Evaluation of immunopathologic effects of aqueous extract of *Echinacea purpurea* in mice after experimental challenge with *Pasteurella multocida* serotype A

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Summary

In order to assess the immunopathological effects of aqueous *Echinacea purpurea* extract (EPE) on mice experimentally challenged with *Pasteurella multocida* serotype A, forty female BALB/c mice were randomly divided into four groups. The groups included a control group (received sterile distilled water 2 times/week for 2 weeks, intraperitoneally and then 100 µl sterile saline intranasally), a PMA group (received sterile distilled water as the control group and after 2 weeks, 5.6×10^3 CFU/ml of *P. multocida* serotype A, intranasally), an EPE+PMA group (received *E. purpurea* extract intraperitoneally 2 times/week for 2 weeks and then challenged as the PMA group) and an EPE group (received *E. purpurea* extract as EPE+PMA group and then 100 µl sterile saline intranasally). After 24 and 48 h post challenge, half of the animals in each group were sacrificed and analyzed for bacterial counts in their lungs and livers, TNF_α serum levels and histapathological changes. The results showed significant differences in lung bacterial counts between PMA and EPE+PMA groups. TNF_α serum level was significantly higher in the PMA group. Histopathological examination revealed infiltration of neutrophils in alveolar septa and hyperemia in the PMA group. In addition, the criteria of bronchopneumonia were partially recovered in the EPE+PMA compared to the PMA group. According to the results, it seems that *E. purpurea* extract has an immunomodulatory effect and can be used to prevent or control of pneumonia caused by *Pasteurella*.

Key words: *Echinacea purpurea*, *Pasteurella multocida*, Mice, TNF_α, Histopathology

Introduction

Pasteurella multocida is a gram negative bacterium causing widespread infections in various domestic animals; snuffles in rabbits, pneumonia and haemorrhagic septicaemia in cattle, sheep and goats and fowl cholera in chickens. Pasteurellosis, an infection with *Pasteurella* sp. which is found in humans and animals, is an important respiratory disease, especially in animals with high economic importance (Kuhnert and Christensen, 2008). Serotype *P. multocida* is one of the nasopharyngeal commensal pathogens associated mainly with respiratory diseases in animals (Vasfi Marandi and Mittal, 1997).

Echinacea purpurea (purple cone flower) has been used traditionally in North America for the treatment of various symptoms of “colds and flu”, as well as the treatment of respiratory diseases, candidiasis, and wound healing (Bauer, 1998; Barrett, 2003; Sharma *et al*., 2010). *Echinacea* extracts have been shown to have nonspecific immunomodulatory properties *in vitro* (Bauer and Wagner, 1991), including increased phagocytosis (Stotzem *et al*., 1992), increased cytokine production (Burger *et al*., 1997), increased natural killer cell activity (See *et al*., 1997) and increase immunoglobulin G levels in rats (Rehman *et al*., 1999). Previous studies suggest the potential use of *Echinacea* for controlling bacterial infections (Sharma, 2010). Sharma *et al*. (2010) reported that respiratory bacteria such as *Streptococcus pyogenes*, *Hemophilus influenzae* and *Legionella pneumophila* were also readily inactivated by *Echinacea*, and their pro-inflammatory responses caused by different cytokines secreted by bronchial epithelial cell cultures in response to infection were reversed. According to their results, *Staphylococcus aureus* (methcillin-resistant and sensitive strains) and *Mycobacterium smegmatis* were less sensitive to the antibacterial effects of *Echinacea* but their pro-inflammatory responses were completely reversed. Therefore, they reported that *Echinacea* to have a dual action against respiratory bacteria: one being its anti-bacterial action, a bactericidal effect against some of the bacteria incriminated in upper respiratory infections, and the other an anti-inflammatory effect which could reverse inflammation caused by these bacteria.

Despite the *in vitro* immunomodulatory and bactericidal activity of *E. purpurea* extract on the immune system and on different bacteria, the principle role and *in vivo* activity of this plant on the immune system following experimental *P. multocida* infection have not been addressed. Therefore, an *in vivo* study was designed to investigate the immunopathologic effects of

E. purpurea on the experimental infection of *P. multocida* serotype A in mice.

Materials and Methods

Echinacea

Echinacea purpurea powder was obtained from aerial vegetative parts (Goldaru Co., Isfahan, Iran). *Echinacea* aqueous extract was prepared using distilled water incubated at 37°C in a water bath for 2 h. The suspension was filtered using sterile 0.45 µm PVDF syringe filter units. The filtered extract was stored at 4°C prior to use.

