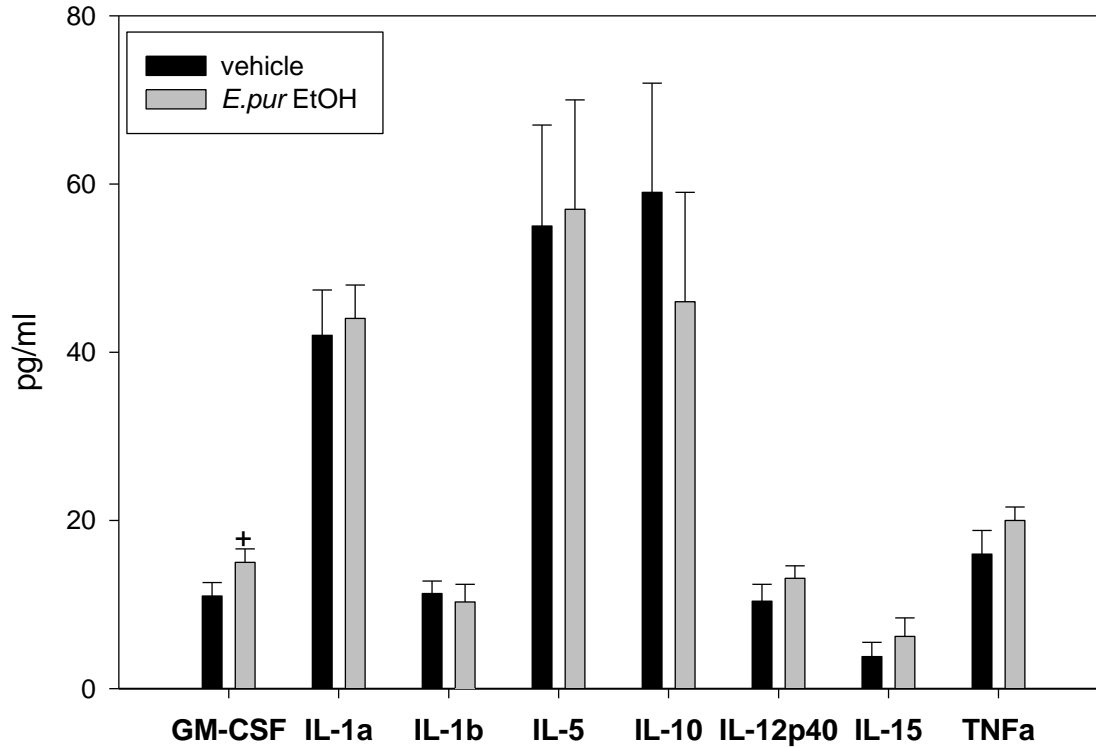


Figure 4a: Cytokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E.pur* EtOH= *E. purpurea* ethanol extract. (+p<0.1)

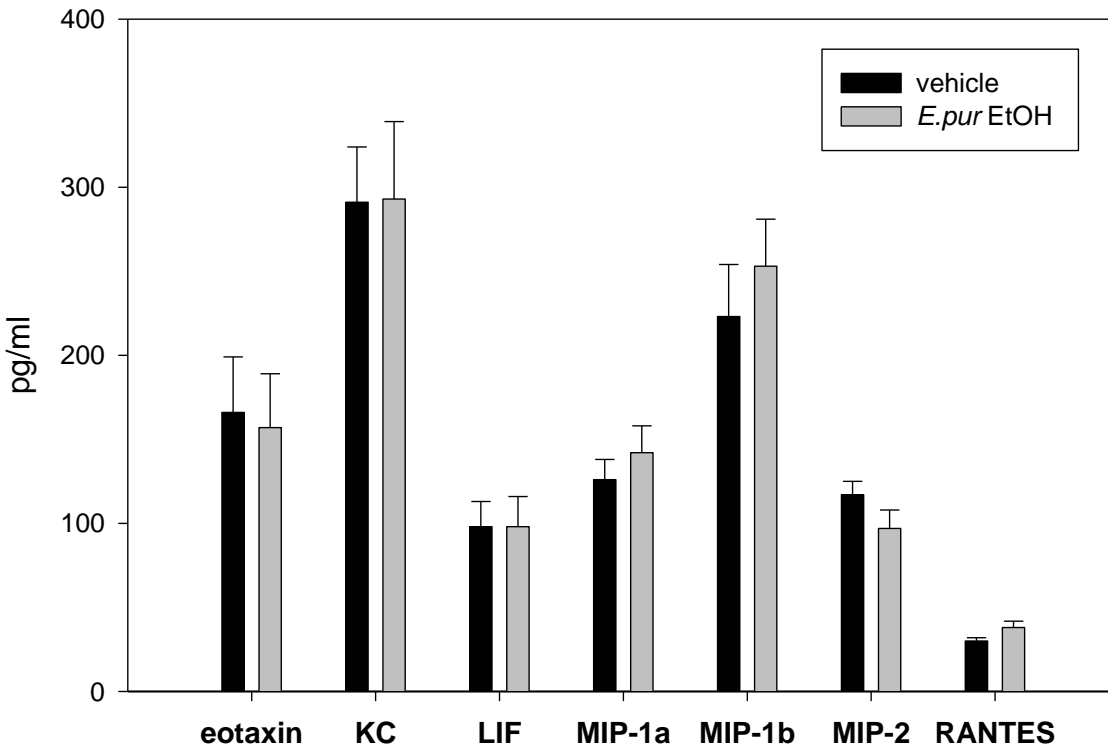


Figure 4b: Chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E.pur* EtOH= *E. purpurea* ethanol extract. There were no significant differences between groups.

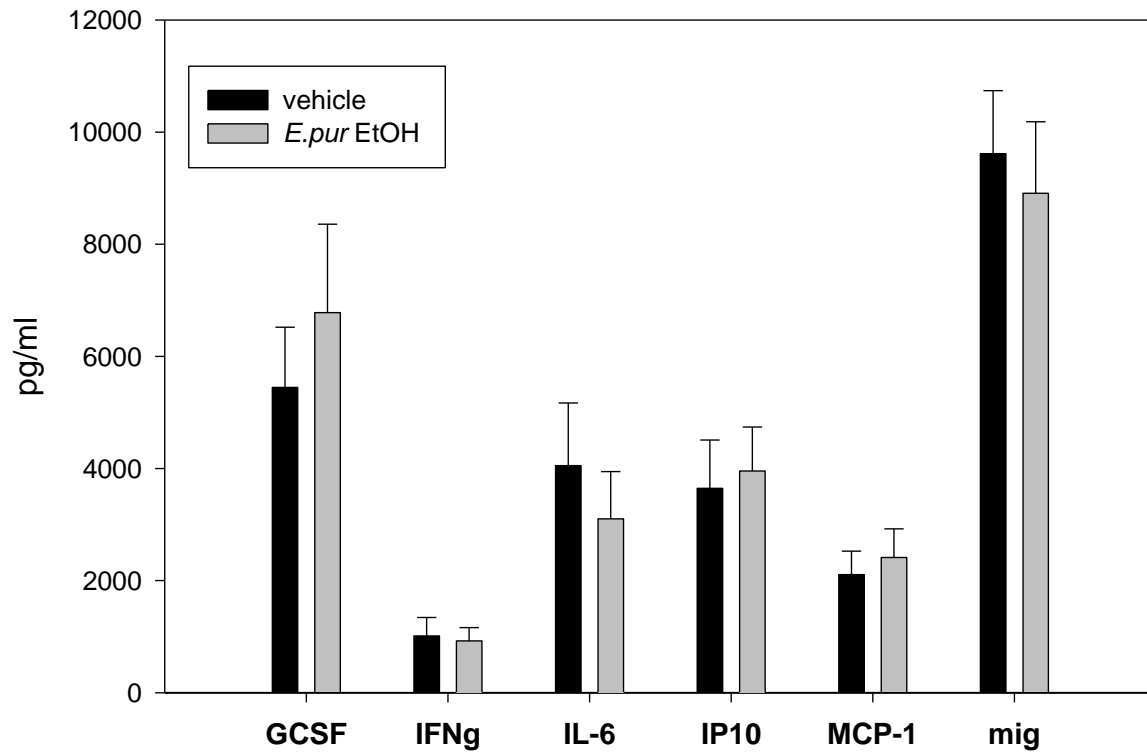


Figure 4c: Chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E.pur* EtOH= *E. purpurea* ethanol extract. There were no significant differences between groups.

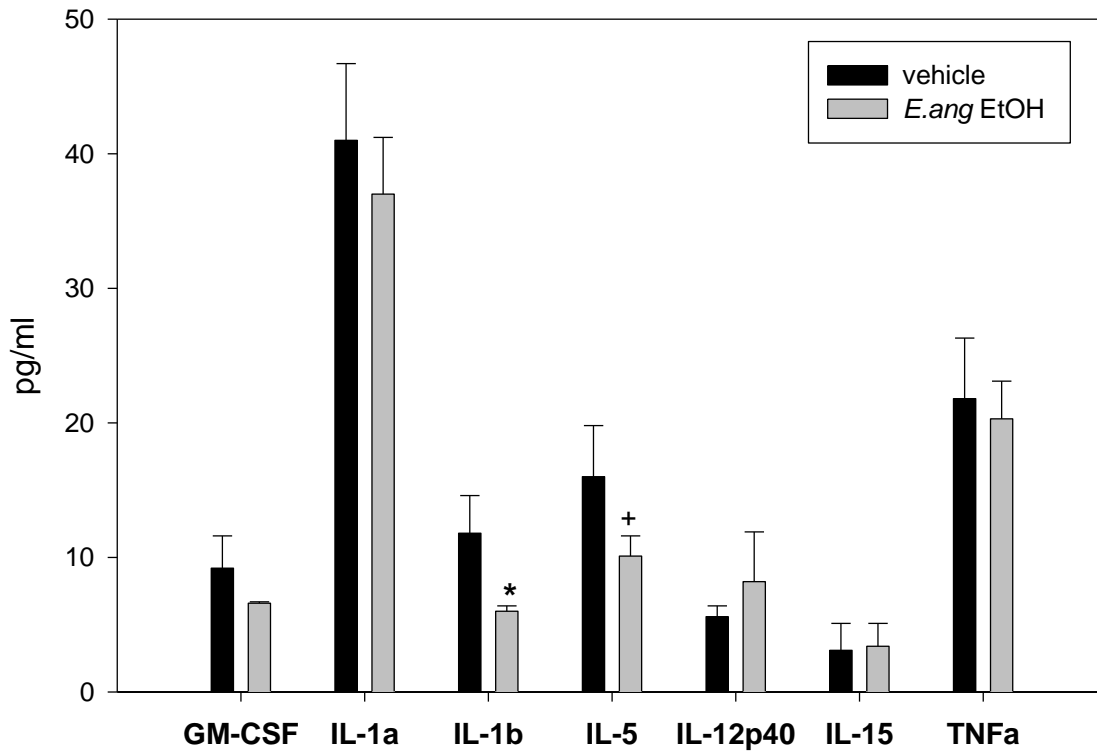


Figure 5a: Cytokine profile in BAL fluid at day 7 post infection using Ethanol extract of *Echinacea angustifolia*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 7 days of infection with influenza and was analyzed using 32-plex kit. *E.ang* EtOH= *E. angustifolia* ethanol extract. (*- $p < 0.05$; +- $p < 0.1$)

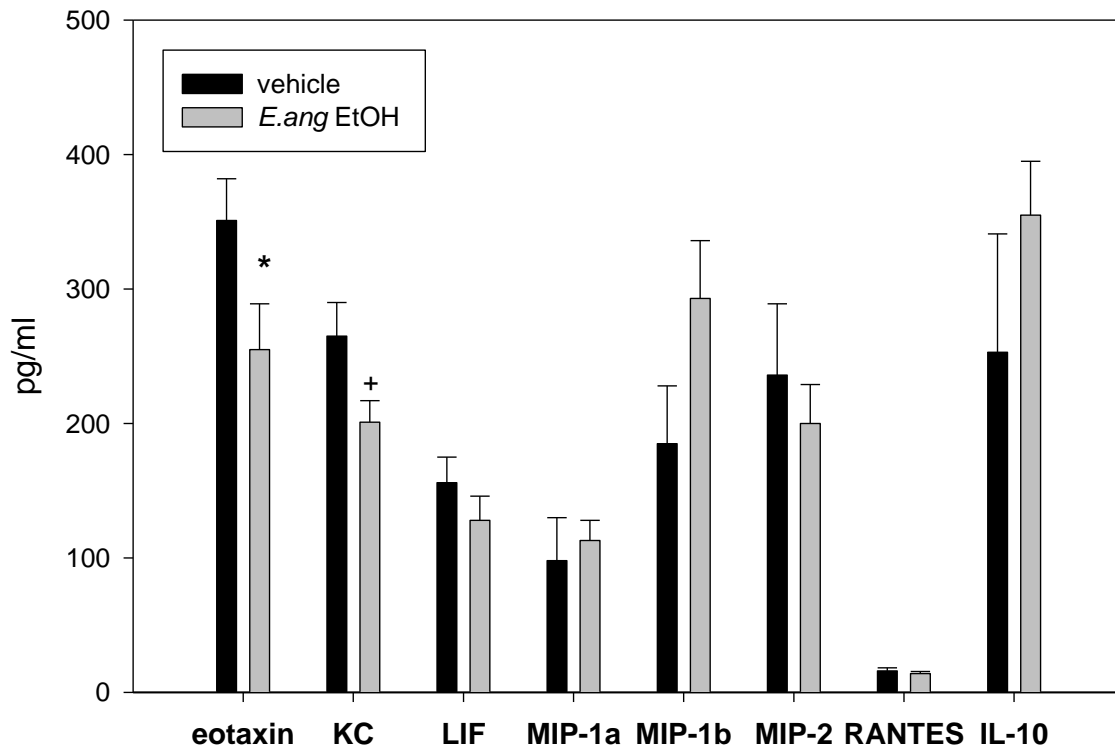


Figure 5b: Cytokine profile in BAL fluid at day 7 post infection using Ethanol extract of *Echinacea angustifolia*. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 7 days of infection with influenza and was analyzed using 32-plex kit. *E.ang* EtOH= *E. angustifolia* ethanol extract. (*- $p < 0.05$; +- $p < 0.1$)

