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Echinacea stimulates macrophage function in the lung and spleen of normal rats

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Abstract

Echinacea plant extract has been used for immunostimulation for many years but the evidence supporting its therapeutic potential is still controversial. Using male Sprague-Dawley rats (425–475 g), an *in vivo* study was conducted to examine the immunomodulatory effects of preparations of Echinacea containing its components cichoric acid, polysaccharides and alkylamides in different concentrations. The rats were gavaged orally with these preparations, two times/day for 4 days. Phagocytic activity of alveolar macrophage was increased with increasing concentrations of the Echinacea components. A trend of increase in TNF-α and nitric oxide release by the alveolar macrophages following an *in vitro* stimulation with LPS was also evident. An enhanced release of cytokines (such as TNF-α and IFN-γ) in response to Echinacea components, was also apparent in rat's spleen macrophage, but at higher concentrations. These results suggest that the Echinacea preparations containing optimal concentrations of cichoric acid, polysaccharides and alkylamides are potentially effective in stimulating an *in vivo*, non-specific immune response in normal rats.

Keywords: Echinacea; Alveolar macrophages; Splenocytes; Phagocytosis

1. Introduction

The genus Echinacea (purple coneflower) is a North American indigenous plant. Its extract has been traditionally used in the treatment of various conditions, such as bacterial/viral infections, cancer, seizures and AIDS [1]. In recent years, it has become one of the popular herbal products in North America and Europe as an immune promoter, particularly for the prevention and treatment of upper respiratory tract infections.

According to many *in vitro* studies, Echinacea-induced immunomodulation appears to occur through stimulation of non-specific immune system [2,3]. It has, thus, been shown to stimulate the phagocytic activity of the macrophages [4–6], increase the production of IL-1, IL-6 and TNF-α by the macrophages [7], and enhance the natural killer function of human peripheral blood mononuclear cells [8]. However, in contrast to these *in vitro* studies the results from the *in vivo* and the clinical trials have been highly variable [9,10]. One of the reasons for this discrepancy could be that the Echinacea extract had not been standardized in terms of its active constituents. The lipophyllic alkylamides, polar caffeic acid derivatives such as cichoric acid, and polysaccharides have been suggested to be the potential bioactive constituents of Echinacea [11].

Using healthy rats, an *in vivo* study was designed to investigate the dose related effects on the immunomodulatory potential of an extract of Echinacea containing various levels of its bioactive components.

2. Materials and methods

2.1. Echinacea extracts

Cichoric acid, polysaccharide and alkylamide fractions were obtained from *Echinacea purpurea* plants by water-ethanol extraction of the roots or the aerial parts. The frac-

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Table 1
Composition of the echinacea extracts*

Extracts	B	C	D	E
		μg/kg/d		
Cichoric acid	40	120	800	2,000
Polysaccharides	1000	3000	20,000	50,000
Alkylamides	4	12	80	200

* The extracts were prepared at the Research and Development Laboratory of 'Natural Factors', Burnaby, BC, Canada. Fractions enriched in cichoric acid, polysaccharides or alkylamides were obtained by water-ethanol extraction of roots or aerial parts of *Echinacea purpurea* plants and were used to make above extracts in 50% ethanol.

tions were purified to >95% purity and were used to make 4 extracts B-E in 50% ethanol (Table 1). An average per kilogram 'basal dose level' was determined for each component through a survey of various products. Since a great deal of controversy exists regarding the effectiveness and quality of various Echinacea products, formulations containing different concentrations of the components were tested. Extract B was made to provide the basal dose level of the components (cichoric acid, polysaccharides and alkylamides at a concentration of 40, 1000 and 4 μg/Kg/day, respectively). The extracts C, D and E contained cichoric acid, polysaccharides and alkylamides at levels 3, 20 and 50 times the basal dose level, respectively. The extracts were prepared at the Research and Development laboratory of 'Natural Factors' (Burnaby, BC, Canada) and labeled with their identities concealed until the completion of the study.

2.2. Animals

Male Sprague-Dawley rats weighing 425–475 g were obtained a week before the start of the experimental treatments. Throughout the study the animals were fed a pelleted chow diet (Purine Lab Rodent diet no. 5001, Purina, Richmond, IN, USA) and were kept in separate plastic cages in a well-ventilated room maintained at 21 ± 2°C with a 12-h light dark cycle. After a week of acclimatization, the animals were randomly divided into 5 groups of 6 rats each. Groups B-E received the four extracts of Echinacea (B-E) and group A received 50% ethanol and served as control. All the extracts (100 μl) were administered to the animals by oral gavage twice a day for 4 days. The study had received an approval by the University of Alberta Animal Welfare Committee.