Bacteria

Pasteurella multocida serotype A was prepared from Razi Vaccine and Serum Research Institute (Iran, Karaj). The bacterium was cultured in blood agar medium containing 5% sheep blood. Bacteria identity was confirmed morphologically and biochemically. The bacteria were cultured overnight in 100 ml TSB medium at 37°C. The culture was centrifugated at 4000 rpm for 10 min and washed twice with sterile saline and resuspended in sterile PBS. The bacterial suspension OD_{600} was adjusted to 0.1 and counted by surface plate method (Quinn *et al*., 2002).

Determination of LD_{50} and ID_{50}

Female BALB/c mice with an age range of 6-8 weeks were obtained from Jundishapur Animal Laboratory Centre, Ahvaz, Iran. The storage condition of mice was standardized. Before determining LD_{50}, the pathogenicity of the *P. multocida* serotype A strain was tested in healthy mice by intraperitoneal inoculation of 0.5 ml PBS containing 3.5×10^7 CFU/ml of *P. multocida* serotype A. Subsequently, the determination of the LD_{50} for *P. multocida* serotype A was calculated by Reed and Muench's method (Reed and Muench, 1938). Briefly, appropriate bacterial volumes (100 µl) from serially tenfold dilutions of *P. multocida* serotype A prepared in sterile PBS were intranasally inoculated in six mice per group. The number of deaths was recorded. Animals which did not die from infection were killed by cervical dislocation according to animal ethics, and bacterial isolation was carried out on their lungs and livers. LD_{50} and ID_{50} of *P. multocida* serotype A were then calculated by the Reed and Muench method (Reed and Muench, 1938).

Experimental infection

Forty female BALB/c mice (25-27 g) were kept under controlled conditions. Food and water were allowed *ad libitum*. A commercial pelleted diet was used during the experiments. The animals were allowed to adapt to the laboratory conditions for 2 weeks before the beginning of the experiment. All animals were randomly assigned to four groups (10 mice per group). In the control group, mice received 0.5 ml sterile distilled water two times/week for two weeks followed by 100 µl sterile saline intranasally. In the second group (PMA), mice received sterile distilled water similar to the control group and after 2 weeks, 5.6×10^3 CFU/ml of *P. multocida* serotype A in a total volume of 100 µl, was administered intranasally. The mice were maintained in an upright position for 30 sec after inoculation. The third group (EPE+PMA) were injected with 0.5 ml of *E. purpurea* extract (40 mg/ml) intraperitoneally two times/week for two weeks and then challenged with *P. multocida* serotype A, similar to the PMA group. The fourth group (EPE) received 0.5 ml *E. purpurea* extract (40 mg/ml) intraperitoneally two times/week for two weeks and then 100 µl of sterile saline intranasally.

Blood sampling

After weighing and sacrificing mice at 24 and 48 h post-bacteria-challenge in the PMA and EPE+PMA groups, blood samples were collected from the animals by cardiocentesis (five mice at 24 and five mice at 48 h post infection). About 0.3 ml blood was collected in sterile microtubes without anticoagulant for serum separation. Serum samples were taken and stored frozen at -20°C for TNF_α assays.

Histopathology

After necropsy and weighing the lungs, parts of lungs, livers and spleens were fixed in 10% neutral buffered formalin for histopathological examination. Remaining lung and liver tissues were used for bacterial isolation. Specimens in formalin were processed routinely and embedded in paraffin wax. Tissue sections of 5 µm thickness were routinely stained with haematoxylin and eosin (H&E). The lesions were scored (-) as none; (+) as minimal; (++) as moderate and (+++) as severe.

Bacteriological examination

Approximately 0.1 g of each lung and liver was removed aseptically after necropsy at 24 and 48 h post challenge. The tissue was homogenized in 900 µl sterile normal saline and a serially tenfold dilution to 10^7 was prepared. Subsequently, 50 µl of each dilution was cultured on sheep blood agar plates and incubated at 37°C for 24 h. The number of colonies was counted and the number of bacteria was reported as tissue CFU g^{-1}.