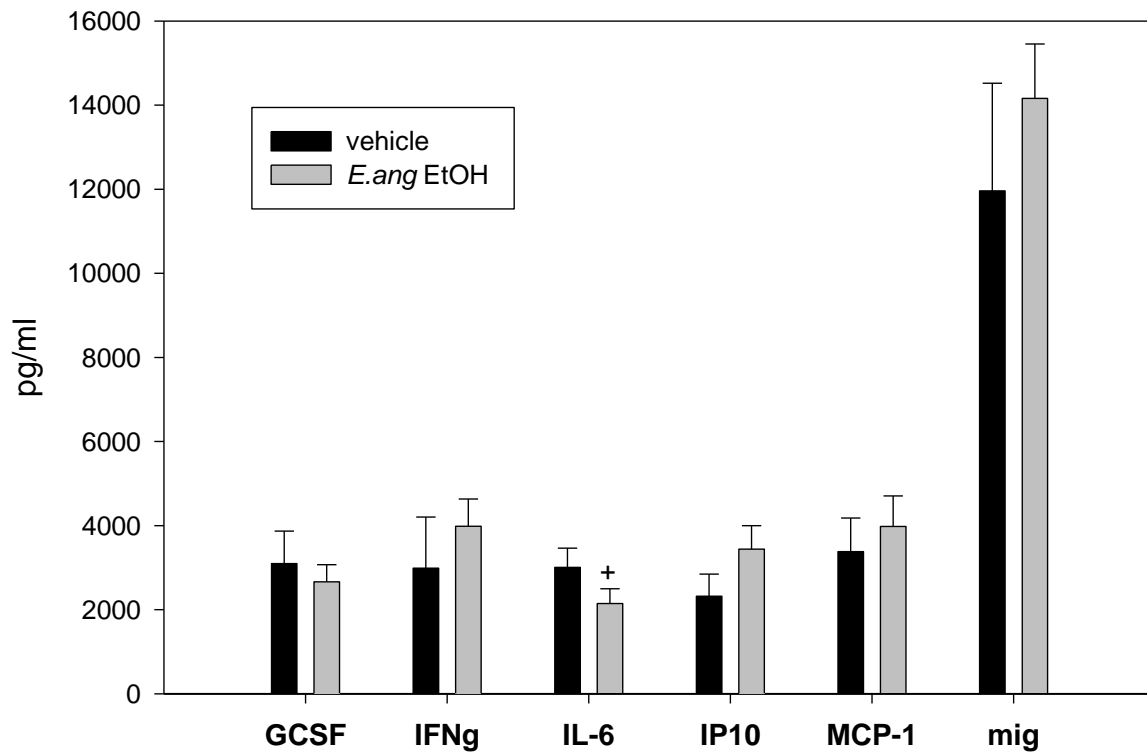


Figure 5c: Cytokine profile in BAL fluid at day 7 post infection using ethanol extract of *Echinacea angustifolia*. 10-12 mice per group were used. Ethanol 5% was used as vehicle and *E.ang* EtOH- ethanol extract of *E. angustifolia*. (*- $p < 0.05$; +- $p < 0.1$)

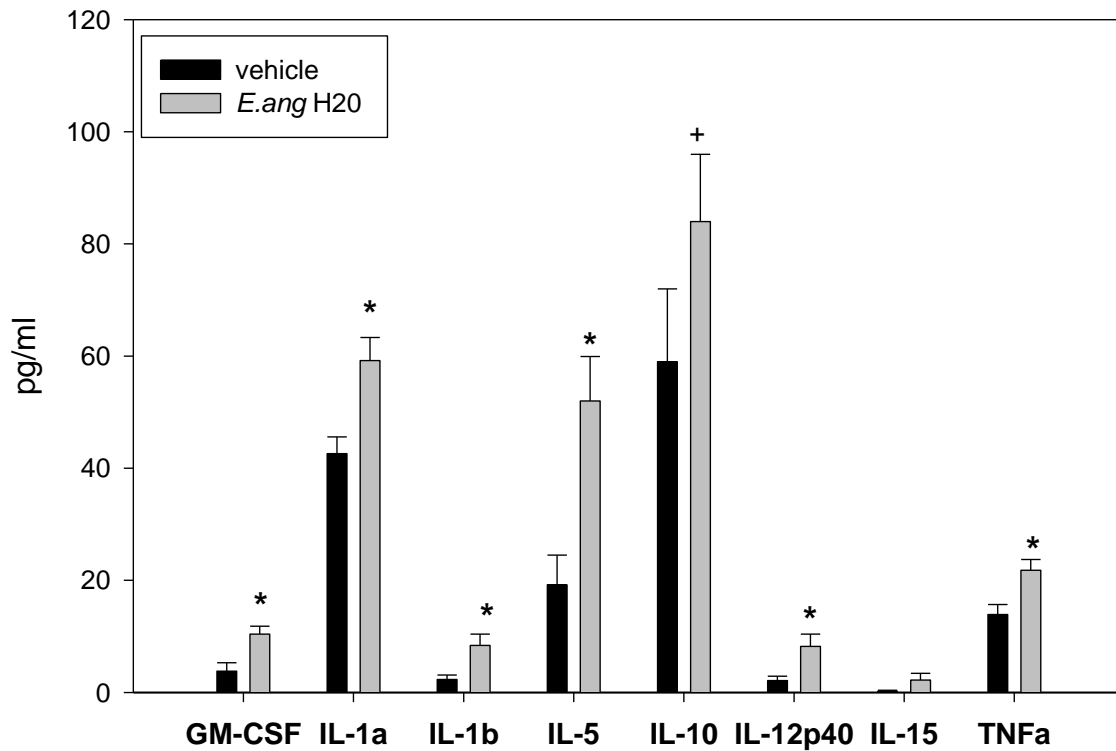


Figure 6a: Cytokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia*. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. Significant increase in wide range of cytokines and chemokines was observed in group gavaged with extract. *E. ang* H2O= *E. angustifolia* water extract. (*- $p < 0.05$; +- $p < 0.1$)

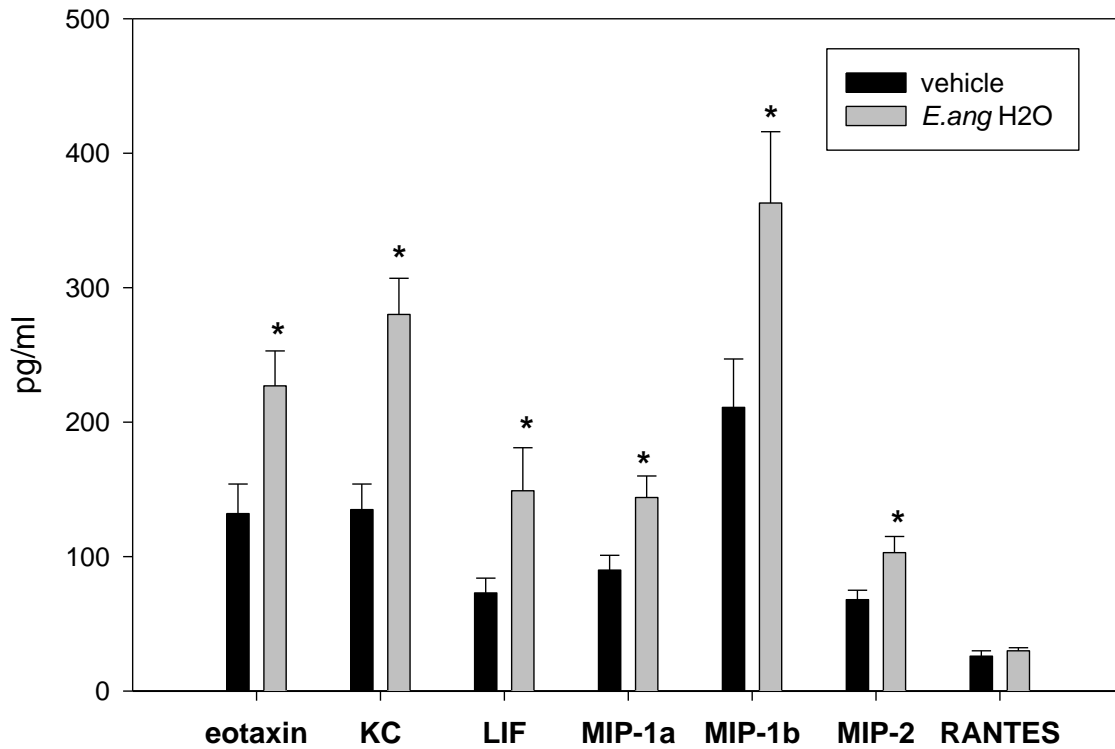


Figure 6b: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia*. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. Significant increase in wide range of cytokines and chemokines was observed in group gavaged with extract. *E. ang* H2O= *E. angustifolia* water extract. (*-p<0.05)

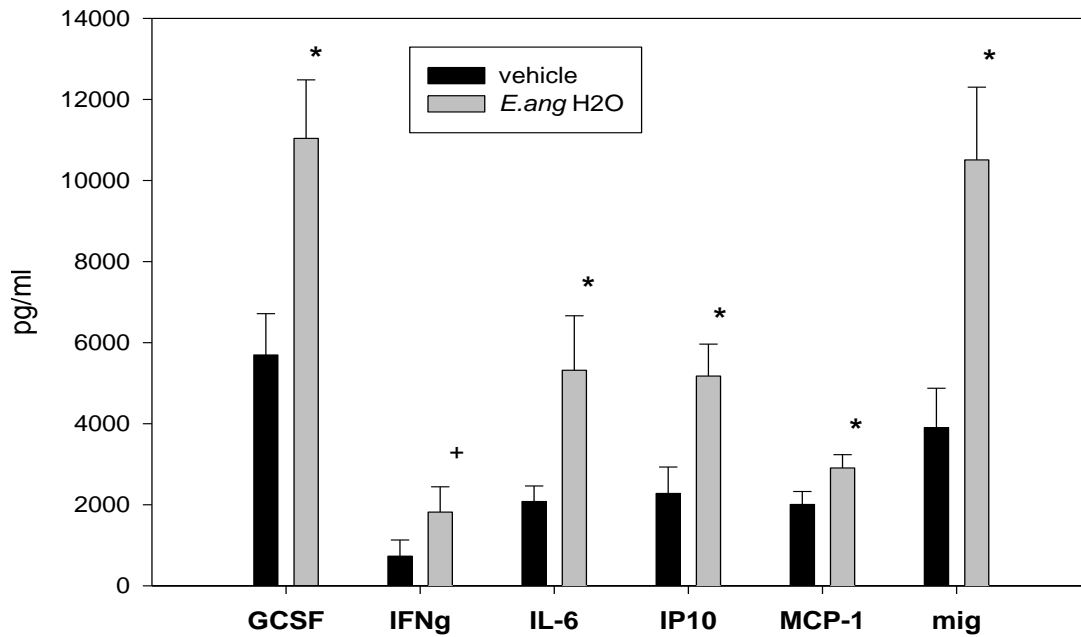


Figure 6c: Cytokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia*. Vehicle group was gavaged with water 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. Significant increase in wide range of cytokines and chemokines was observed in group gavaged with extract. *E. ang* H2O= *E. angustifolia* water extract. (*-p<0.05; +-p<0.1)