2.3. Sample collection

The body weights of the animals were recorded at the beginning and at the end of the experimental treatment. After 4 days of gavaging, the animals were sacrificed by a lethal pentobarbital injection. Alveolar macrophages (AM) were obtained from the animals by bronchoalveolar lavage (BAL) [12]. The exsanguinated animals were lavaged via a polyethylene cannula inserted into the trachea. The BAL cells (AM) were collected by 3 × 20 ml washes with ice-cold sterile PBS (0.1 M, pH 7.4). The AM were washed twice and then resuspended in adequate volume of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) to give a concentration of 1×10^6 cells/ml.

The spleens were also removed from the animals under sterile conditions and placed in chilled sterile petri dishes containing Krebs-Ringer Hepes buffer (KRH) with 0.5% (w/v) bovine serum albumin (BSA). The immune cells were isolated from the spleen by pressing them through nylon mesh (100 um) [13]. The red blood cells were lysed using a lysis buffer (NH_4Cl 155 mM, EDTA 0.1 mM, $KHCO_3$ 10 mM, pH 7.4) under sterile conditions. The cells were washed three times with ice cold KRH with 0.5% BSA and

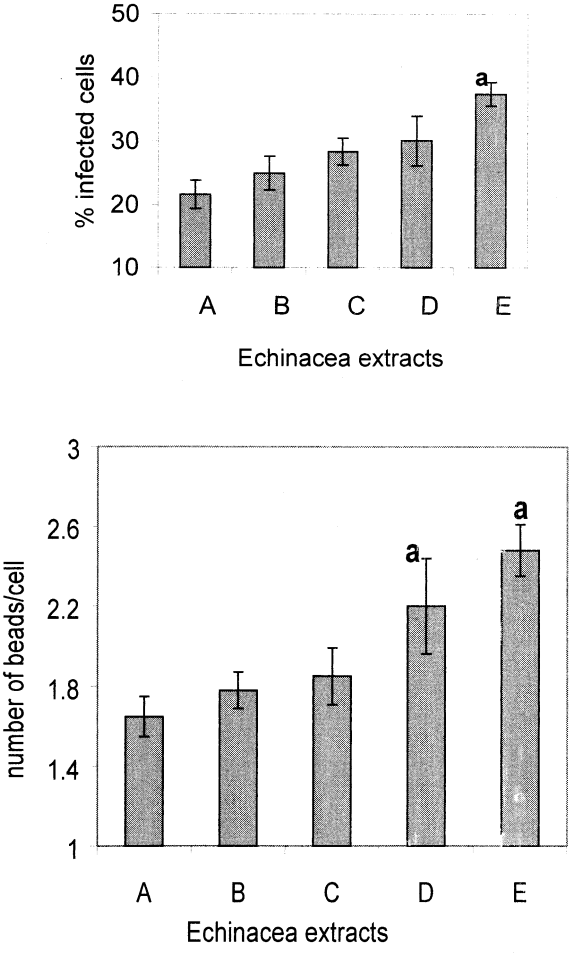

Fig. 1. (a and b). Effect of oral administrations of the Echinacea extracts to rats for 4 days on the phagocytic activity of the alveolar macrophages. Values are means for six animals, with their standard errors indicated by vertical bars. Bars not sharing a common superscript letter are significantly different at $P < 0.05$. A: 50% ethanol, B: Echinacea extract providing cichoric acid, polysaccharides and alkylamides at a concentration of 40, 1000 and 4 ug/Kg/day, respectively. C, D and E: Echinacea extracts having the concentrations of the three components at level 3, 20 and 50 times the level in the extract B, respectively.