Serum TNF_α analysis

A commercial ELISA kit (Boster Biological Technology, LTD) was used to determine TNF_α serum concentration by following the procedures recommended by the manufacturer.

Statistical analysis

Data were analyzed using SPSS (version 16, SPSS, Inc., Chicago, IL, USA). Lung index results together with bacterial counts and serum TNF_α profiles were compared between groups and subgroups (24 and 48 h) using analysis of variance and LSD tests. Semi-quantitative scoring of histopathological changes was analyzed by Kruskal-Wallis tests.

Results

Clinical observation

Clinical observations of mice in the PMA group included the inactivity of mice, depression, weakness, and tangled hair. Three mice died during 24 h, and two mice at 48 h post challenge. In the EPE+PMA group, one mouse died at 24 and 48 h post challenge. In the EPE and control groups the mice were healthy and appeared normal. No significant variation in lung indices of test groups were observed in comparison to the control group (P>0.05). Lung index data are shown in Table 1.

Lung and liver bacterial isolation and count

To assess the *in vivo* effects of *E. purpurea* extract on mice experimentally infected with *P. multocida*, bacterial counts of lung and liver homogenates of different groups were measured (Table 1). Mean bacterial counts for different groups are summarized in Table 1. In the PMA group, the average number of *P. multocida* at 24 and 48 h post challenging was 81.7×10^9 and 95.9×10^9 CFU/g in the lungs and 5.5×10^9 and 7.6×10^9 CFU/g in the livers. In the EPE+PMA group, the average number of *P. multocida* in the lungs at 24 h post challenging was 6.2×10^9 CFU/g and in 48 h, 2.6×10^9 CFU/g. In the livers, the average number of bacteria at 24 h post challenging was 0.7×10^9 CFU/g and 2.8×10^9 CFU/g at 48 h. No bacteria were isolated from lungs and livers in the control and EPE groups. Bacterial counts in the PMA group were higher at 48 h than 24 h. In the EPE+PMA group, bacterial count was higher in 24 h than 48 h. Significant differences were found between PMA and EPE+PMA groups in 24 and 48 h (P<0.0001).

Pathological observation

The histopathologic study of PMA and EPE+PMA groups revealed different lesions in the lungs, livers and spleens. The predominant changes in the lungs of the PMA group after 24 and 48 h were acute bronchopneumonia with diffuse and severe infiltration of polymorphonuclear cells within alveolar septa and alveolar lumen. Hyperemia was obvious in both subgroups (24 and 48 h), scored as moderate to severe. Seroproteinaceous exudates containing fibrin were detected within alveolar lumen, bronchioles and around vessels (Fig. 1A). In one mouse, bacterial colonies were seen with high magnification. Lungs of mice in the EPE+PMA group showed mild to moderate infiltration of neutrophils and moderate hyperemia (Fig. 1B). Significant differences were found in neutrophils infiltration between PMA and EPE+PMA (P<0.05). The lungs of control and EPE groups showed normal histological structure. Liver lesions in the PMA group were cell swelling and diffuse infiltration of neutrophils in sinusoidal spaces (Fig. 1C). Also, blood vessels were engorged with red and white blood cells. In EPE + PMA livers, the infiltration of inflammatory cells was decreased (Fig. 1D). Spleen histopathological changes in the PMA group were seen as the accumulation of neutrophils in the marginal zone of white pulp (Fig. 1E). The spleen of EPE + PMA mice showed less infiltration of neutrophils (Fig. 1F).

Serum TNF_α profile

To determine whether EPE had immunomedulator effect on inflammatory cytokine, TNF_α, serum TNF_α was measured in all groups. Serum TNF_α concentrations are presented in Table 2. Serum TNF_α concentration was found to be higher in the PMA and EPE+PMA groups. However, the concentration of TNF_α in the EPE+PMA group was lower than that of the PMA group. The results of the data analysis showed that the differences between serum TNF_α concentrations were significant for the PMA and EPE+PMA groups (P<0.05), the PMA and EPE groups and the PMA and the control groups (P<0.01). The differences between the EPE and EPE+PMA groups, the EPE and the control groups and the EPE+PMA and the control groups was not significant (P>0.05).

