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Alcohol extracts of Echinacea inhibit production of nitric oxide and tumor necrosis factor-alpha by macrophages *in vitro*

ZILI ZHAI[1,2], **DEVON HANEY**[1,3], **LANKUN WU**[4], **AVERY SOLCO**[5], **PATRICIA A. MURPHY**[5], **EVE S. WURTELE**[4], **MARIAN L. KOHUT**[6], and **JOAN E. CUNNICK**[1,2,3]

1*Department of Animal Science, Iowa State University, Ames, IA, USA*

2*Neuroscience Interdepartmental Graduate Program, Iowa State University, Ames, IA, USA*

3*Interdepartmental Microbiology Program, Iowa State University, Ames, IA, USA*

4*Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*

5*Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, USA*

6*Department of Health and Human Performance, Iowa State University, Ames, IA, USA*

Abstract

It has been suggested that Echinacea has anti-inflammatory activity *in vivo*. Nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-1beta are important mediators in the inflammatory response. The effect of alcohol extracts of *E. angustifolia* (EA), *E. pallida* (EPA) and *E. purpurea* (EP) on the production of these inflammatory mediators in both LPS-stimulated RAW 264.7 macrophages *in vitro* and murine peritoneal exudate cells (PECs) *in vivo* were investigated. As macrophages produce these inflammatory mediators in response to pathogenic infection, parallel cultures of macrophages were studied for phagocytosis and intracellular killing of *Salmonella enterica*. EPA and EP *in vitro* inhibited NO production and TNF-α release in a dose-dependent manner. RAW 264.7 cells treated with EA or EP showed decreased killing over 24 h, although EA enhanced bacterial phagocytosis. Upon bacterial infection, RAW 264.7 cells produce high levels of NO; however, an Echinacea-mediated decrease in NO production was observed. Echinacea alcohol extracts administered orally at 130 mg/kg per day for seven days had a weak effect on NO production and phagocytosis by LPS-stimulated PECs. The results indicated that all Echinacea species significantly decreased inflammatory mediators *in vitro*, however, only EA and EP reduced bacterial killing. Oral administration of Echinacea alcohol extracts did not adversely affect the development and anti-bacterial function of inflammatory PECs *in vivo*, however, NO production was decreased during bacterial infection of PECs.

Keywords

Echinacea; macrophage; nitric oxide; phagocytosis; tumor necrosis factor-alpha

Introduction

The genus Echinacea is a popular natural immunostimulant. Reports indicate that Echinacea increases phagocytosis by neutrophils and macrophages, and stimulates these inflammatory cells to produce proinflammatory cytokines and free radicals (Stimpel et al. 1984; Steinmuller et al. 1993; Burger et al. 1997; Goel et al. 2002), which are responsible for the antimicrobial

Correspondence: Joan E. Cunnick, Interdepartmental Microbiology Program, 207 Science I, Iowa State University, Ames, IA 50011, USA. Tel: –1 515 294 2070. Fax: –1 515 294 6019. E-mail: jcunnick@iastate.edu.

activity. Contrary to these reports, Echinacea is also used as an anti-inflammatory agent. The anti-inflammatory effects have been demonstrated using murine inflammatory models, such as carrageenan-induced paw edema and abraded skin (Tubaro et al. 1987; Raso et al. 2002; Speroni et al. 2002). These seemingly contradictory, but interdependent biological activities of Echinacea products are, to a very large extent, related to their immunomodulating properties and may be due to the variability in composition of the herb products utilized in each study (Percival 2000). Several active components have been identified in Echinacea, including lipophilic alkamides, polar caffeic acid derivatives (mainly echinacoside and cichoric acid) and high molecular weight polysaccharides (Barnes et al. 2005). In the current herbal market, Echinacea products are a mixture of many varieties and may be blended with other herbs in various forms, making it difficult to clarify which components play a predominant role in the immunomodulatory activity. The interaction between the components of Echinacea crude preparations is also unclear. The effects of Echinacea products might also depend on the host condition. Echinacea may enhance immune function in the body with a weakened immune system (Currier & Miller 2000; Bodinet et al. 2002), but suppress it when an inflammatory response predominates. Our previous animal study found that Echinacea treatment restored mild stress-induced immune changes to normal levels (Zhai et al. 2007).

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

In this study, the effects of alcohol extracts of the three commonly used Echinacea species – *E. angustifolia* (EA), *E. pallida* (EPA), and *E. purpurea* (EP) – were further examined both *in vivo* and *in vitro* by determining the production of inflammatory mediators, NO, TNF-α and IL-1β in activated macrophages. Parallel cultures of *ex vivo* macrophages and the macrophage cell line RAW 264.7 cells were studied for phagocytosis and intracellular killing of *Salmonella* in order to understand if Echinacea alcohol extracts compromise the antibacterial function of macrophages through inhibition of inflammatory mediators.