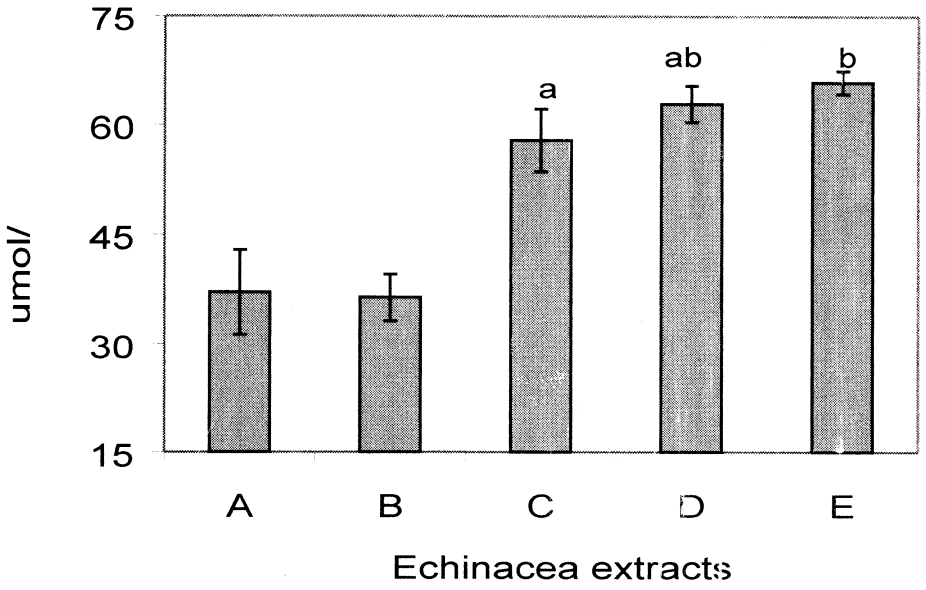

Fig. 2. Effect of oral administrations of the Echinacea extracts to rats for 4 days on LPS-induced Nitric oxide release by the alveolar macrophages. Values are means for six animals, with their standard errors indicated by vertical bars. Bars not sharing a common superscript letter are significantly different at $P < 0.05$. A: 50% ethanol, B: Echinacea extract providing cichoric acid, polysaccharides and alkylamides at a concentration of 40, 1000 and 4 ug/Kg/day, respectively. C, D and E: Echinacea extracts having the concentrations of the three components at level 3, 20 and 50 times the level in the extract B, respectively.

were finally suspended in RPMI supplemented with 4% FCS at a concentration 1.5×10^6 cells/ml.

2.4. Phagocytosis assay

AM (0.25×10^6) in RPMI were incubated at 37°C in a humidified atmosphere of 5% CO_2 in well chamber slides (Lab-Tek 11 Chamber slide w/cover, Nalgene Nunc International) for 3 hr for adherence. The non-adherent cells were then washed off and 200 uL fresh medium (RPMI-1640 containing 10% FCS) was added. The assay for phagocytosis was performed by adding latex beads to the wells in a ratio of 10 beads/cell and continuing the incubation for another 1h at 37°C. Phagocytosis was terminated by washing the wells 5 times with RPMI with 10% FCS. The slides were stained with Hemastaining kit (Hema 3 stain set, Fisher Scientific). The percentage of infected cells and the phagocytic index (number of beads per infected cell) were determined by microscopic examination under the oil immersion.

2.5. Measurement of nitric oxide (NO) and cytokine production

The AM in RPMI with 10% FCS at a concentration of 0.5×10^6 cells/ml were incubated with or without LPS (*E. coli*, 10 μg) for 60 h in a humidified atmosphere at 37°C in the presence of 5% CO_2. After 60 h, the cells were centrifuged at 2000 rpm for 10 min at 4°C. Cell-free supernatants were collected and stored at −70°C for later analysis of NO and TNF-α.

The spleen cells were incubated with and without phorbol myristate acetate (PMA, 40 ng) and ionomycin (0.5 nmol) for 60 h. The cells were then centrifuged at $228 \times g$ and the cell free supernatants were collected and stored at −70°C for the later analysis of TNF-α, IFN-γ and IL-2.

Nitric oxide (NO) production was determined by measuring its stable metabolic end product, nitrite by colorimetric reaction using Griess reagent [14]. Concentrations of the cytokines in the cell culture supernatants were determined by enzyme-linked immunosorbent assay (OptEIA ELISA kits, Pharmingen, San Diego, CA).

2.6. Statistical analysis

The data was subjected to one-way ANOVA using Statistical Analysis System version 7.0. The treatment means were compared using Duncan's multiple range test and the differences were considered to be significant if the associated P value was <0.05 [15].

