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RESEARCH ARTICLE

Echinacea and trypanasomatid parasite interactions: Growth-inhibitory and anti-inflammatory effects of *Echinacea*

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Abstract

Context/objective: Herbal preparations derived from various species and parts of *Echinacea* (Asteraceae) have been advocated for various medical applications, as a result of the many antimicrobial and immunomodulatory activities attributed to them.

Materials and methods: In order to investigate their effects on parasites, four preparations of *Echinacea*, with distinct chemical compositions, were evaluated for growth inhibition of three species of trypanosomatids: *Leishmania donovani, Leishmania major*, and *Trypanosoma brucei*. In addition one *Echinacea* preparation was tested for anti-inflammatory activity in cell culture models designed to measure pro-inflammatory cytokines induced by *L. donovani*.

Results and discussion: All *Echinacea* preparations inhibited growth of the organisms, though with different relative potencies, and in some cases morphological changes were observed. However, there was no obvious correlation with the composition of the marker compounds, alkylamides, caffeic acid derivatives, and polysaccharides. *L. donovani* stimulated the production of the pro-inflammatory cytokines IL-6 and IL-8 in human bronchial epithelial cells and in human skin fibroblasts, but in both cases the standardized ethanol extract of *E. purpurea* (L.) Moench (Echinaforce®) abolished the stimulation, indicating anti-inflammatory activity of this extract.

Conclusions: Thus various *Echinacea* extracts can inhibit the proliferation of these parasites and at least one can reverse the pro-inflammatory activity of *Leishmania donovani*.

Keywords: Leishmania *sp.; Trypanosoma brucei; pro-inflammatory cytokines; IL-6; IL-8*

Introduction

Echinacea (Asteraceae) is one of the most popular herbal medicines for the treatment of cold and flu symptoms, and other respiratory disorders. In laboratory and animal studies extracts of several *Echinacea* species, particularly *E. purpurea* (L.) Moench. and *E. angustifolia* (D.C.), have been shown to possess a wide variety of bioactivities, including immune modulation, anti-inflammatory, antiviral, and antibacterial properties (Barnes et al., 2005; Vimalanathan et al., 2005; Sharma et al., 2008 Vohra et al., 2009). However, similar studies on the effects of *Echinacea* on parasites have not been reported.

Leishmaniasis and trypanosomiasis are diseases caused by protozoans belonging to the Trypanosomatidae family: *Leishmania* and *Trypanosoma*. There are four forms of Leishmaniasis depending on the species of *Leishmania* responsible: cutaneous, diffuse cutaneous, mucocutaneous, and visceral Leishmaniasis (WHO, 2008a). Likewise there are two forms of trypanosomiasis depending on the species of *Trypanosoma*

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responsible: African trypanosomiasis (sleeping sickness) and American trypanosomiasis (Chagas disease).

Leishmania are mainly transmitted by the bite of an infected female phlebotomine sandfly (WHO, 2008a). In the sandfly, *Leishmania* are in the promastigote stage (flagellated protozoans), which later transform into amastigotes (non-flagellated protozoans) after they are engulfed by macrophages.

Similar to *Leishmania, Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodensiense* (parasites responsible for African trypanosomiasis), or *Trypanosoma cruzi* (parasite responsible for American trypanosomiasis) are transmitted to humans by the bite of an infected tsetse fly (*Glossina* genus) or an infected assassin bug (subfamily *Triatominae*) respectively (WHO, 2008b). Procyclic (non-infective) trypanosomes multiply in the insect vector whereby they transform into metacyclic (infective) trypanosomes that are then injected into humans after a blood meal.

Both parasites cause widespread disease, with hundreds of thousands of new cases each year. Although drugs are available for the treatment of different stages of the diseases, they are frequently associated with severe side effects (Hay et al., 2008; WHO 2008a, 2008b). However, some recent studies have examined antiparasitic properties of several plant extracts, with promising results (Atawodi et al., 2003; Braga et al., 2007; Gamboa-Leon et al., 2007; Desrivot et al., 2007; Hay et al., 2008). But similar studies with *Echinacea* have not been reported.