Materials and methods

Preparation of alcohol extracts

The plants EA, EPA and EP were harvested in the USDA North Central Regional Plant Introduction Station (Ames, IA, USA) with the identification numbers PI 631285, PI 631293 and PI 631307, respectively. Alcohol extracts from the dried roots were prepared as previously described (Zhai et al. 2007) followed by evaporation until dry. These dry residues were subsequently dissolved in ethanol and phosphate buffed saline (PBS) to desired concentrations for *in vitro* assays or dissolved in ethanol and Nanopure water for animal studies. The extracts

used for *in vitro* assays were further filter-sterilized using a 0.45-μm microfilter (Costar, Corning, NY, USA). Aliquots of alcohol extracts were stored at −80°C (for *in vitro* assays) or −20°C (for animal studies) and freshly thawed for each experiment. The endotoxin levels were evaluated in aliquots using the Limulus Amebocyte Lysate Test (BioWhittaker, Inc., Walkersville, MD, USA) according to the manufacturer's specifications for a microplate assay, and were detected to be below the limit of detection (0.1 EU/ml). Phytochemical analysis was performed to detect alkamides and caffeic acid derivatives in aliquots using high performance liquid chromatography (HPLC) as described previously (Wu et al. 2004).

Animals

Animal care and experimental procedures were approved by the Iowa State University Committee on Animal Care. Male BALB/c mice, 8 weeks of age, were obtained through Iowa State University Laboratory Animal Resources from Harlan Laboratories (Indianapolis, IN, USA) and allowed to acclimatize to the new environment for 2 weeks. The mice were housed three per cage and provided free access to food and water under a reverse 12 h light/dark regimen (lights off at 09:00 h).

Echinacea administration *in vivo*

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

Peritoneal exudate cell collection

Some 12–15 h after the last gavage, the mice were euthanized by CO_2 asphyxiation, and PECs were immediately collected by peritoneal lavage with ice-cold EDTA (0.1%; w/v) in Hank's balanced salt solution (Fortier & Falk 1994). For each group, PECs were pooled and washed twice with RPMI 1640 medium supplemented with 2 mM glutamine, 25 mM Hepes, 50 μg/ml gentamicin and 10% heated-inactivated fetal bovine serum (complete medium).

Macrophage cell line

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

Cell viability assay

Mitochondrial reduction of MTS, a tetrazolium compound, to a colored formazan was determined as an indicator of cell viability. RAW 264.7 cells and PECs were seeded at 4×10^4 cells per well in 96-well tissue culture plates with or without 1 μg/ml lipopolysaccharide (LPS; *E. coli* 055:B5, L6529; Sigma, St. Louis, MO, USA). For cultures of RAW 264.7 cells, one of the alcohol extracts was added at 0–500 μg/ml at the same time the cells were plated in the absence or presence of LPS. After overnight incubation, MTS (Promega, Madison, WI, USA) was added and cultures then successively incubated for 3 (PECs) or 1 (RAW 264.7 cells) h. The extent of formazan formation was determined photometrically at absorbance 490 nm

using a Bio Kinetics Reader (Bio-Tek Instruments, Winooski, VT, USA). The absorbance of cell-free complete medium with or without vehicle or Echinacea alcohol extracts was used as a blank and subtracted from the value of the corresponding treatment groups.

Growth and freezing of *Salmonella*

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

Gentamicin protection assays

The engulfment and killing of *Salmonella* were performed using both RAW 264.7 cells and PECs. The protocol for each cell type was identical with the exception that Echinacea alcohol extracts were added to cultures of RAW 264.7 cells. PECs were collected from mice that had been gavaged with one of the alcohol extracts *in vivo*. RAW 264.7 cells and PECs were seeded at a concentration of 4×10^5 cells per well (1 ml) in 24-well plates and incubated overnight. After harvesting the culture supernates for NO and cytokine assays, described below, cells were washed three times in gentamicin-free complete medium. *Salmonella* were then added to the adherent RAW 264.7 cells and PECs at a multiplicity of infection (MOI) of 100 bacteria per cell. Following 2 h of infection, cells were washed in warm complete medium containing 50 μg/ml of gentamicin and maintained in gentamicin-containing complete medium for the remainder of the experiment to kill all extracellular bacteria. At 0, 4 and 24 h post-infection, one set of parallel cell cultures were washed with sterile PBS to remove gentamicin and then lysed in distilled water to release the intracellular bacteria. Intracellular bacteria were enumerated by serial dilution and plate count on LB agar. Lysate dilutions on LB agar were incubated overnight at 37°C and counts were expressed as colony forming units (CFU)/ml. CFU at the 0 h time point reflects the amount of phagocytosis by the cultured RAW 264.7 cells and PECs, while the reduction in CFU at 4 and 24 h time points reflects the killing of intracellular bacteria by the cells. As Echinacea treatment induced some changes in eukaryotic cell growth (see Figure 1), the intracellular bacteria quantitation was adjusted based on the parallel MTS assay to normalize each treatment group to the same cell count before bacterial infection by using the formula: number of intracellular bacteria = number of viable bacteria counted × (control cells MTS OD)/(treated cells MTS OD).