3. Results

Neither weight gain nor food intake of the rats was affected by oral administration of the Echinacea extracts for 4 days. Fig. 1 (a and b) illustrates the effects of Echinacea on the phagocytic activity of the AM. The percentage of actively phagocyting AM increased in a dose-dependent fashion when the concentration of the three components in the Echinacea extracts was increased and the extract E produced the most significant effects ($P < 0.05$). Increasing the concentrations of cichoric acid, polysaccharides and

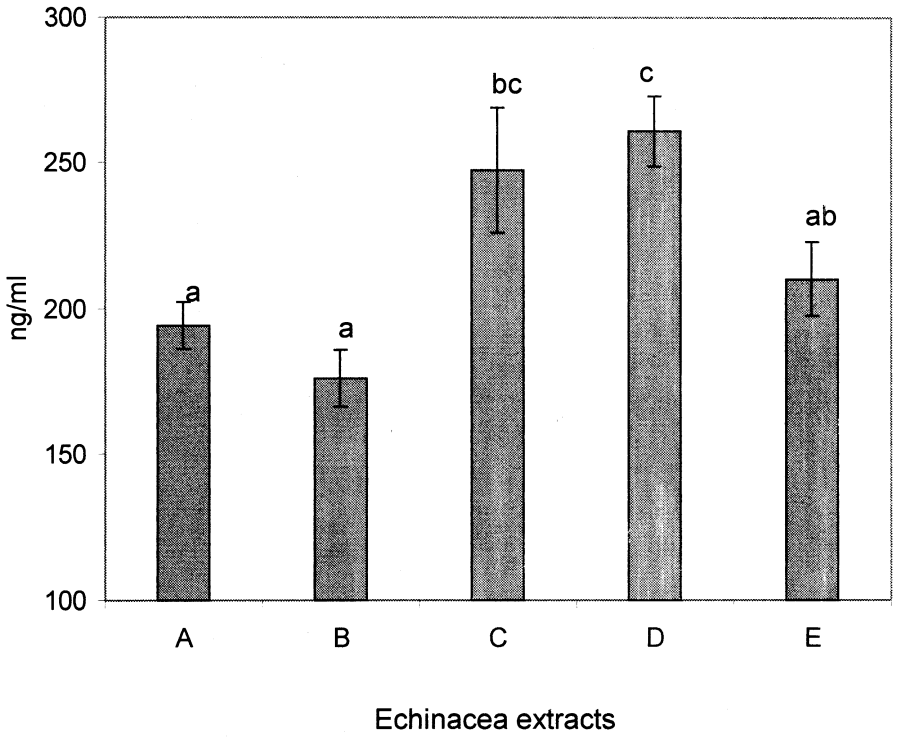

Fig. 3. Effect of oral administrations of the Echinacea extracts to rats for 4 days on LPS-induced TNF-α release by the alveolar macrophages. Values are means for six animals, with their standard errors indicated by vertical bars. Bars not sharing a common superscript letter are significantly different at $P < 0.05$. A: 50% ethanol, B: Echinacea extract providing cichoric acid, polysaccharides and alkylamides at a concentration of 40, 1000 and 4 ug/Kg/day, respectively. C, D and E: Echinacea extracts having the concentrations of the three components at level 3, 20 and 50 times the level in the extract B, respectively.

alkylamides in the extracts also resulted in dose-related effects on the phagocytic index of the AM and the effects obtained with the extracts D and E were significantly different ($p < 0.05$) from the extracts B, C or the control.

The effects of the Echinacea extracts on NO and TNF-α release by the AM after an *in vitro* stimulation with LPS were also assessed. In the absence of any stimuli, neither TNF-α nor NO was secreted at a detectable level. Stimulation with LPS caused a significant increase in the release of TNF-α and NO by the AM. The oral administration of the extracts containing the increasing concentrations of the three components resulted in a dose-related increase in NO production. The rats gavaged with the extract E showed an 80% increase ($P < 0.05$) in NO release (Fig. 2) in comparison to control animals. Increasing the concentrations of the three components in Echinacea extracts also resulted in an increase in TNF-α release by the AM (Fig. 3).

The effects of oral administration of the Echinacea extracts on the stimulated production of cytokines TNF-α, IFN-γ and IL-2 by the spleen cells are shown in Table 2. Increasing the concentrations of cichoric acid, polysaccharides and alkylamides in the Echinacea extracts led to increases in the release of TNF-α and IFN-γ by the spleen cells. The effects were statistically significant with the extract E ($P < 0.05$). However, none of the extracts had any effect on the stimulated IL-2 release by the splenocytes.