We therefore decided to investigate possible antiparasitic properties of several standardized commercial preparations of *Echinacea*. In addition, since we know that *Echinacea* can act as an anti-inflammatory agent in cells exposed to various viruses and bacteria (Sharma et al., 2006, 2008, 2009), we tested for possible anti-inflammatory activity (anticytokine activity) in cells exposed to the parasites.

Materials and methods

Echinacea extracts

Commercial *Echinacea* preparations from the aerial and/or root portions of *Echinacea purpurea* and *Echinacea angustifolia* (EA, EP-2, and EP-3) were obtained from J.T. Arnason (University of Ottawa), who also performed the HPLC analyses (Table 1). Complete details on the origin and chemical analyses of these preparations were given in Vohra et al. (2009). EA, EP-1, and EP-2 were coded respectively as brands D1-60, IL-1 and Il-12 (Vohra et al., 2009). EP-1 was a standardized commercial extract of *E. purpurea* (Echinaforce®, A. Vogel/Bioforce, Roggwil, Switzerland). It was also analyzed by HPLC. The samples were stored at 4°C in tubes wrapped with foil (to prevent light exposure) during the experimental study. These extracts were chosen based on their representation of various types of commercial *Echinacea* extracts and different compositions of marker compounds (Vohra et al., 2009). EP-1 and EA were ethanol extracts containing alkylamides (PID 8/9) and little or no polysaccharide. On the other hand, EP-2 and EP-3 were aqueous extracts rich in polysaccharides but lacking alkylamides. Caffeic acid derivatives were present in accordance with expected compositions (Bauer, 1998; Binns et al., 2002). Table 1 shows the origins of the extracts and the composition of these standard markers.

Parasite cultures

L. donovani promastigotes were cultured at 26°C in M199 medium (Sigma, M4530, St. Louis MO) supplemented with 10% fetal bovine serum (FBS) (Sigma), 25 mM HEPES (Stem Cell Technologies), penicillin and streptomycin (100μg/mL) (Stem Cell Technologies Vancouver, BC, Canada), 25 mM adenosine, hemin (6 μg/mL) and folic acid. Exponentially growing cells were maintained

Table 1. Composition of *Echinacea* extracts.

	EP-1 *E. purpurea* aerial + 5% root	EA *E. angustifolia* root	EP-2 *E. purpurea* aerial	EP-3 *E. purpurea* aerial
Ethanol content (v/v)	65%	48%	0	0
Dry weights (mg/mL)	160	91	100	100
Caffeic acid	0	27.3*	19.4	22.3
Caftaric acid	264.4	73.1	546.6	372.8
Chlorogenic acid	40.2	4.4	20.8	17
Cichoric acid	313.8	90.7	755.6	828
Cynarin	0	173.5	7.1	5.6
Echinacoside	6.9	557	24.2	22.4
Alkylamides (PID 8/9)	36.3	620.9	0	0
Polysaccharide (mg/mL)	Trace	0	1.92	2.4

*Concentrations expressed as μg/mL (except polysaccharides), means of 4 determinations.

at 26°C in non-vented culture flasks (Corning Inc. New York, USA). Procyclic *T. brucei,* and the growth medium, were obtained from Terry Pearson (University of Victoria, BC). They were cultured at 26°C.