Nitrite assay

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

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

Cytokine assays

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

Statistical analysis

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

Results

Phytochemical analysis

Echinacea alcohol extracts contain several chemical components. Lipophilic alkamides and hydrophilic caffeic acid derivatives are two groups of the most studied metabolites. The concentrations of phytochemicals identified and quantified using HPLC are listed in Table I. Alkamides were the major phytochemicals detected, especially in EA and EP alcohol extracts. Note that amides 7 and 8 appear in the present EPA extracts though they were undetectable in our previously prepared alcohol extracts (Zhai et al. 2007). Among the caffeic acid derivatives, echinacoside was found in higher quantities in both EA and EPA extracts, while cichoric acid was the main form in EP extract.

In vitro experiments

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

To assess the effect of Echinacea alcohol extracts on NO production, RAW 264.7 cells were exposed to LPS plus alcohol extracts for 24 h. The alcohol extracts reduced NO production in a dose-dependent manner (Figure 2A). In comparison to the vehicle control, EPA at 100 μg/ml significantly decreased the production of NO (p = 0.009). When the herbal concentration was raised to 200 μg/ml, NO production was significantly inhibited by all three alcohol extracts (p values ≤0.042). None of the three alcohol extracts at the concentrations tested interfered with the reaction between nitrite and the Griess reagent (data not shown).

RAW 264.7 cells activated by LPS secrete high levels of TNF-α (reaching up to 9000 pg/ml). The effect of Echinacea alcohol extracts on the TNF-α levels was evaluated in the same culture supernates analyzed for the NO levels. All three extracts had a similar inhibitory effect on TNF-α secretion as on NO production (Figure 2B). When compared to the vehicle control, both EPA and EP at a concentration of 100 μg/ml significantly decreased TNF-α secretion (p<0.02 and

$p<0.0001$, respectively). EA, EPA and EP at the highest concentration tested of 200 μg/ml also inhibited TNF-α secretion (p values <0.04).

After incubation with Echinacea alcohol extracts overnight, RAW 264.7 cells were assessed for phagocytosis (CFU at 0 h) and bacterial killing (reduction in CFU at 4 and 24 h) after the addition of *Salmonella*. Figure 3 shows the survival of bacteria in RAW 264.7 cells pretreated with one of the three alcohol extracts in the absence of LPS. Incubation of RAW 264.7 cells with EA at 100–200 μg/ml enhanced the subsequent phagocytosis (p values < 0.04), but inhibited bacterial killing at 4 (p values < 0.026) and 24 h (p values < 0.026) post-infection. EPA displayed no significant effects on bacterial phagocytosis, but increased bacterial killing over 24 h incubation ($p = 0.002$ at 200 μg/ml). EP had no effect on bacterial phagocytosis, but later decreased bacterial killing. EP at 100–200 μg/ml significantly inhibited bacterial killing 4 h post-infection (p values < 0.037). At 24 h post-infection, EP at 10 μg/ml also showed an inhibitory effect on bacterial killing ($p = 0.011$).

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

Ex vivo experiments

Parallel studies were performed using proteose peptone-elicited PECs. After mice were gavaged with Echinacea alcohol extracts for seven consecutive days, PECs were harvested and incubated overnight in the absence or presence of LPS. There were no significant differences in cell number as determined by the MTS assay (Figure 5A) and NO production (Figure 5B) between the vehicle control and any of the Echinacea treatment groups. In addition, no significant differences in the secretion of cytokines IL-1β and IL-10 in culture supernates of PECs in the presence of LPS were associated with oral administration of the extracts compared to the vehicle control (Figure 5C). For all these assays of PECs (cell viability, NO production and cytokines), no significant difference was observed between the two control groups (the no gavage control and the vehicle control).

After incubation of PECs overnight without LPS stimulation, cells were further assessed for phagocytosis and bacterial killing as well as NO production at 0, 4 and 24 h post-infection. In comparison to the no gavage control, the vehicle control displayed significantly decreased phagocytosis ($p < 0.02$) (Figure 6A). However, one of the three extracts, EPA, showed a marginal increase in bacterial phagocytosis over the vehicle control ($p = 0.052$). With increasing time after infection, there were no significant differences in bacterial killing between the vehicle control and any of other groups. Measurement of NO production post-infection showed that mice gavaged with the vehicle displayed increased potential in NO production and a significant increase in the vehicle control was found at 4 h after infection compared to the no gavage mice ($p < 0.05$) (Figure 6B). Conversely, three Echinacea treatments showed, to some extent, decreased NO production when compared to the vehicle control, and EP exhibited a significant decrease in NO production compared to the vehicle control ($p < 0.03$) at 4 h, but not 24 h post-infection.