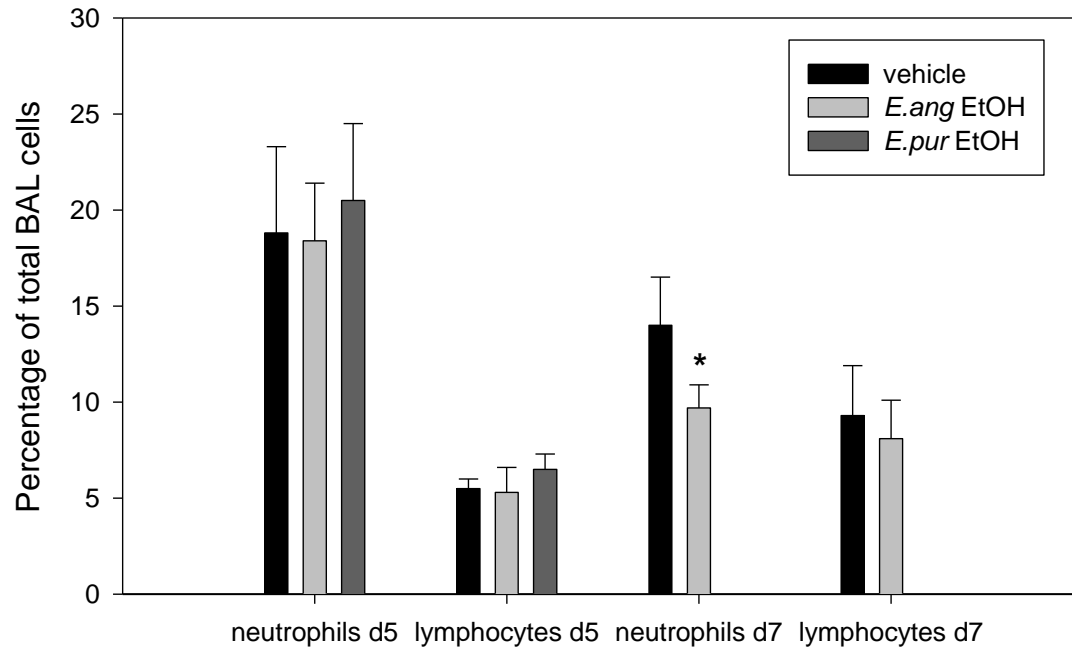


Figure 7a: Cell population in BAL Fluid after treatment with ethanol extract of *E. angustifolia* and *E. purpurea* at day 5 and day 7 of infection with influenza virus. BAL cells were stained for surface markers and determined by flow cytometry. 10-12 mice per group were used. Ethanol 5% was used to gavage vehicle group. *E. angustifolia* EtOH= *E. angustifolia* Ethanol extract, *E. purpurea* EtOH= *E. purpurea* Ethanol extract. Note that at Day 7 experiment, *E. purpurea* Ethanol extract was not tested. (*-p<0.05)

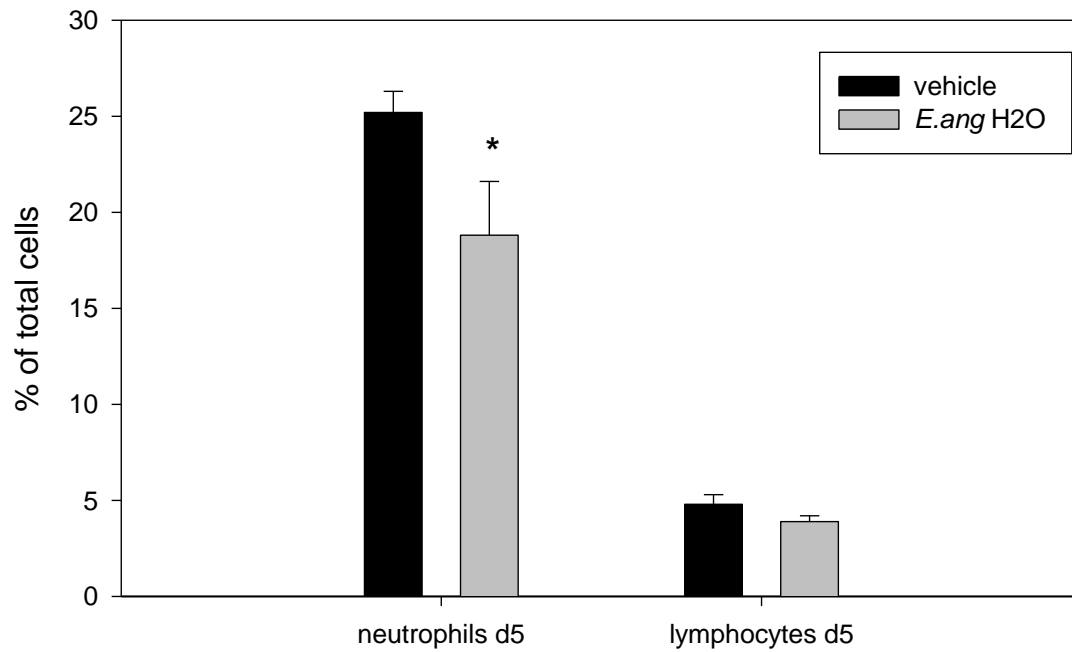


Figure7b: Cell population in BAL Fluid after treatment with water extract of *E. angustifolia* at day 5 of infection with influenza virus. BAL cells were stained for surface markers and determined by flow cytometry. 10-12 mice per group were used. Water was used to gavage vehicle group. *E. ang* H2O= *E. angustifolia* water extract. (*-p<0.05)

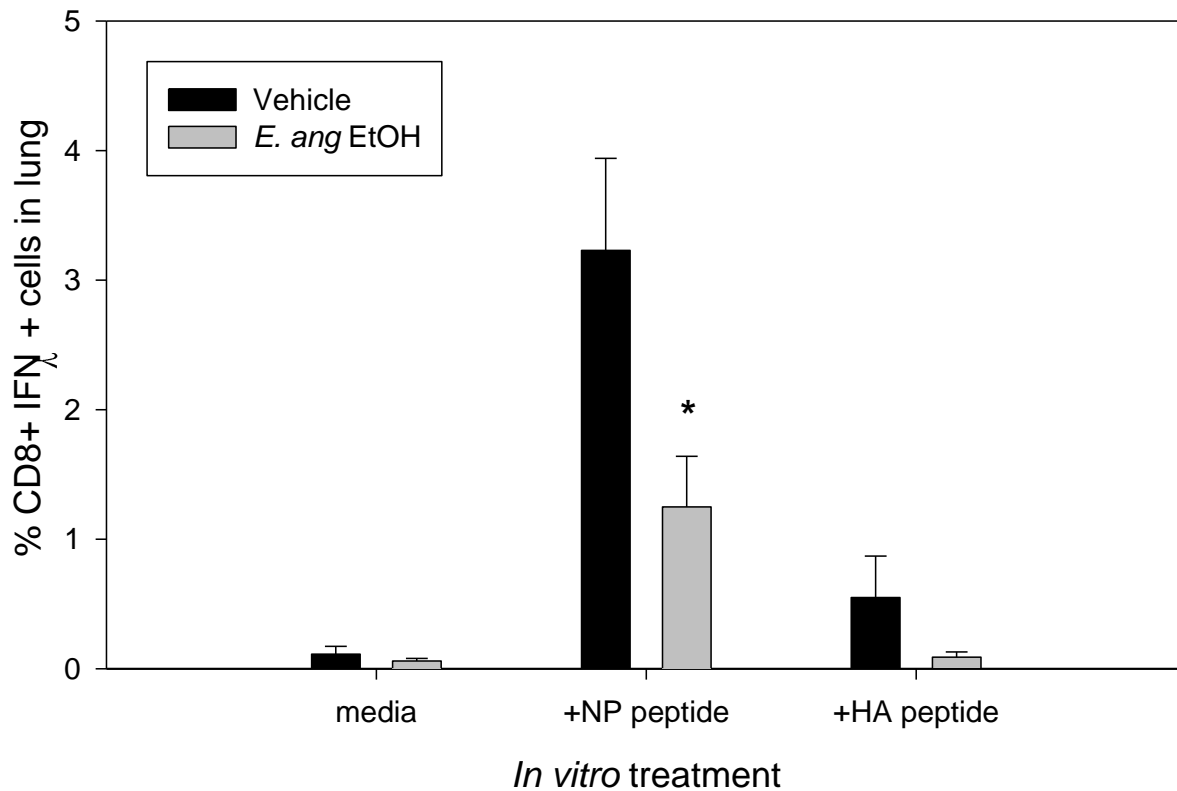


Figure 8a: Percentage of CD8⁺ cells producing IFN γ in response to *in vitro* stimulus in lung of mice infected with Influenza at Day 7 Post infection and gavaged with Ethanol Extract of *E. angustifolia*. 10-12 mice per group were used and lungs were collected for intra-cell staining. Stimuli in vitro was from Influenza virus specific peptides- HA and NP. Staining was done CD8⁺ surface marker and intra cell for IFN γ production and samples run under flow cytometry. Vehicle= ethanol 5%, *E. ang* EtOH- ethanol extract of *E. angustifolia*.

(*-p<0.05)

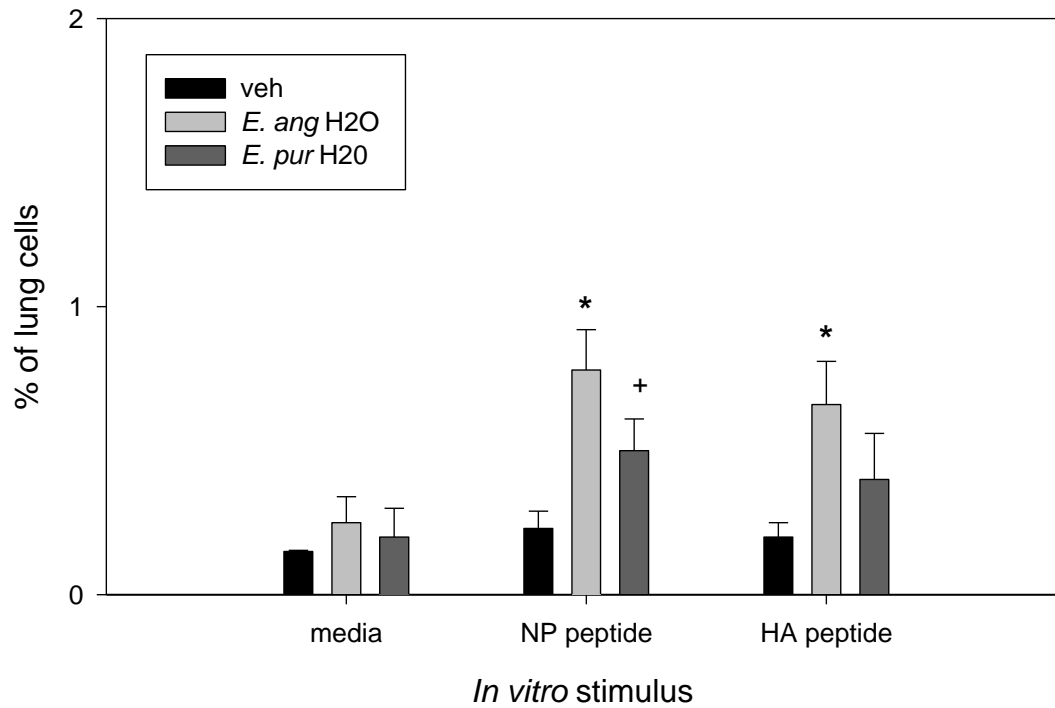


Figure 8b: Percentage of CD8⁺ cells producing IFN γ in response to *in vitro* stimulus in lung of mice infected with Influenza at Day 7 post infection and gavaged with water extract of *E. angustifolia* and *E. purpurea*. Treatment groups contained 10-12 mice. Stimuli *in vitro* was influenza virus specific peptides- HA and NP. Vehicle group was gavaged with water. *E. ang* H2O= *E. angustifolia* water extract, *E. pur* H2O= *E. purpurea* water extract. (*-p<0.05; +-p<0.1)

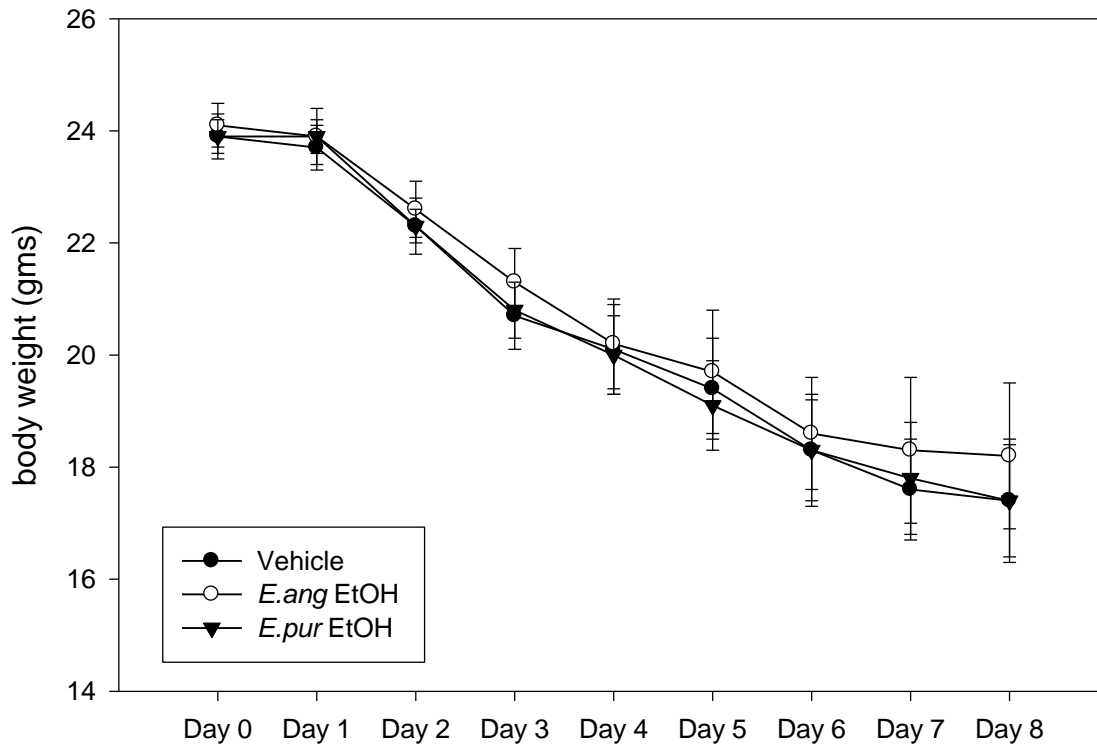


Figure 9a: Body weight graph of mice infected with influenza virus for day 8 post infection.