Proliferation assay

Exponentially growing Leishmania promastigotes (day 3 growth) were resuspended at 2×10^6 cells/mL after hemocytometer counting with Trypan blue (Sigma). The cell suspension (1 mL) was pipetted into each well of a 24-well plastic tissue culture plate (Corning). *Echinacea* extracts were then pipetted directly into the wells and immediately mixed with the pipette tip to prevent possible local concentration effects. Possible secondary effects of ethanol on the organisms were also assessed by adding equivalent amounts of 65% or 48% ethanol solutions (95% ethanol diluted accordingly in sterile distilled water) in accordance with the EP-1 and EA extracts, which contained 65% and 48% ethanol, respectively. The plates were then sealed around the edges with parafilm to limit airflow and incubated at 26°C for 24, 48, or 72 h, after which cell proliferation was assayed using hemocytometer counting with Trypan blue. Growth controls were assessed by culturing *Leishmania* in the absence of *Echinacea* or ethanol. The effect of *Echinacea* or ethanol on cell proliferation was then expressed as the percent of the control cultures for each time point.

Trypanocidal activity of *Echinacea* was assayed in a similar manner described above; procyclic *T. brucei* were resuspended at 2×10^6 cells/mL after hemocytomer counting with Trypan blue (Sigma). This cell suspension (0.5 mL) was pipetted into 1.5 mL sterile microfuge tubes. Microfuge tubes were used because *T. brucei* do not grow well in the 24-well tissue culture plates used for *L. donovani*. Means and standard deviation (SDM) values were calculated for all replicate samples.

Anti-inflammatory activity

Details of the test system were described previously (Sharma et al., 2008, 2009). BEAS-2B human epithelial cells, originally obtained from ATCC (American Type Culture Collection, Rockville, MD), were grown in Dulbecco MEM (DMEM) in 10% fetal bovine serum. For the experiments, cells were sub-cultured and grown to confluency in 6-well trays, after which the medium was changed to DMEM without serum. Under these conditions the cultures remained viable for at least 5 days. Human skin fibroblasts (courtesy of Aziz Ghahary) in their sixth passage were also cultivated in DMEM with 10% serum. No antibiotics or antimycotic agents were used. Rhinovirus type 1A (RV1A from ATCC) was propagated and assayed, by plaque assay, in H-1 cells (Sharma et al., 2008). The stock virus had a titer of 1×10^8 pfu/mL.

Cells were infected with $58\text{-}64 \times 10^6$ /mL of *L. donovani* (approximately 50 organisms per epithelial cell), or with 1 pfu/cell of RV1A as positive control, for 1 h, followed by *Echinacea* or medium for 48 h. Cell free supernatants were then removed for cytokine assay by ELISA which was carried out according to the instructions supplied by the companies (either R&D Systems, Minneapolis, MN, for IL-8, or e-Bioscience, San Diego CA, for IL-6). Absorbance readings at 540 nm were converted to pg/mL cytokine by means of standard curves.

Results

The growth and viability of each organism were measured in the presence and absence of each of the test *Echinacea* extracts, or the appropriate solvent controls, and also the ability of selected extracts to inhibit *Leishmania donovani* induced pro-inflammatory cytokine secretion. The origins and phytochemical characteristics of the extracts are summarized in Table 1.

Inhibition of growth of Leishmania *promastigotes*

Figure 1 shows the inhibitory effect of increasing concentrations of aqueous *Echinacea* extract EP-3 on the growth of *L. donovani*. At 1:1000 dilution the cell number was indistinguishable from the untreated control; but increasing concentrations of the extract resulted in proportionately greater inhibition in growth at all three time points examined. At 1:10 dilution (10 mg/mL) the cell numbers were only 21%, 12%, and 8% of controls at 24, 48, and 72 h, respectively (Figure 1). Furthermore, at