Discussion

This study demonstrates that Echinacea alcohol extracts *in vitro* inhibit LPS-induced generation of NO and TNF-α in macrophages. Although NO and TNF-α are needed for clearing

and containing bacterial infection, excessive NO and TNF-α are implicated in a pathological role in inflammatory responses. Such an inhibitory property may endow Echinacea alcohol extracts as an effective anti-inflammatory remedy. In the present study, we found evidence that Echinacea can decrease inflammatory mediators *in vitro*, but that the effect *in vivo* was observed only in comparison with the vehicle control group, which exhibited an excessive production of NO during *Salmonella* infection. Interestingly enough the excessive NO production and suppressed NO production did not correlate with any biological effects of bacterial killing in the PECs.

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

The NO and TNF-α inhibitory potential of the three alcohol extracts *in vitro* is generally EPA > EP > EA. The basis of this difference in the modulation of NO and TNF-α production may rest on the distinct amounts and types of phytochemicals between the three herbal preparations. HPLC data showed that EPA and EP had higher levels of echinacoside or cichoric acid, respectively, and they share high levels of some individual alkamides, such as alkamide 2 that occurs in relatively small amount in EA. These herbal components may be quantitatively important as modulators of NO and TNF-α production. Further studies are needed to address the respective role of individual phytochemicals and their interaction in the modulation of inflammatory mediators.

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

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

The opposite effects of Echinacea alcohol extracts on the non-activated and activated macrophages were also seen in cell numbers with the use of the MTS assay (Figure 1). In comparison to the vehicle, Echinacea resulted in decreased cell number in the presence of LPS. While macrophages are in a resting state, Echinacea stimulated, to some extent, an increase in cell number. Echinacea alcohol extracts might stimulate the non-activated macrophages (e.g. increase cell number and/or function), but reduce macrophage proliferation when they are activated.

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

RAW 264.7 cells closely resemble the murine peritoneal macrophages in their response to inflammatory stimuli and pathogenic microbes. We expected that Echinacea alcohol extracts could exert a similar, although not identical, effect on PECs as they did on the macrophage-like RAW 264.7 cells. Unfortunately, a weak *in vivo* effect on inflammatory PECs was seen. The only notable change in PECs was an EP-mediated reduction in NO production at the 4-h time point after *Salmonella* engulfment. Interestingly, the reduction in NO production did not result in a significant decrease in bacterial killing. The *in vivo* effect in this regard might be largely influenced by several factors, such as the metabolism of the phytochemicals, the activation state of PECs and the complex *in vivo* environment (e.g. constitutive expression of cytokines, growth factors and hormones (Pruett et al. 2005)). Although inflammatory agents, such as proteose peptone and thioglycollate, are commonly applied to induce an inflammatory response resulting in an influx of strongly activated macrophages into the peritoneum and thus an increased peritoneal macrophage yield, it is unclear as to how and to what extent these inflammatory agents affect the functional state of macrophages (Fortier & Falk 1994). On the other hand, lack of an inhibitory effect of orally administered Echinacea alcohol extracts on bacterial phagocytosis and killing by PECs may indicate that Echinacea will not adversely affect these important innate immune functions.