10-12 mice were assigned to each treatment group, infected with influenza virus and gavaged and weighed throughout the course of infection. Vehicle group was gavaged with 5% ethanol and extract group was gavaged with either *E. ang* EtOH or *E. pur* EtOH extract. *E. ang* EtOH= ethanol extract of *E. angustifolia*, *E. pur* EtOH= ethanol extract of *E. purpurea*.

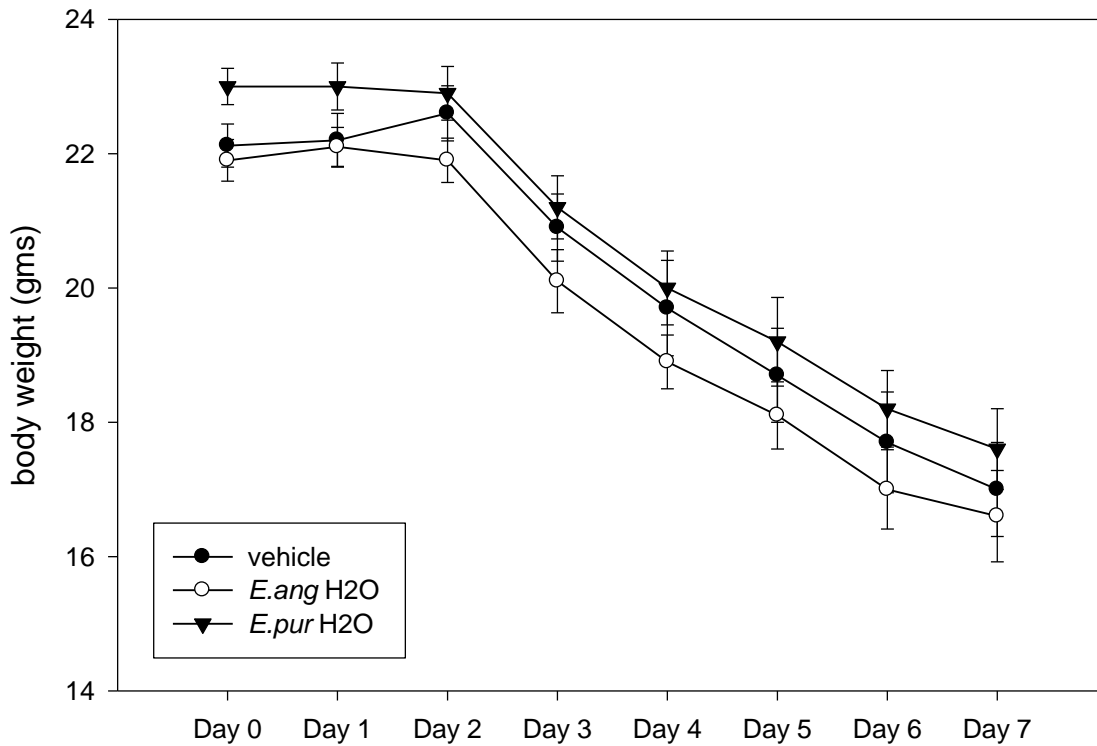


Figure 9b: Body weight graph of mice infected with influenza virus for day 7 post infection.

10-12 mice were assigned to each treatment group, infected with influenza virus and gavaged and weighed throughout the course of infection. Vehicle group was gavaged with water and extract group was gavaged with either *E. ang* H2O or *E. pur* H2O. *E. ang* H2O= water extract of *E. angustifolia*, *E. pur* H2O= water extract of *E. purpurea*.

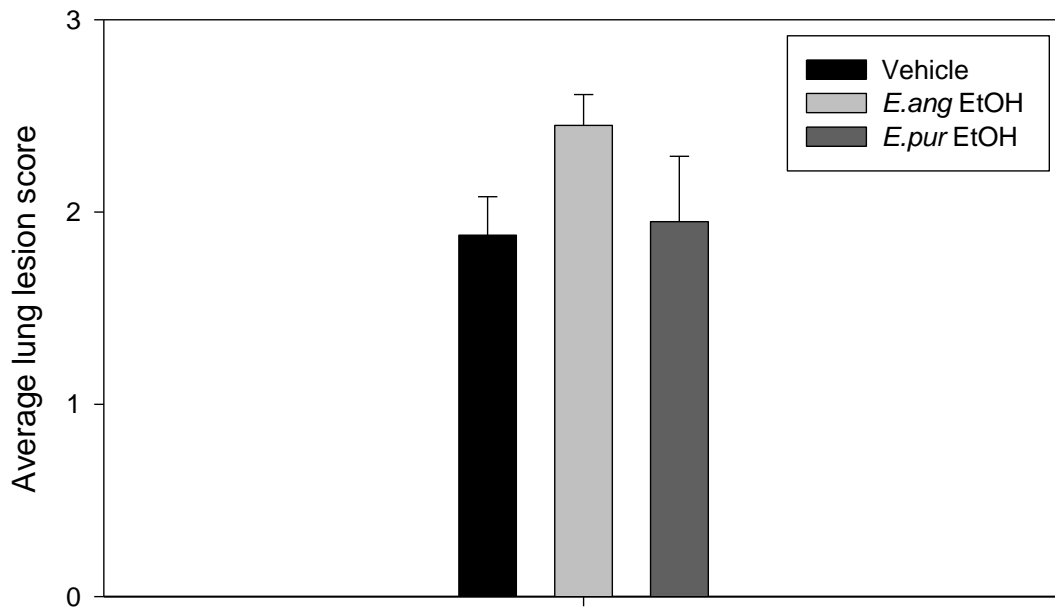


Figure 10: Lung lesion score graph of mice treated with ethanol extract of *Echinacea* for 8 day after infecting with influenza virus. Each group was assigned with 12 mice. Vehicle group was treated with 5% ethanol only. Lung lobes were collected at day 8 of infection and fixed for histopathology exam. *E. ang* EtOH= ethanol extract of *E. angustifolia*, *E. pur* EtOH= ethanol extract of *E. purpurea*.

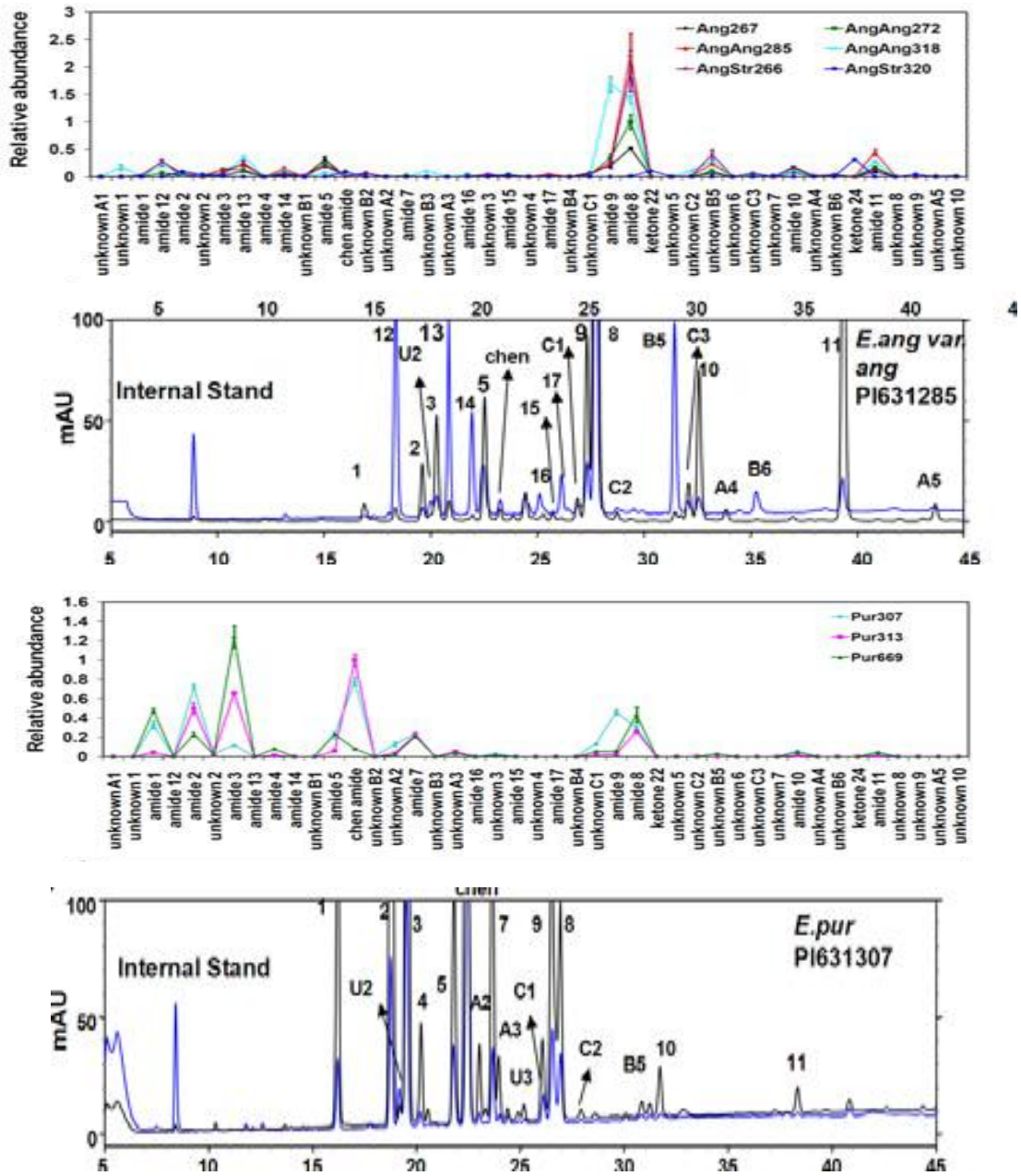


Figure 11: Relative abundance of lipophilic metabolites in *E. angustifolia* (PI631285) and *E. purpurea* (PI631307). Reference: Lankun Wu, Dissertation (2008)

CHAPTER THREE: CONCLUSION

Taking into consideration all of the findings from multiple experiments, we can make general comments on the immune modulating properties of *Echinacea* extracts in an *in vivo* model of influenza infection. The overall results based on experiments using two *Echinacea* species (*E. angustifolia* and *E. purpurea*) show that water extracts improve the survival rate of infected mice and are more immunomodulatory as compared to ethanol extracts, even within the same species. Improvement of survival rate in water extract-treated mice may be explained by the early increase of inflammatory mediators resulting in a more rapid or potent innate cell recruitment. As a result, there may be increased antigen presenting cell activation and/or T helper cell activation that are essential for optimal CD8⁺ effector function and expansion. Our results have shown that water extract treatment of infected mice resulted in an increased influx of influenza specific CD8⁺ effector cells to the lungs at day 8 of infection. CD8⁺ effectors play critical role in viral clearance and potentially improving the survival rate. The effects on CD8⁺ cell activation as assessed by IFN γ production may result from an *Echinacea*-induced activation of innate host defenses that occurs in the first several days of infection. Further support for this possibility will be tested in future studies in which CD8⁺ effector cell-mediated viral clearance is measured. These results suggest that different solvents used in the extraction process may result in the retention of different bioactive components of *Echinacea*. Polysaccharides and some polyphenols have been shown to be present in water extracts while alkamides are main component of ethanol extract. Other data from the botanical center clearly shows that alkamides and some polyphenols are present in the ethanol extracts used in these experiments. The alkamides and polyphenols may account

for the anti-inflammatory activity observed in ethanol extract treated mice at later phases of infection (reduced neutrophils and reduced IFN γ ⁺ CD8⁺ cells in the lungs). One major limitation of these findings is that information regarding the potentially bioactive constituents in the water extracts is not currently available. However, the *in vivo* results with water extracts show immune stimulatory activity which is consistent with literature showing that polysaccharides (and polyphenols) present in water extracts are the immunostimulatory component of *Echinacea* extract. These results are consistent with earlier studies in different *in vivo* and *in vitro* models of infection.

It is interesting to note that none of extracts used have any evidence of anti-viral activity during early phase of infection. However, if the mechanism by which *E. angustifolia* water extracts improve survival is through an enhancement of CD8⁺ effector function, then an improvement in symptoms and/or viral clearance may not be detectable until the later phases of infection (day 8-12). Another potential limitation of these studies is that only specific time points have been evaluated (days 1, 5, 7, 8 post-infection), and it is possible that the immune alterations that were observed do not translate into significant clinical improvements until the very later stages of infection and recovery (days 9-14). Finally, it is possible that an interaction of components in the rodent chow with constituents in the plant extracts may play a role in affecting host response. The experiments to address these questions are the focus of future research.