Table 1: Summary of lung index and bacteriological findings following intranasal infection of *Pasteurella multocida* in mice

Groups	Time of sacrificing after infection (h)	The average index of lung	The average number of *P. multocida* in lung (CFU/g)	The average number of *P. multocida* in liver (CFU/g)
Control	24	0.96 ± 0.18	0	0
	48	0.89 ± 0.23	0	0
PMA	24	1.33 ± 0.36	81.7×10^9	5.5×10^9
	48	1.25 ± 0.57	95.9×10^9	7.6×10^9
EPE+PMA	24	1.09 ± 0.36	6.2×10^9	0.7×10^9
	48	1.14 ± 0.54	2.6×10^9	2.8×10^9
EPE	24	1.01 ± 0.03	0	0
	48	0.98 ± 0.14	0	0

Table 2: Means ± SEM of serum TNF_α values following intranasal infection of *Pasteurella multocida* in mice

Groups	Control		PMA		EPE+PMA		EPE	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
OD_{450}	0	0	0.721	0.252	0.237	0.053	0	0
Concentration of TNF_α (pg/ml)	33.4±19.6	56.2±19.8	2963.6±2261.8	910.2±651.8	844.1±1264.0	247.0±440.4	39.6±25.0	41.4±26.8

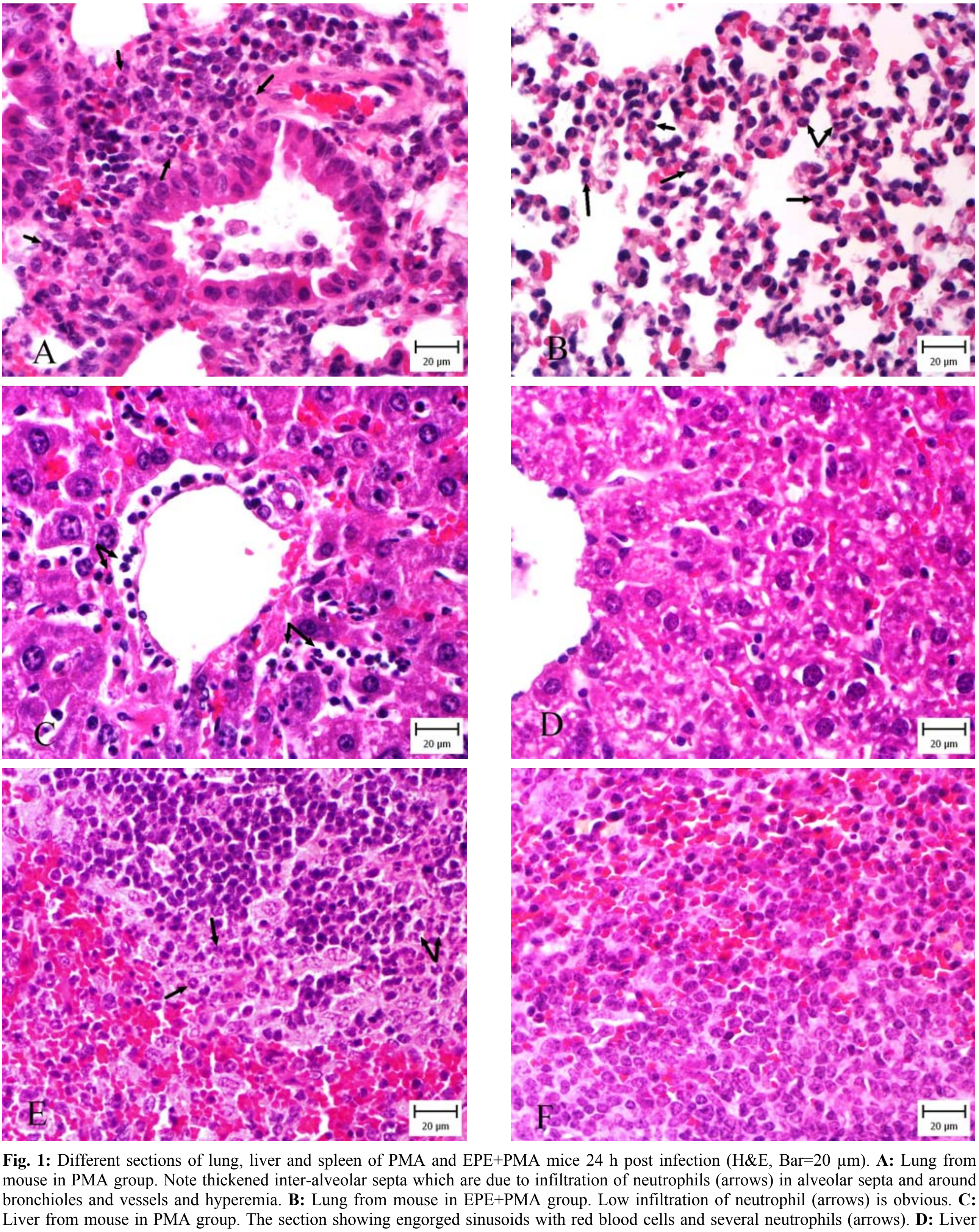

Fig. 1: Different sections of lung, liver and spleen of PMA and EPE+PMA mice 24 h post infection (H&E, Bar=20 μm). **A:** Lung from mouse in PMA group. Note thickened inter-alveolar septa which are due to infiltration of neutrophils (arrows) in alveolar septa and around bronchioles and vessels and hyperemia. **B:** Lung from mouse in EPE+PMA group. Low infiltration of neutrophil (arrows) is obvious. **C:** Liver from mouse in PMA group. The section showing engorged sinusoids with red blood cells and several neutrophils (arrows). **D:** Liver from mouse in EPE+PMA group. Reduced number of neutrophils is clear. **E:** Spleen from mouse in PMA group. Note numerous neutrophils around the white pulp. **F:** Spleen from mouse in EPE+PMA group. Decreased numbers of neutrophils are observed.

Discussion

Mouse models of *P. multocida* serotype A infection provide a well established experimental model to study immunopathologic responses to infection. *Echinacea purpurea* is widely used as a self-prescribed agent against upper respiratory tract infections such as the common cold (O'Hara *et al*., 1998). To characterize *in vivo* antibacterial and potential immunomodulatory effects of *Echinacea*, a mouse model of live *P. multocida* infection was used in the present study. In this model, clinical signs, lung and liver bacterial cultures, histopathological lesions and serum TNF_{α} levels were studied.