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

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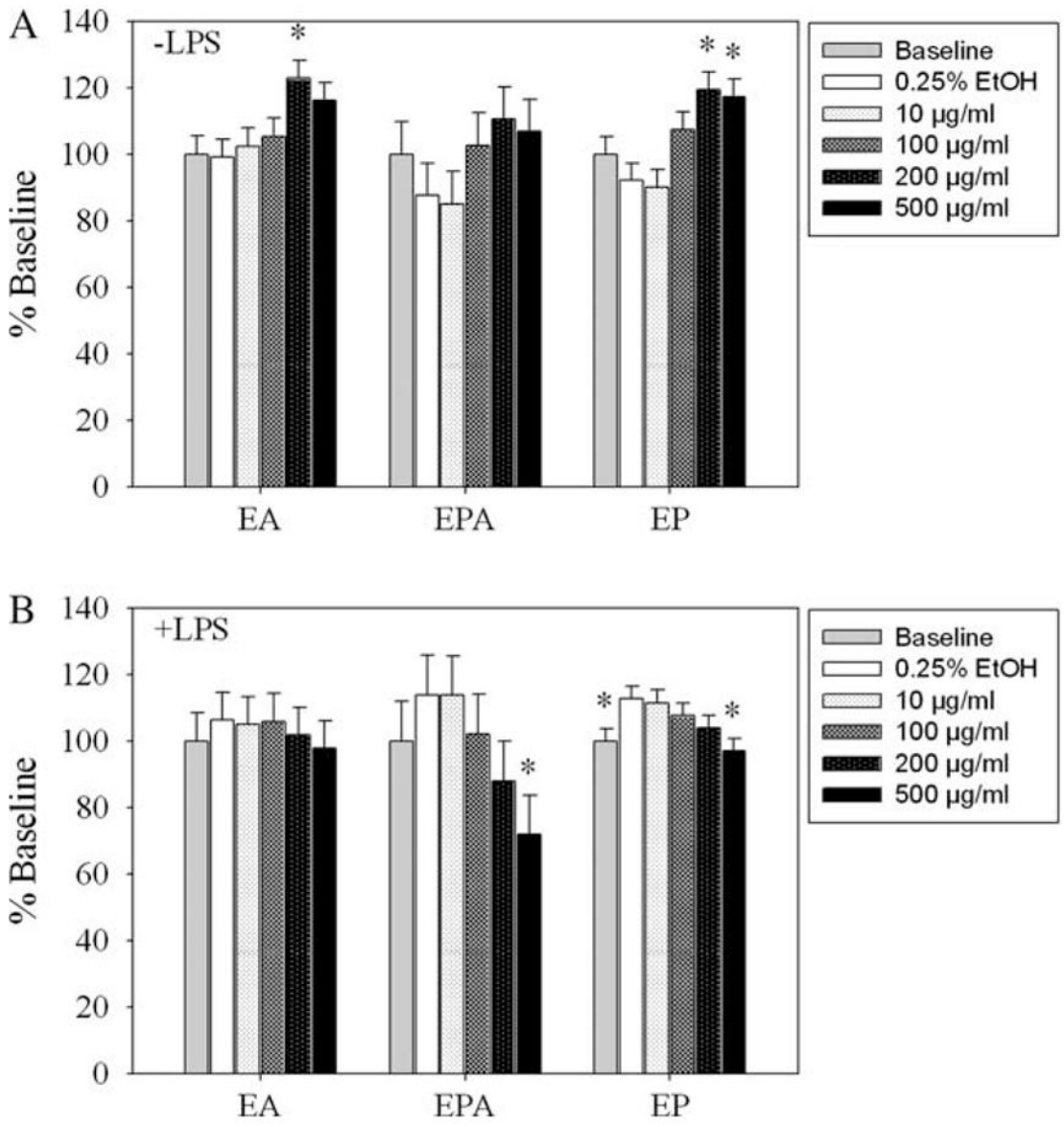

Figure 1.