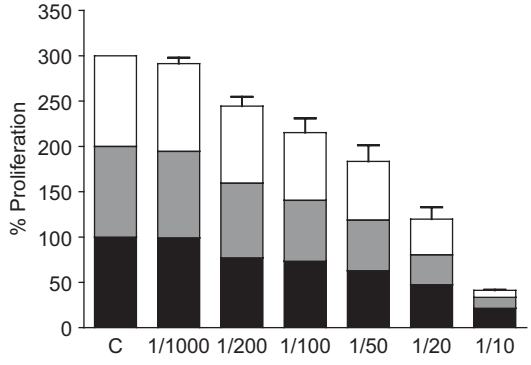

Figure 1. Effect of aqueous *Echinacea* extract EP-3 on the extracellular proliferation of *L. donovani*. Exponentially growing *L. donovani* were incubated for 24, 48 and 72 h with the indicated dilutions of *Echinacea* extract EP-3. Control *Leishmania* were grown under identical conditions without *Echinacea*. At each time point the numbers of motile surviving parasites were counted by Trypan blue exclusion. The data shown are representative of three independent experiments: lower black bars, 24 h; grey bars, 48 h; upper white bars, 72 h.

this concentration the treated cells appeared rounded with minimal movement. Similar results were obtained for the other aqueous *Echinacea* extract EP-2 (data not shown).

In comparison, *L. major* appeared to be somewhat more resistant to EP-3, in spite of their equivalent concentrations (Table 1). In this case the final cell counts at 24, 48, and 72 h were 56%, 54%, and 35% of controls, respectively. In addition the treated cells appeared to have normal morphology though slower movements.

The ethanol extracts also showed inhibition of *L. donovani* and *L. major* proliferation. Figure 2 shows the results for *L. donovani* and extract EA. Dilutions of 1:500 and 1:200 showed little effect, but inhibition was evident in concentrations above 1:100 (0.9 mg/mL). The ethanol solvent was also inhibitory at 1:20 or greater concentrations (>5% v/v). At 1:50 the cell numbers were unaffected by ethanol, but *Echinacea*-treated cell numbers were reduced to 30%, 12%, and 7% of controls at 24, 48, and 72 h, respectively (Figure 2). Corresponding values for *L. major* were 59%, 26%, and 10%. Morphologically, the cells treated with 1:50 dilution (1.8 mg/mL) were rounded and slower moving compared with control cells. The other ethanol extract, EP-1, showed similar responses (data not shown).

Inhibition of growth of trypanosomes

Figure 3 shows the inhibitory effect of extract EP-3 on proliferation of *T. brucei*. Progressive inhibition was evident with increasing concentrations of extract, and at 1:50 or greater (>2 mg/mL) the effect was completely trypanocidal. Under these conditions the cells were non-motile and curled into balls. Extract EP-2 showed similar results (data not shown).

Figure 4 shows the effects of the ethanol extract EA. The higher concentrations were again trypanocidal, although the ethanol solvent itself also contributed to the inhibitory effect at the highest concentration tested (1:10). The other ethanol extract EP-1 gave similar results (data not shown).

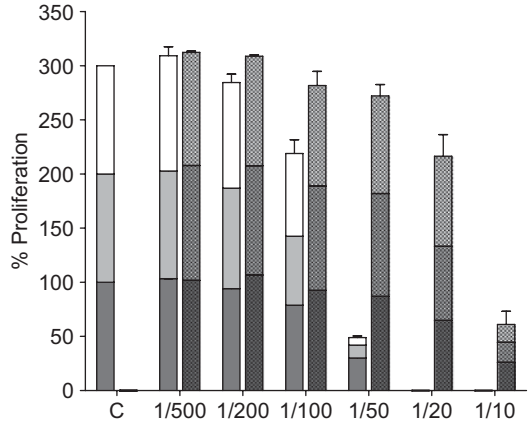

Figure 2. Effect of ethanol *Echinacea* extract EA on the extracellular proliferation of *L. donovani*. Exponentially growing *L. donovani* were incubated for 24, 48 and 72 h with the indicated dilutions of EA (left hand bars; lower black bars, 24 h; middle grey bars, 48 h; upper white bars, 72 h). Controls consisted of dilutions of ethanol corresponding to those in the diluted extracts (right hand bars; lower bars, 24 h; middle bars, 48 h; upper bars, 72 h). At each time point the numbers of motile surviving parasites was counted by Trypan blue exclusion. The data shown are representative of three independent experiments.