In this research, the experimental challenge was the intranasal administration of *P. multocida* in mice. The pathological study 24 and 48 h post infection showed acute bronchopneumonia which is in agreement with other studies (Praveena *et al*., 2010; Pors *et al*., 2011).

Clinical observation of the EPE+PMA group showed better conditions in comparison with the PMA group. In addition, the bacterial count of the EPE+PMA group had significant differences with that of the PMA group (P<0.05). This result was also supported by histopathological observations in that the EPE+PMA group showed fewer lesions in comparison to the PMA group. This finding indicated that the improvement may be due to bactericidal or immunomodulatory effects of *E. purpurea* extract, a finding which is in agreement with previous reports (Sharma *et al*., 2010). According to many *in vitro* studies, *E. purpurea* stimulates macrophage activity, leading to the stimulation of non-specific immune responses. Goel *et al*. (2002) reported an evident increase in TNF_{α} and nitric oxide (NO) release by the alveolar macrophages following *in vitro* stimulation with LPS. A predominant mechanism by which alveolar macrophages destroy infectious agents is releasing cytokines such as NO. Alveolar macrophages also produce a variety of cytokines, such as IL_1, IL_6, TNF_{α} and cytotoxic products which can be effective against infectious agents (Luettig *et al*., 1989). It has been made clear that the polysaccharide components of *Echinacea* increase phagocytosis and chemotaxis in macrophages (Sitimpel, 1984; Luettig, 1989) and neutrophils (Wagner *et al*., 1991). Goel *et al*. (2002) reported that phagocytic activity of alveolar macrophages improved with increasing concentrations of *Echinacea* components.