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

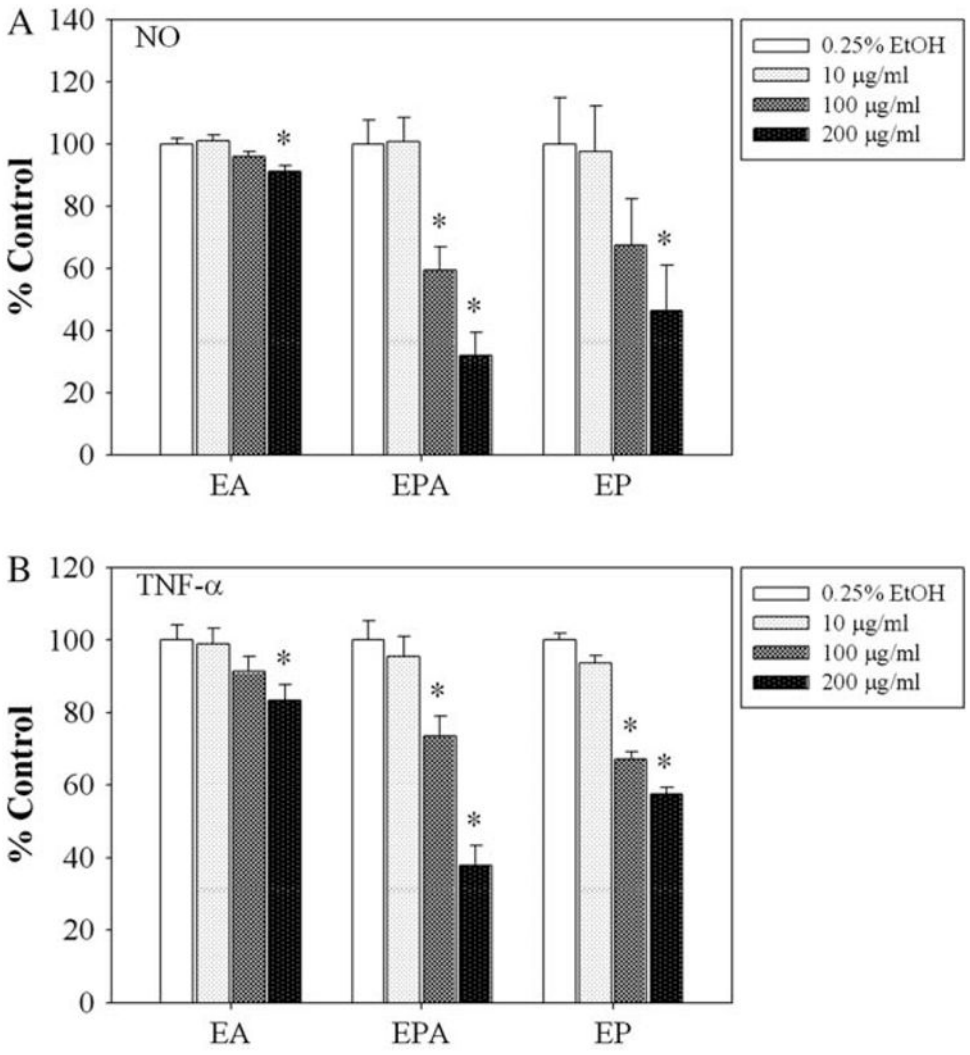

Figure 2.

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

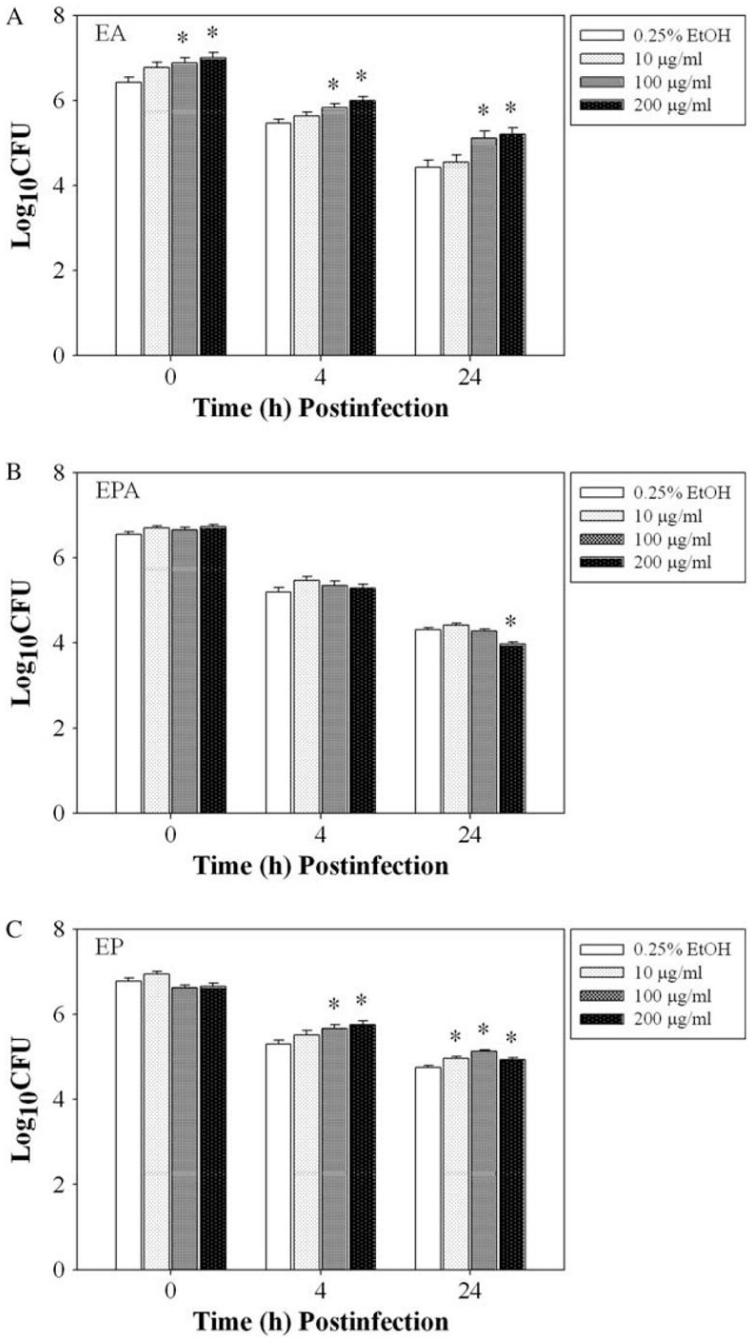

Figure 3.