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APPENDIX

Introduction

Preliminary experiments using both ethanol and water extracts of *E. angustifolia* and *E. purpurea* were conducted at low viral dose (~5% mortality). Infection at the low virus resulted in highly variable responses within the lung. Viral titer varied by more than a log of 3 and cytokine levels varied by greater than 10fold between infected mice. With the large degree of variability, it was difficult to detect treatment effects although some general patterns emerge such that water extracts tended to increase cytokines and chemokines in the BAL. Extract treatment did not reduce viral load in the lungs. At this low dose of infection, weight loss did not occur.

Water and ethanol of both *E. angustifolia* and *E. purpurea* were used for treatment of mice. Mice were gavaged 24 and 48 prior to infection, and every 24 hours after infection up until the time of euthanization. Lung viral titer quantification was performed after 5 day post infection in all the experiments, and cytokines in the BAL fluid were also assessed at 5 days post-infection.

There was no significant difference of viral load in extract treatment group than in vehicle group with any of the extract from both *Echinacea* species (Fig 1a & 1b). The effect of extract treatment on cytokine and chemokine profile in BAL fluid of mice infected with low virus dose at day 5 post infection was also analyzed using 32-plex kit. Treatment with ethanol extract of *E. angustifolia* significantly ($p < 0.05$) decreased some of the cytokines and chemokines (LIX, RANTES, IP-10, IL-6) in lungs while MCP-1 and IL-5 show a trend ($p < 0.1$) toward decrease as compared to vehicle group (Fig 2a, 2b & 2c). On other hand,

treatment with ethanol extract of *E. purpurea* has no effect on cytokine and chemokine in lungs than vehicle group (3a, 3b & 3c). BAL cell population was also not affected by ethanol extract treatment and was similar to vehicle group (Fig 6).

Similarly with water extract at day 5 post infection, no significant effects of either *E. angustifolia* (4a, 4b & 4c) or *E. purpurea* (5a, 5b & 5c) treatment on any of the cytokines or chemokines was measured. Also, no effect of ethanol or water extracts was observed with respect to body weight of mice infected with low virus dose (Fig 7a & 7b).

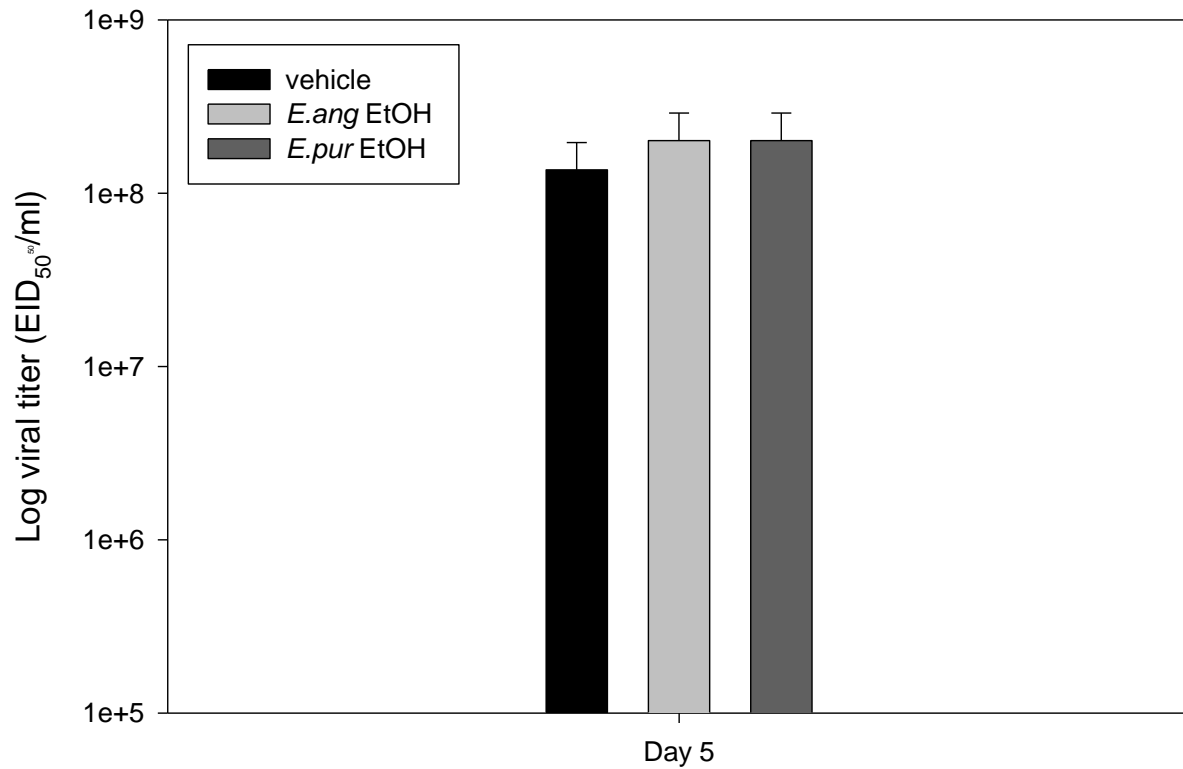
Figures for Appendix

Figure 1a: Viral titer at day 5 post infection using ethanol extract of *Echinacea angustifolia* and *Echinacea purpurea* at low dose of viral infection. 10-12 mice per group was used and lungs were collected to run RT-PCR for quantification of virus. Ethanol 5% was used as vehicle. *E. ang* EtOH= *E. angustifolia* ethanol extract, *E. pur* EtOH= *E. purpurea* ethanol extract.

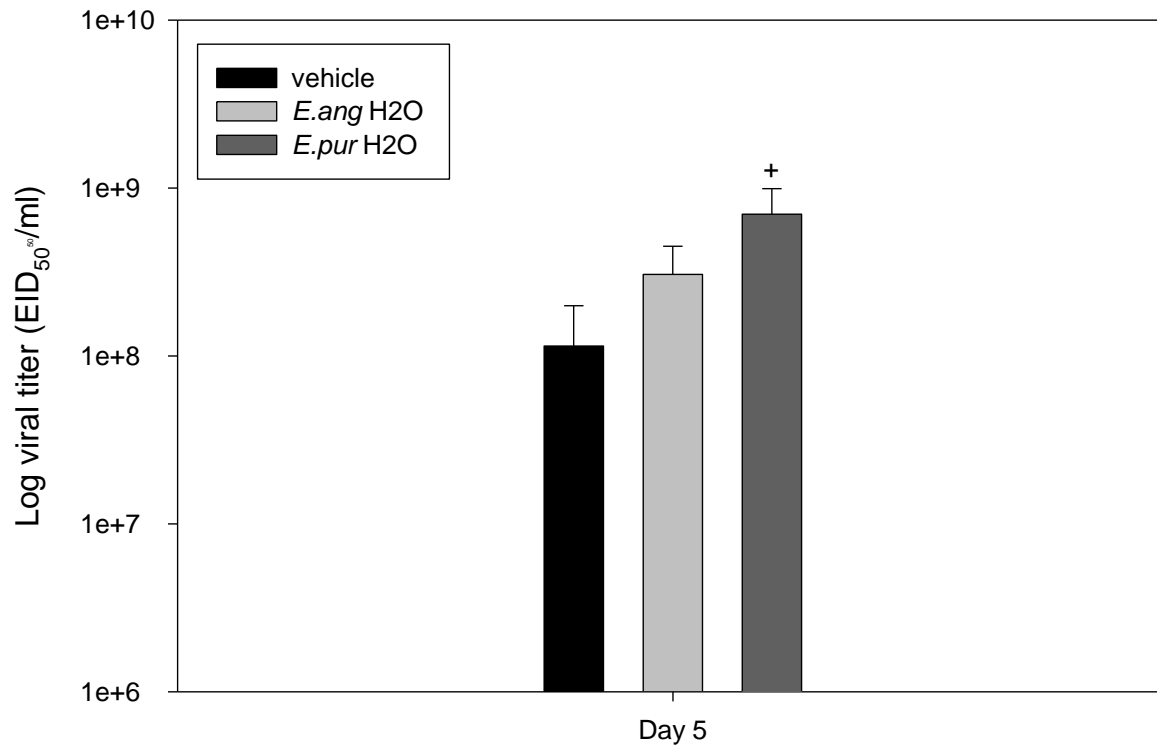


Figure 1b: Viral titer at day 5 post infection using water extract of *Echinacea angustifolia* and *Echinacea purpurea* at low dose of viral infection. 10-12 mice per group were used and lungs were collected to run RT-PCR for quantification of virus. Water used as vehicle. *E. ang* H2O= *E. angustifolia* water extract, *E. pur* H2O= *E. purpurea* water extract. (+-p<0.1)

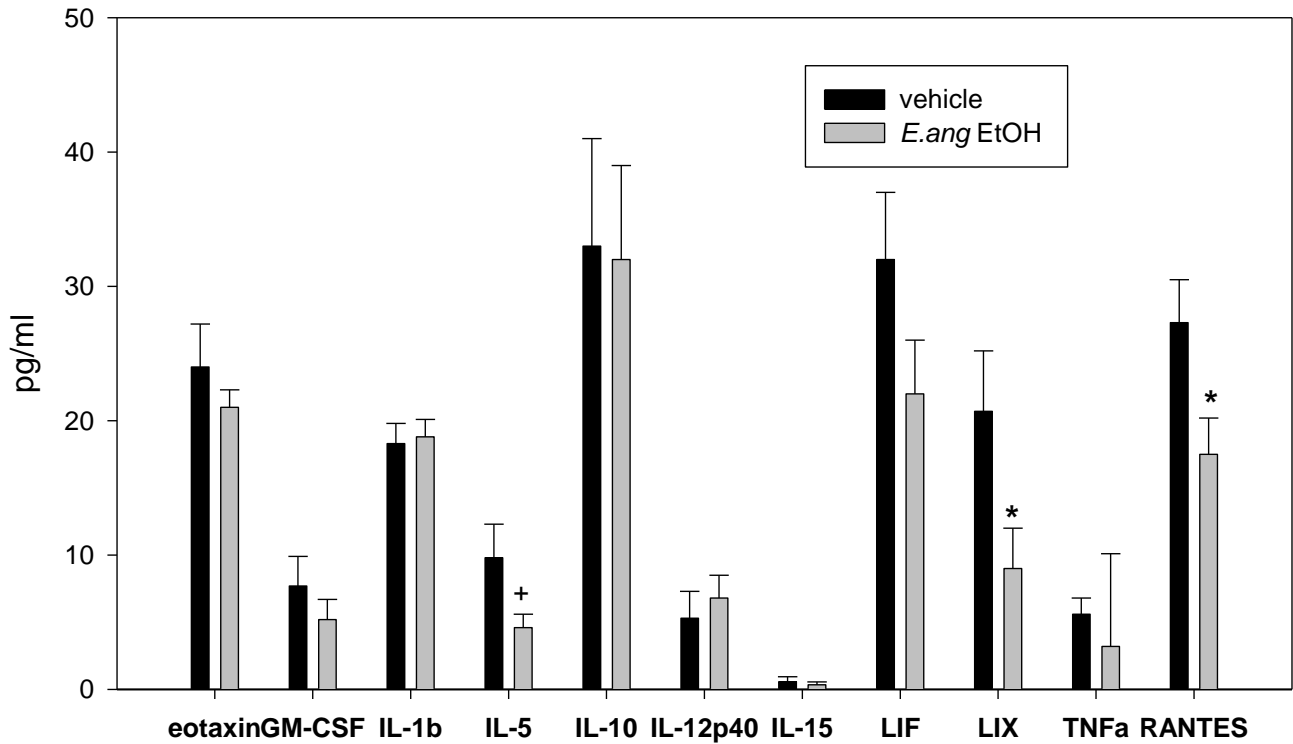


Figure 2a: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia* at low dose of virus infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using multiplex kit. *E. ang* EtOH= *E. angustifolia* ethanol extract. (*- $p < 0.05$; +- $p < 0.1$)