In this study, serum TNF_{α} concentration showed significant differences between the PMA and the control groups. The level of serum TNF_{α} in PMA and EPE groups was also significant (P<0.01). This data indicated that bacterial inoculation increased secretions of cytokines followed by inflammatory reactions. Similar to the present study, Praveena *et al*. (2010) reported higher concentrations of TNF_{α} at 12 and 24 h post infection. LPS and porin proteins isolated from *P. multocida* have been reported to upregulate mRNA expression levels of proinflammatory cytokines in murine splenic lymphocytes (Iovane *et al*., 1998).

Serum TNF_{α} concentrations in the PMA and EPE+PMA groups were found to be significantly different (P<0.05), indicating the decrease of these cytokines in the EPE+PMA group which was also supported by the diminishing inflammatory reaction in lung sections. The histopathological study of the EPE+PMA group showed less infiltration of neutrophils and hyperemia in lungs, meaning that the production of pro inflammatory cytokine, especially TNF_{α} level, has been modulated. Compared to the EPE group, serum TNF_{α} of the EPE+PMA group increased, but the difference was not statistically significant (P>0.05). This change means that *E. purpurea* was able to modulate the secretion of TNF_{α}. As mentioned by other researchers (Rininger *et al*., 2000; Goel *et al*., 2002; Matthias *et al*., 2007), the increasing level of this cytokine in the EPE+PMA group compared to the EPE group may be due to the stimulatory effects of bacteria on increasing TNF_{α} levels in the latter group. Thus far, several experiments have demonstrated the controversial effects of this herb on the immune system. Sullivan *et al*. (2008) showed that activation of peritoneal macrophages by *E. purpurea* polysaccharides resulted in increased production of inflammatory cytokines (TNF_{α}, $IL_{1\alpha}$, IL_6, and IL_{12}) and nitric oxide (NO). Burger *et al*. (1997) demonstrated that by using *E. purpurea* extract in human peripheral blood macrophages IL_1, TNF_{α} and IL_{10} levels can be increased. Goel *et al*. (2002) showed that the amount of TNF_{α} and NO produced by alveolar and spleen macrophages can be increased by using water-ethanol extracts of *E. purpurea*. Chicca *et al*. (2009) showed that ethanolic extracts with alkamide fractions of *Echinacea* stimulate anti-inflammatory cytokines (IL_{10}) and inhibit the secretion of proinflammatory (e.g., TNF_{α}) cytokines from murine macrophage cell lines. Sharma *et al*. (2009) demonstrated that ethanolic extracts of *E. purpurea* caused reductions in IL_6 and IL_8 in a virus infected human bronchial epithelial cell line. Zhai *et al*. (2007) showed that the production of cytokines such as $IL_{1\beta}$, TNF_{α} and NO by macrophages infected with *Salmonella enterica* decreased when cultured with ethanol extracts of *E. purpurea*.

In an *in vitro* study, the alkylamides isolated from *Echinacea* were shown to inhibit 5-lipoxygenase and cyclooxygenase, which are key enzymes for the production of prostaglandins, and important as inflammatory mediators (Muller-Jakic *et al*., 1994). The anti-inflammatory effects of this extract have been shown in Arsenic induced hepatic toxicity (Heidari *et al*., 2011). In the present study, decreased inflammation in the EPE+PMA group was obvious which may be due to the anti-inflammatory effects of *Echinacea* extract. *Echinacea* plant extract has been used for immune stimulations for many years, but the evidence supporting its therapeutical potential is still controversial. In recent years, much effort has been made to identify the potential components in *Echinacea* plant extract and to account for its *in vitro* immunostimulatory effects. Some of these bioactives include polysaccharides, cichoric acid and alkylamides.

In conclusion, intraperitoneal injections of *E. purpurea* extract for 2 weeks before the induction of pneumonia by *P. multocida* prevented bacterial growth in the lung and liver, reduced histopathologic lesions in these organs and modulated the concentration of serum TNF_{α} in challenged mice. Thus, *E. purpurea* extract can be used as an herbal drug for prevention and control of pneumonia due to *P. multocida*. Considering the widespread bactericidal, immunomedulatory and anti-inflammatory properties of *E. purpurea*, it could be a beneficial herbal drug in any respiratory bacterial infection.

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Ethical standards

The experimental and animal care protocols were in compliance with the requirements of the Ethics Committee.

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