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

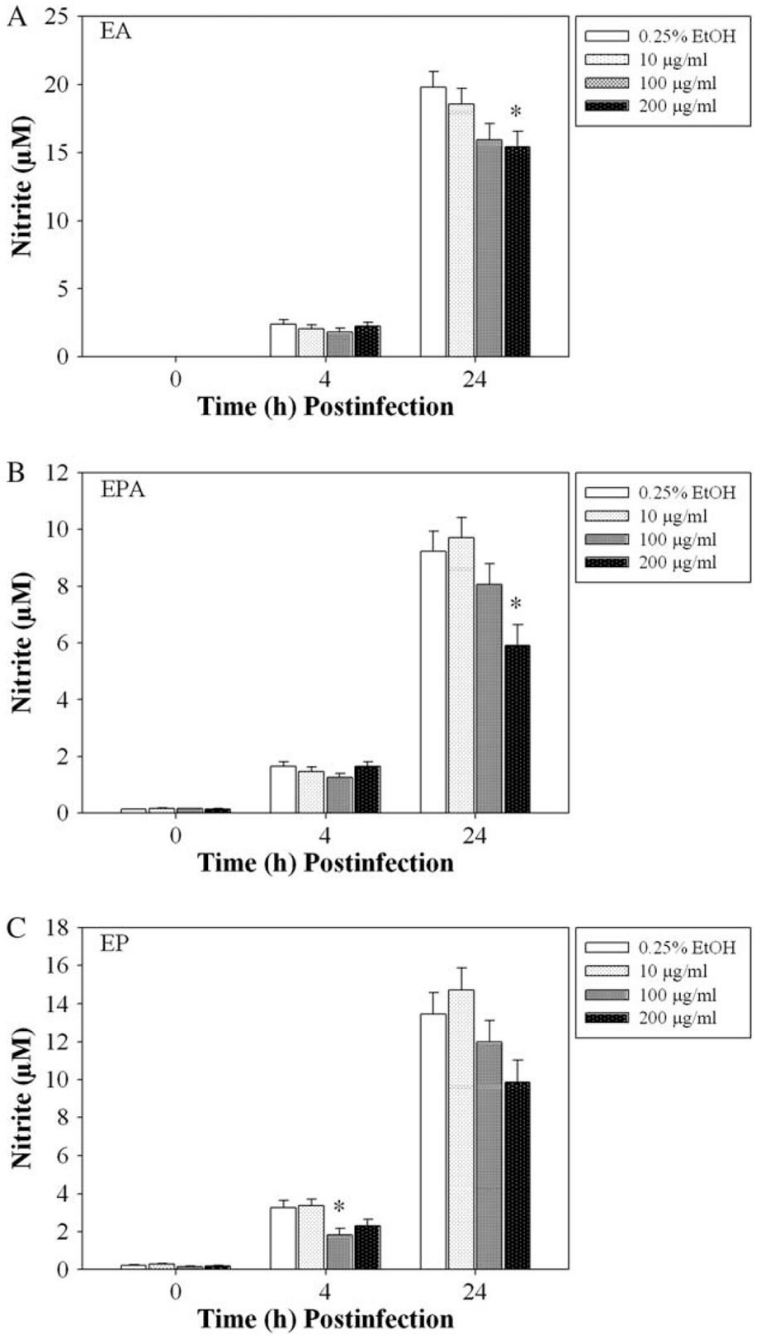

Figure 4.