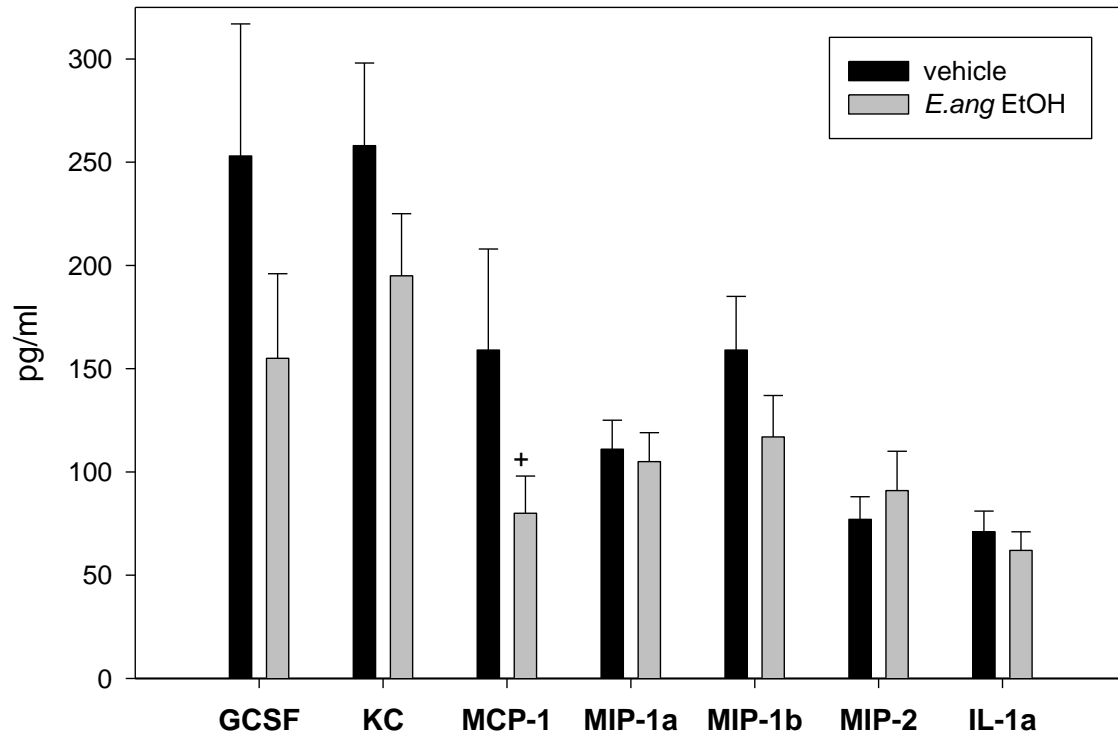


Figure 2b: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia* at low dose of virus infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using multiplex kit. *E. ang* EtOH= *E. angustifolia* ethanol extract. (+-p<0.1)

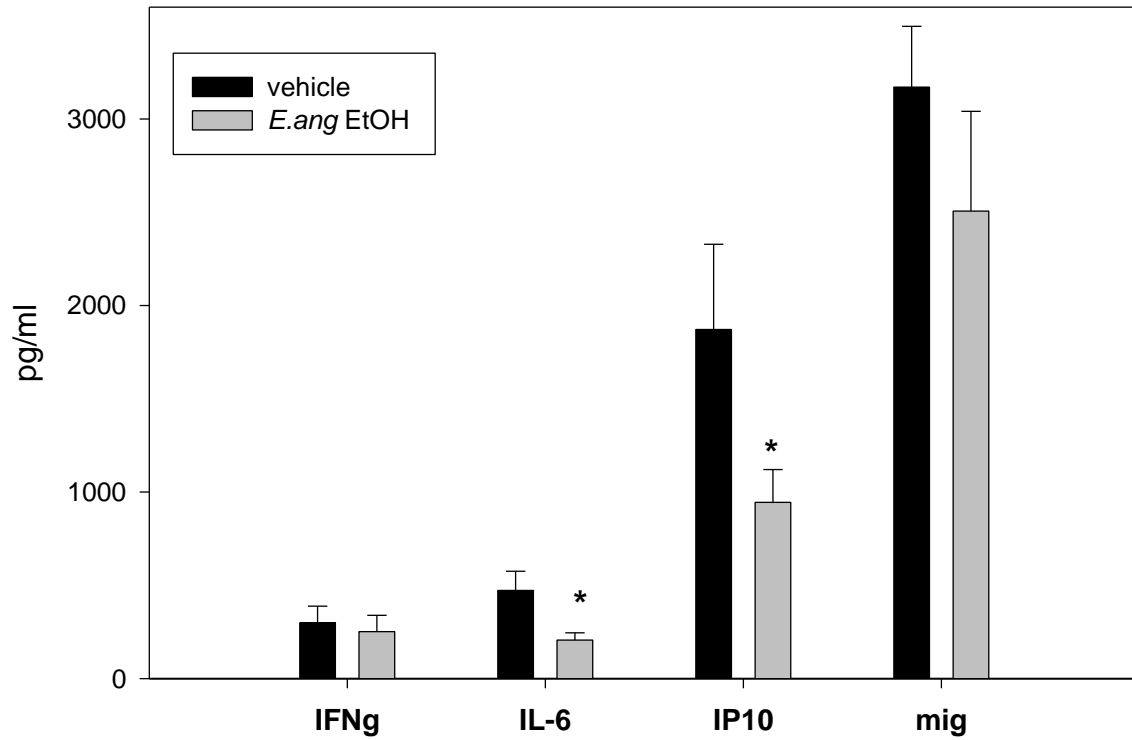


Figure 2c: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea angustifolia* at low dose of virus infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using multiplex kit. *E. ang* EtOH= *E. angustifolia* ethanol extract. (*-p<0.05)

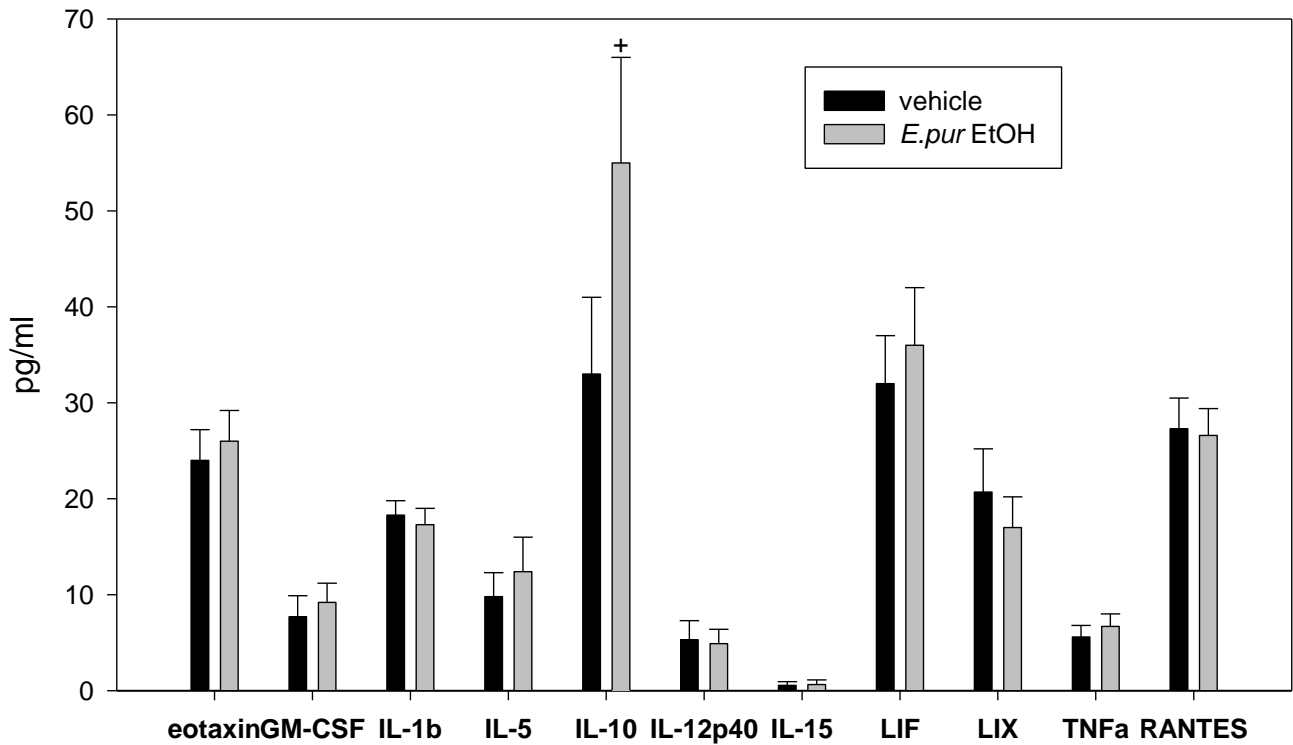


Figure 3a: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* EtOH= *E. purpurea* ethanol extract. (+-p<0.1)

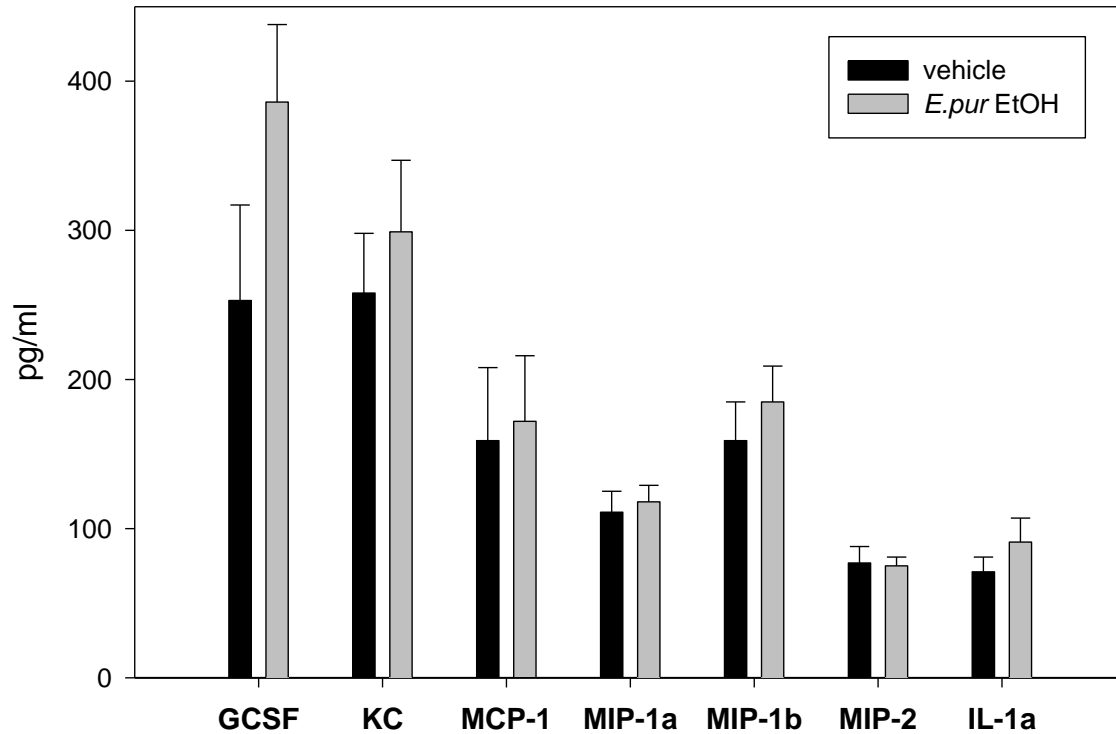


Figure 3b: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* EtOH= *E. purpurea* ethanol extract.

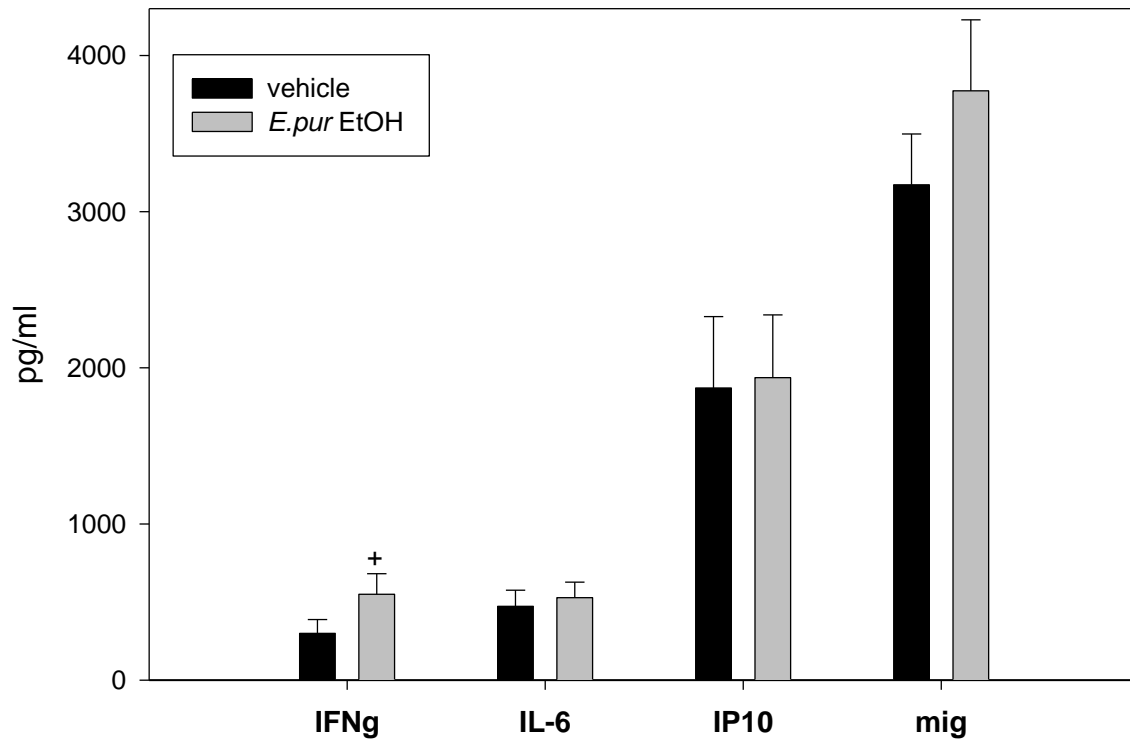


Figure 3c: Cytokine and chemokine profile in BAL fluid at day 5 post infection using Ethanol extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with 5% ethanol. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* EtOH= *E. purpurea* ethanol extract. (+-p<0.1)