4. Discussion

In vitro studies have shown that the commercial preparations of Echinacea extract stimulate cytokine production by human peripheral blood macrophages [7], enhance NK cell function and antibody dependent cytotoxicity of peripheral blood mononuclear cells [8,20,21]. The *in vivo* immune

Table 2
Effect of oral administrations of the Echinacea extracts to rats for 4 days on PMA and Ionomycin induced TNF-α, IFN-γ and IL-2 release by the splenocytes

Echinacea extracts	TNF-α	IFN-γ	IL-2
		nmol/ml	
A	$5.1 \pm 0.8^{b,c}$	16.2 ± 2.6^{b}	80.2 ± 6.6^{a}
B	$5.6 \pm 0.5^{b,c}$	16.6 ± 2.9^{b}	70.5 ± 11.3^{a}
C	$5.3 \pm 0.4^{b,c}$	17.6 ± 2.6^{b}	69.2 ± 2.8^{a}
D	$6.1 \pm 0.8^{a,b,c}$	$19.9 \pm 2.8^{a,b}$	82.7 ± 11.5^{a}
E	8.4 ± 0.7^{a}	26.0 ± 4.5^{a}	83.1 ± 9.3^{a}

n = 6, Means in columns not sharing a common superscript letter are significantly different at $p < 0.05$.

A: 50% ethanol, B: Echinacea extract providing cichoric acid, polysaccharides and alkylamides at a concentration of 40, 1000 and 4 μg/Kg/d, respectively. C, D and E: Echinacea extracts having the concentrations of the three components at level 3, 20 and 50 times the level in the extract B, respectively.

modulatory effects of Echinacea are, however, scanty and inconclusive [16]. This is the first study reporting *in vivo*, quantitative and dose-related effects of an extract of Echinacea, standardized in its contents of cichoric acid, polysaccharides and alkylamides, on immunocompetence in healthy rats. The phagocytic response of the AM, obtained from the rats administered the extracts of Echinacea containing four levels of potential bioactives, increased in a dose-dependent fashion. The effects were evident on both the phagocytic activity as well as phagocytic index. Thus, the overall percentage of the AM phagocytosing latex beads as well as the capacity of individual cells to phagocytose appeared to be increased.

AM, in addition to its phagocytic response to pathogens, also produces a variety of cytokines, such as IL-1, IL-6, TNF-α, and cytotoxic products, including reactive oxygen and nitrogen intermediates, to regulate their own activity and the activity of other immune cells [17]. The present study, therefore, also determined the effects of Echinacea on AM function especially its ability to produce NO and TNF-α. It was of interest that the oral administrations of the Echinacea extracts to rats with the increasing concentrations of cichoric acid, polysaccharides and alkylamides, led to an increased release of NO from the AM. Since release of NO has been known as the predominant mechanism by which AM destroys the infectious agents [18,19], the ability of Echinacea extracts to induce NO release appears to be an important observation. With the exception of the extract E, a dose-related increase in TNF-α release by the AM was also observed. Although Echinacea is widely used for prophylaxis and treatment of upper respiratory tract infections, its effect on alveolar macrophage function is not known. The ability of the Echinacea extracts to induce TNF-α by AM, could then be related to its claimed antiviral effects and therefore, may support its prophylactic and therapeutic effects against common cold [23]. The depression of TNF-α release with the extract E could be due to increased release of NO, inhibiting the release of inflammatory cytokines such as TNF-α by the AM [24].

In the present study the effects of Echinacea on the functional activities of macrophages, natural killer cells and T-cells in the spleen of rats administered orally the extract containing various levels of potential bioactives, were estimated through the measurement of various cytokines. Although, the extracts did not have any statistically significant effect on the IL-2 release by the splenocytes, a trend of dose-related increased release of TNF-α and IFN-γ was observed. The effects obtained with the extract E were significantly different from all the other extracts or the control. This may suggest, that the Echinacea extracts affected only the components of non-specific immune system, involving the macrophages and the NK cells in the spleen of healthy rats, without affecting the T-cell function. Additionally the effects of Echinacea do not appear to be confined to alveolar macrophages. At a higher concentration, the non-specific immune response in the spleen was also stimulated by the extracts.

The results from the present study support the non-specific, immunomodulatory potential of the Echinacea extracts *in vivo*. Although, Echinacea has been widely used as an immune booster herb, its *in vivo* clinical efficacy was still unclear. To bring credibility to this novel herb, it was necessary to conduct a well-controlled *in vivo* study to optimize the concentrations of the principal actives in the preparations so that the products could be pharmacologically consistent. The present study not only provides an *in vivo* evidence of Echinacea's efficacy, but also illustrates a dose-related response of the extracts, which might help to optimize the ratio of these potential bioactive components in the Echinacea preparations for clinical use. Since the Echinacea preparations used were only 95% pure, it may be argued that the immune responses could have been influenced by the 5% unknown component(s). It should be pointed out, however, that no significant differences in immune response were found in between the preparations containing ethanol (control) and the actives in their basal concentrations. Hence, it is likely that the immune stimulating effects of the increased concentrations of polysaccharides, cichoric acid and alkylamides were the true reflection of these bio-actives.

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