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

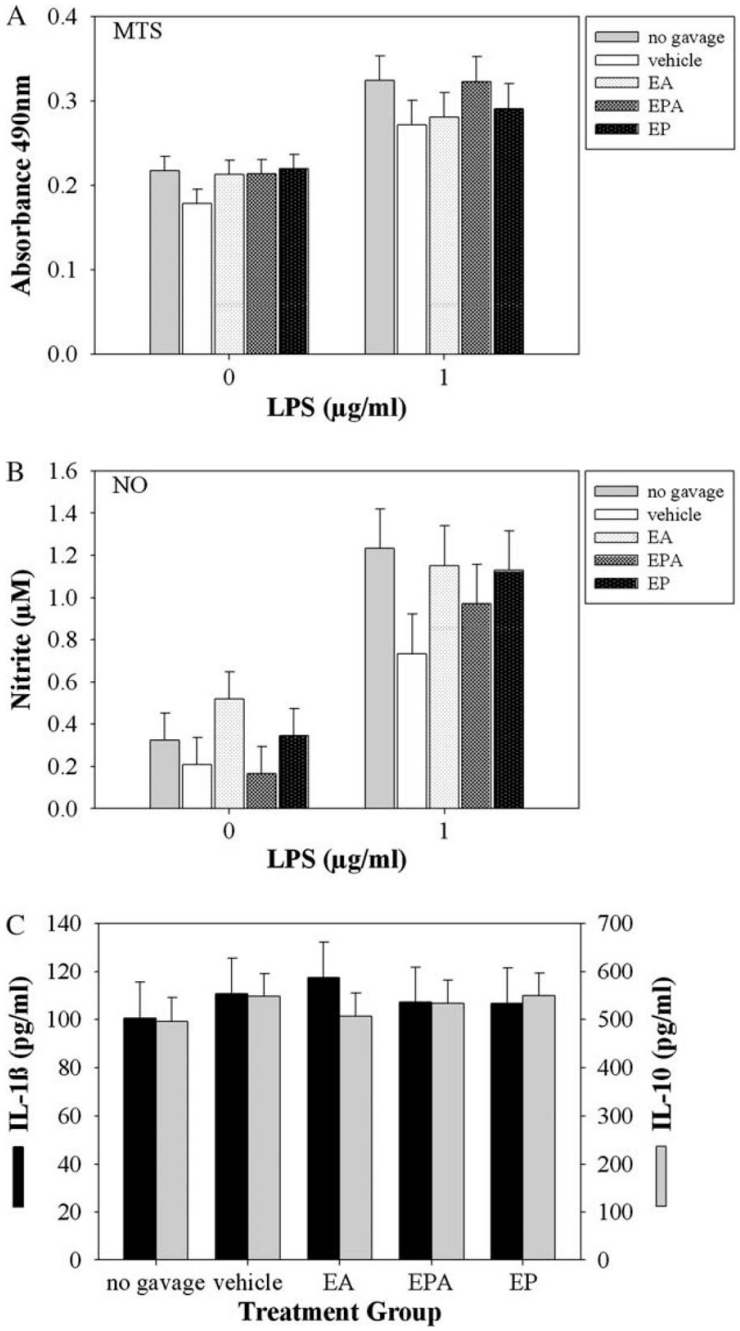

Figure 5.

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

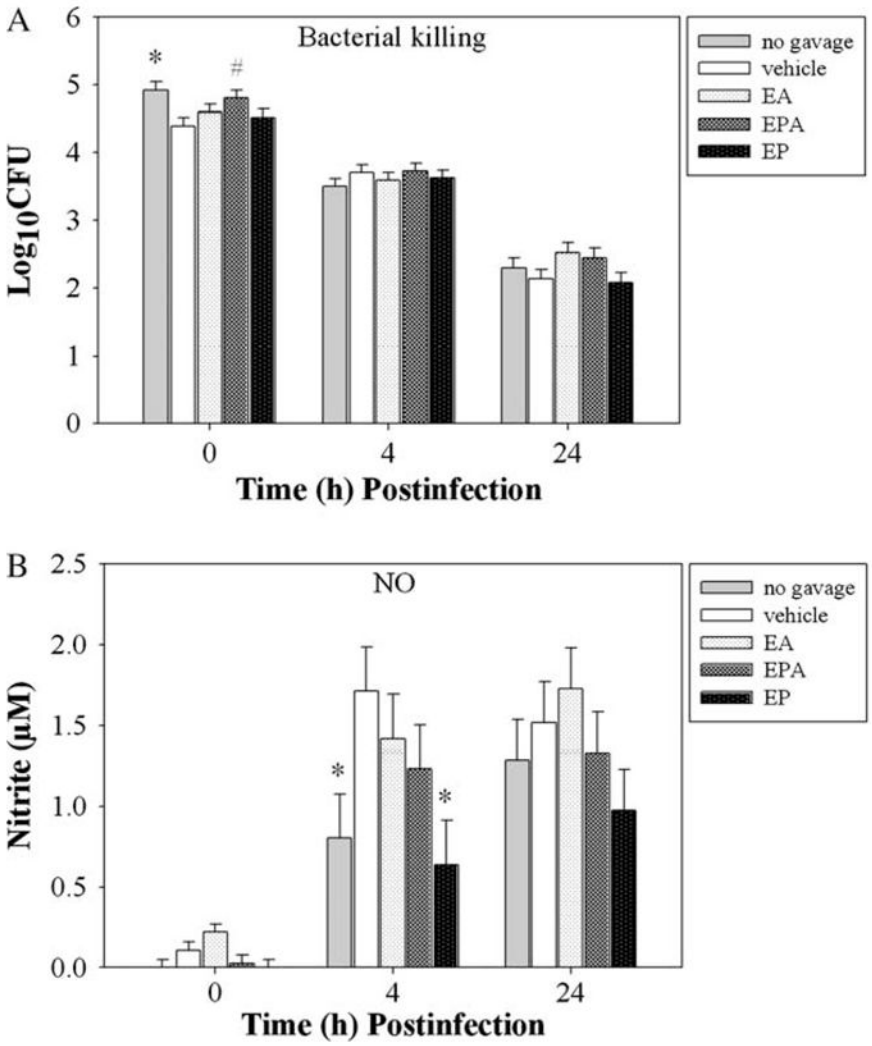

Figure 6.

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

Table I

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

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

* Units are μg metabolite per milligram dried extract.