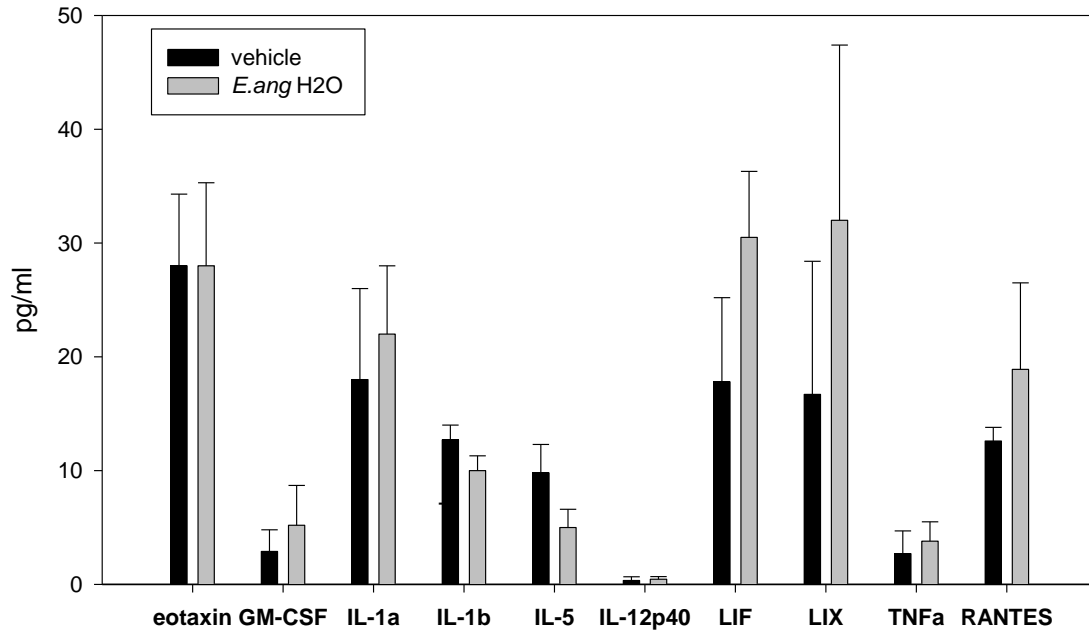


Figure 4a: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. ang* H2O= *E. angustifolia* water extract.

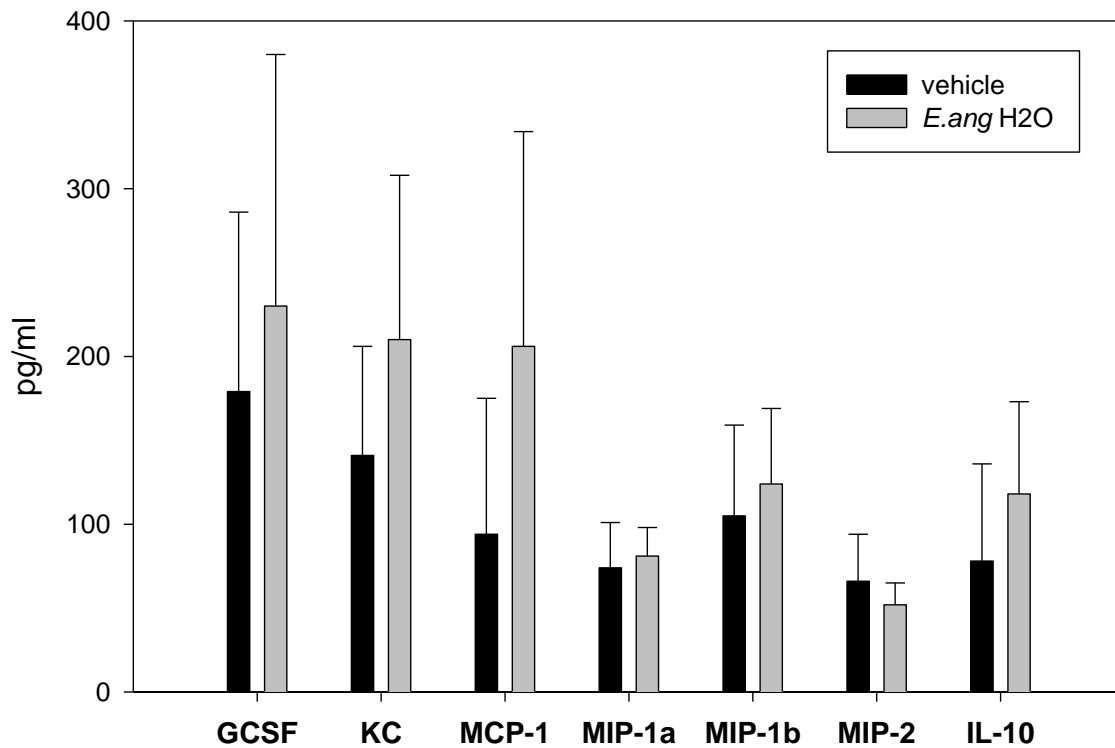


Figure 4b: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. ang* H2O= *E. angustifolia* water extract.

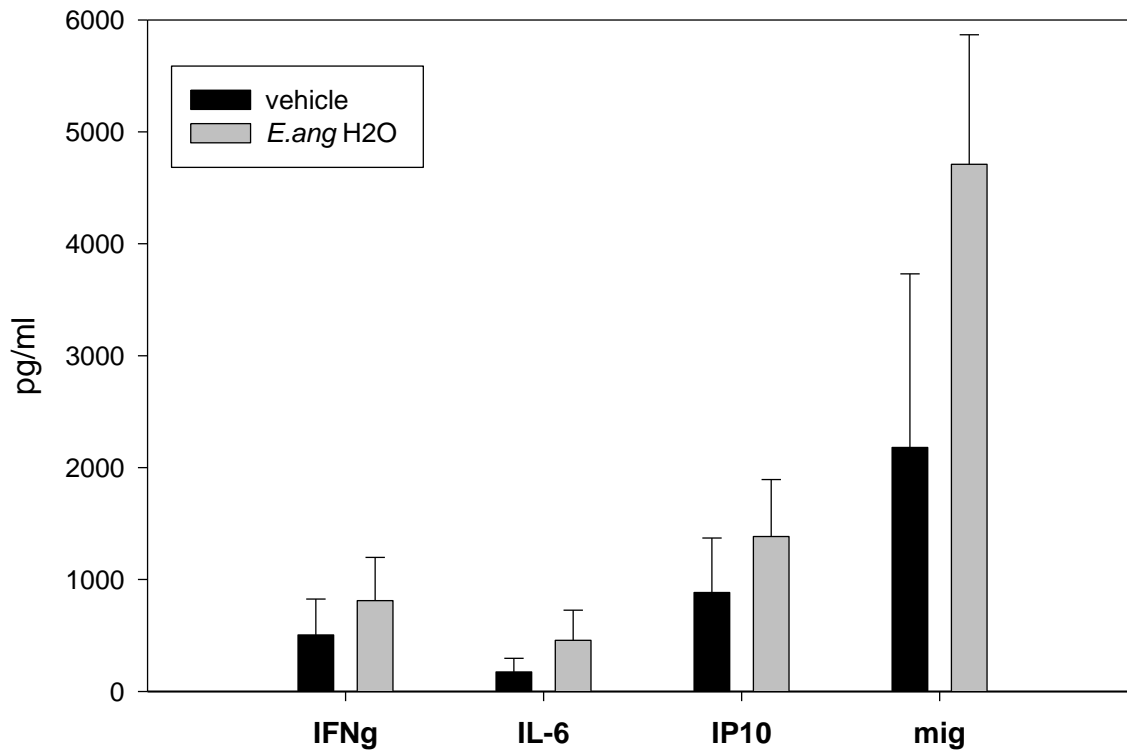


Figure 4c: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea angustifolia* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. ang* H₂O= *E. angustifolia* water extract.

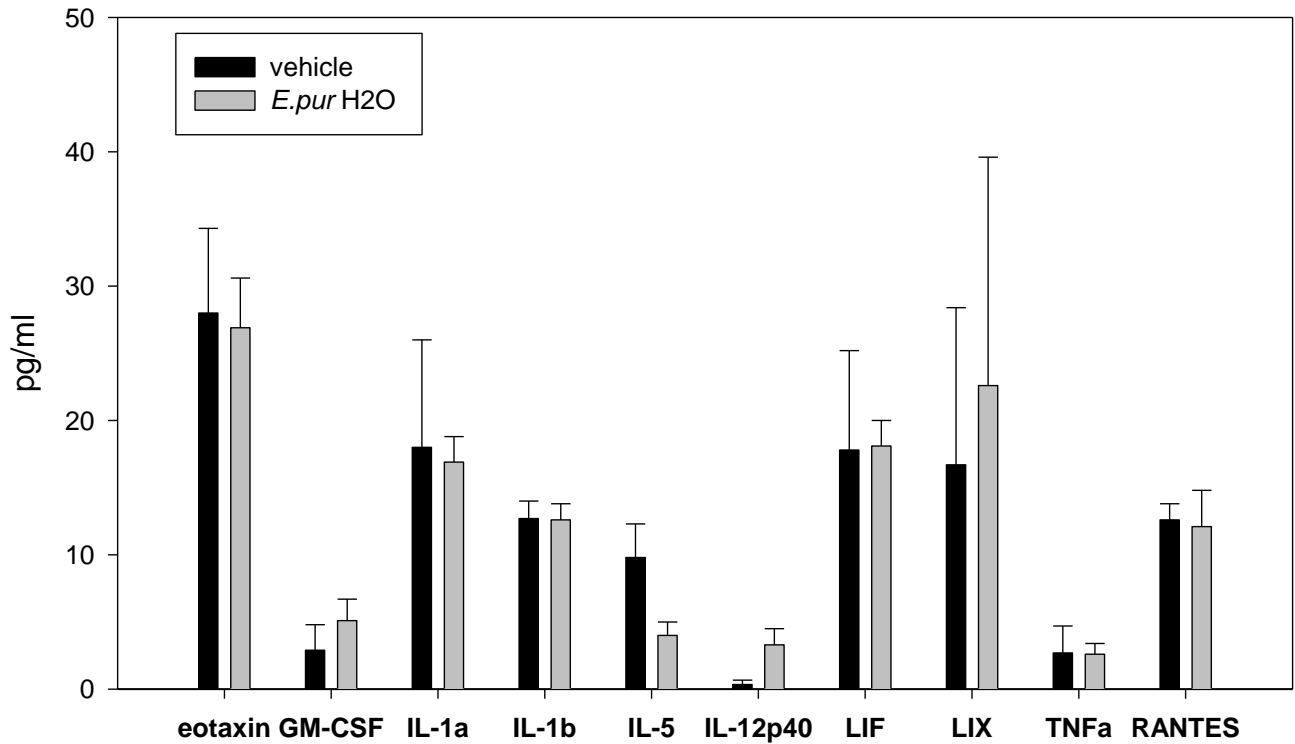


Figure 5a: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* H2O= *E. purpurea* water extract.

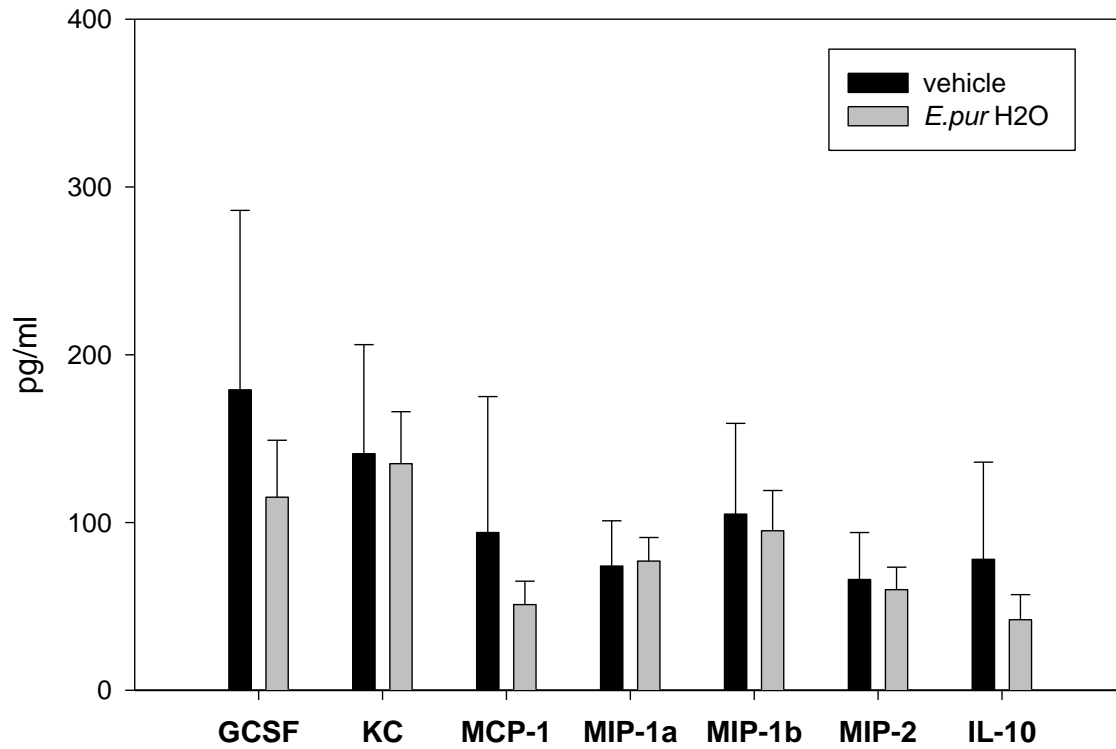


Figure 5b: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* H2O= *E. purpurea* water extract.

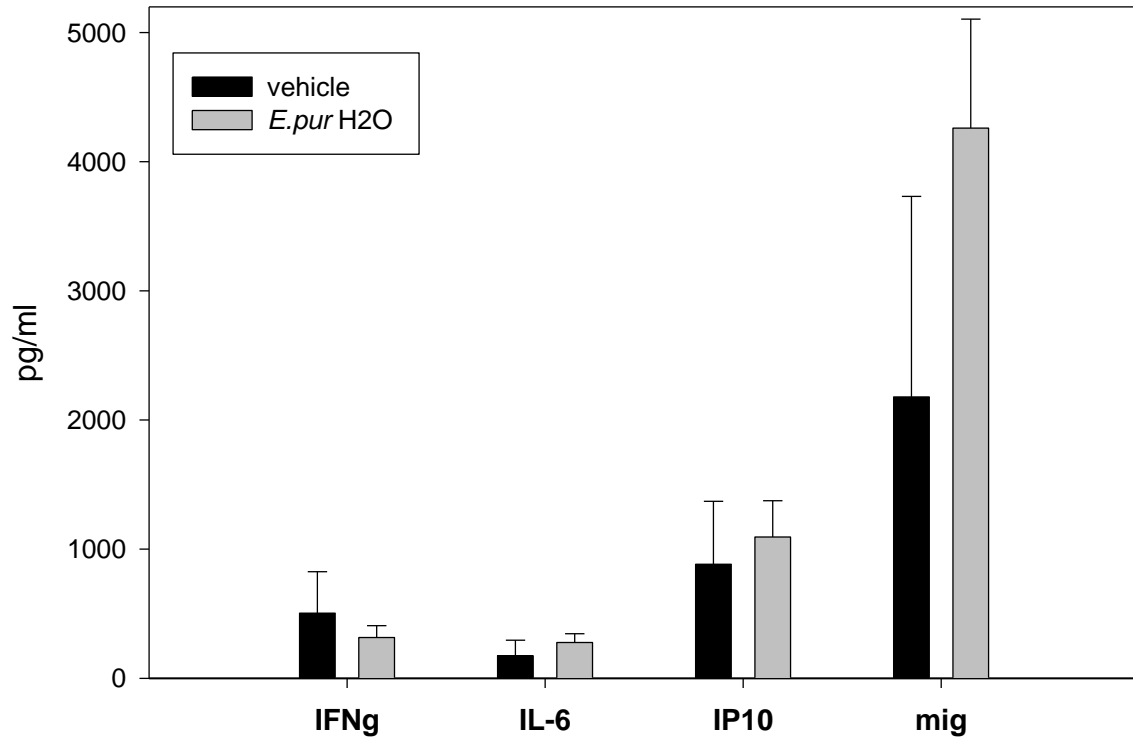


Figure 5c: Cytokine and chemokine profile in BAL fluid at day 5 post infection using water extract of *Echinacea purpurea* at low dose of viral infection. Vehicle group was gavaged with water. 10-12 mice per group were assigned. BAL was collected after 5 days of infection with influenza and was analyzed using 32-plex kit. *E. pur* H2O= *E. purpurea* water extract.

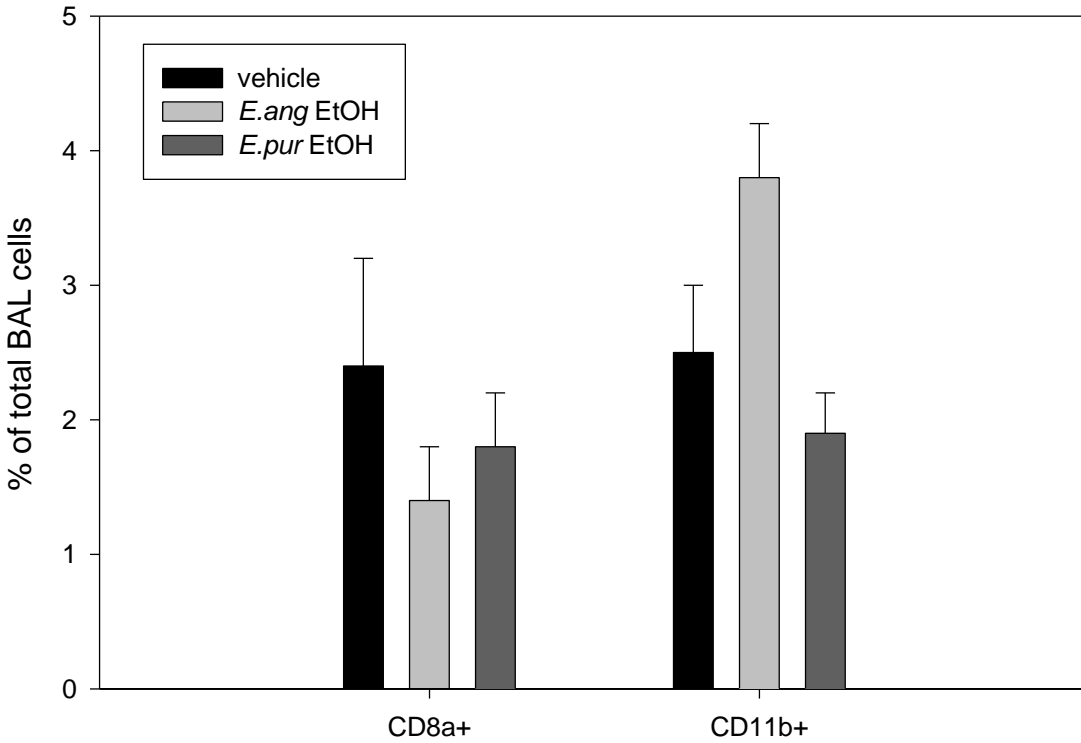


Figure 6: Cell population in BAL Fluid after treatment with ethanol extract of *E. angustifolia* and *E. purpurea* at day 5 of infection with low dose of influenza virus. BAL cells were stained for surface markers and determined by flow cytometry. 10-12 mice per group were used. 5% ethanol was used to gavage vehicle group. *E. ang* EtOH= *E. angustifolia* Ethanol extract, *E. pur* EtOH= *E. purpurea* Ethanol extract.

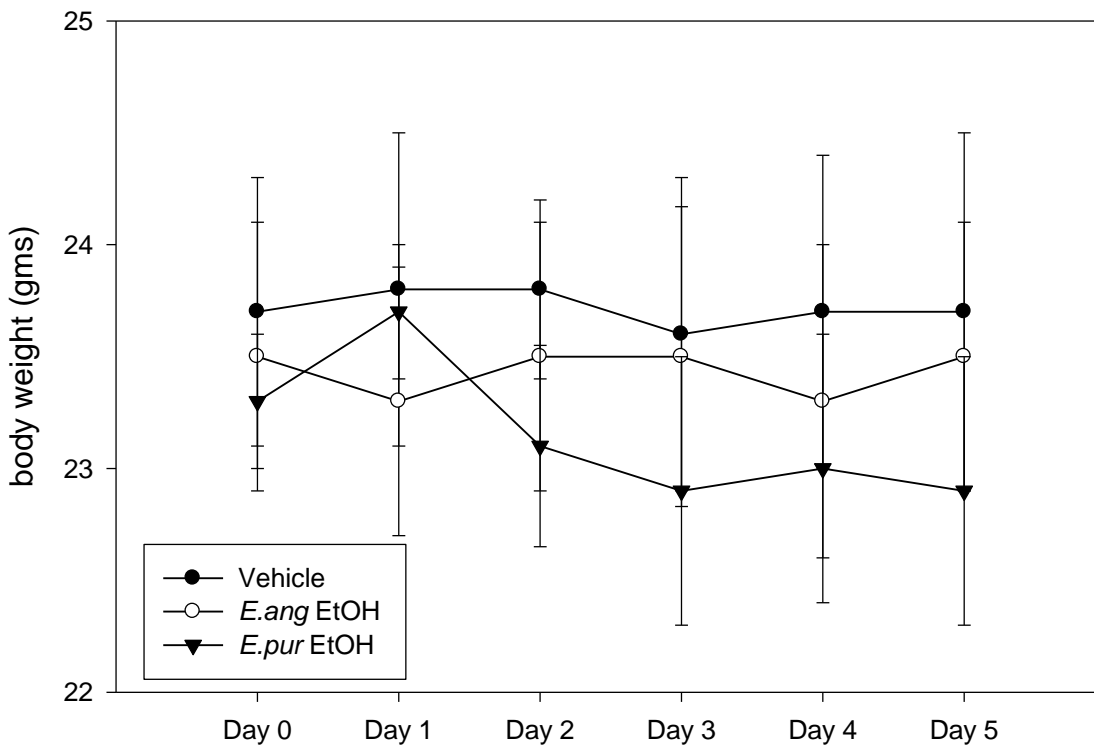


Figure 7a: Body weight graph of mice infected with low dose of influenza virus for day 5 post infection. 10-12 mice were assigned to each treatment group, infected with influenza virus and gavaged and weighed throughout the course of infection. Vehicle group was gavaged with 5% ethanol and extract group was gavaged with either *E. ang* EtOH or *E. pur* EtOH. *E. ang* EtOH= ethanol extract of *E. angustifolia*, *E. pur* EtOH= ethanol extract of *E. purpurea*.

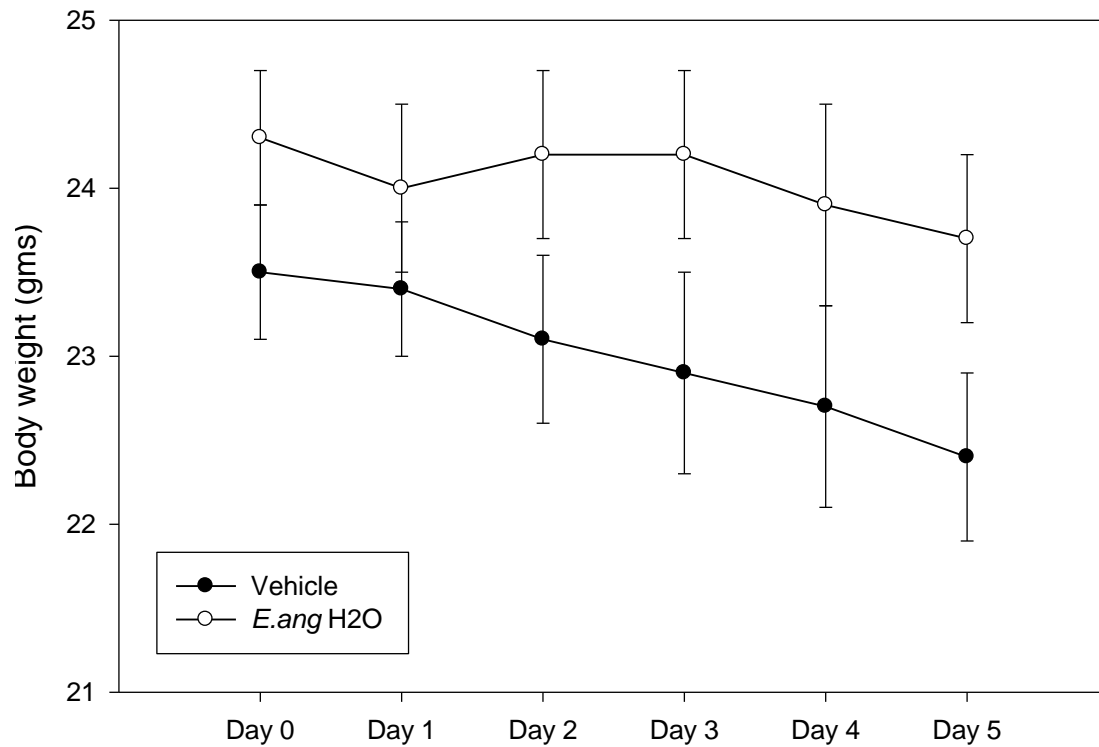


Figure 7b: Body weight graph of mice infected with low dose of influenza virus for day 5 post infection. 10-12 mice were assigned to each treatment group, infected with influenza virus and gavaged and weighed throughout the course of infection. Vehicle group was gavaged with water and extract group was gavaged with either *E. ang* H2O extract. *E. ang* H2O= water extract of *E. angustifolia*.

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