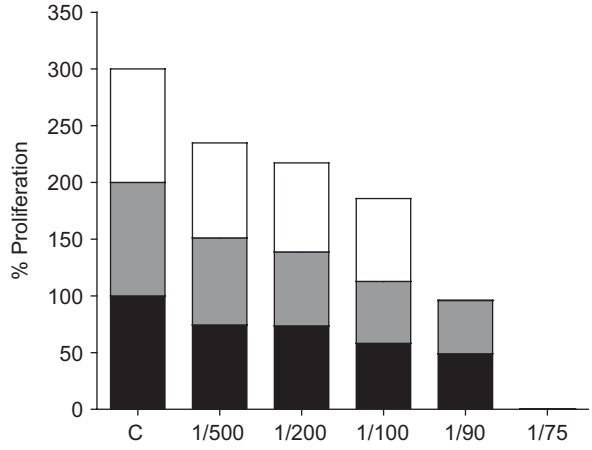

Figure 3. Effect of aqueous *Echinacea* extract EP-3 on the extracellular proliferation of *T. brucei.* Exponentially growing *T. brucei* were incubated for 24, 48 and 72 h with the indicated dilutions of *Echinacea* extract EP-3. Control trypanosomes were grown under identical conditions without *Echinacea*. At each time point the numbers of motile surviving parasites was counted by Trypan blue exclusion. The data shown are representative of three independent experiments: lower black bars, 24 h; grey bars, 48 h; upper white bars, 72 h.

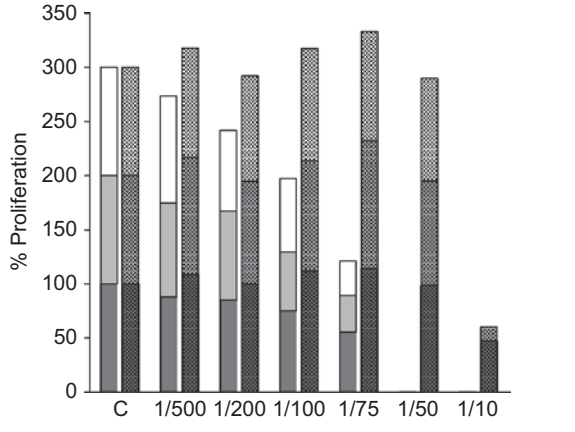

Figure 4. Effect of ethanol *Echinacea* extract EA on the extracellular proliferation of *T. brucei*. Exponentially growing *T. brucei* were incubated for 24, 48 and 72 h with the indicated dilutions of *Echinacea* extract EA (left hand bars; lower black bars, 24 h; middle grey bars, 48 h; upper white bars, 72 h). Controls consisted of dilutions of ethanol corresponding to the diluted extracts (right hand bars; lower bars, 24 h; middle bars, 48 h; upper bars, 72 h). At the end of the experiment, the number of motile surviving parasites was counted by Trypan blue exclusion. The data shown are representative of three independent experiments.

Inhibition of pro-inflammatory activity

In order to investigate the possibility of *Leishmania* induction of pro-inflammatory cytokines and the anti-inflammatory effect of *Echinacea*, we used the model system characterized in our previous reports (Sharma et al., 2006, 2009), in which RV1A and other viruses were shown to induce substantial levels of secretion of IL-6 and IL-8 (CXCL8) at 48 h post infection.

IL-6 and IL-8 secretion were measured by ELISA tests in control uninfected cells, cells plus *Echinacea,* and *L. donovani* or RV1A infected cells with and without *Echinacea*. *L. donovani* induced the secretion of both IL-6 and IL-8, in BEAS-2B bronchial epithelial cells and in human skin fibroblasts, and *Echinacea* was very effective in inhibiting these responses. In many cases the *Leishmania* + *Echinacea* values were indistinguishable from control uninfected cells (Table 2). There were no evident *L. donovani*-induced cytopathic effects within 48 hours post infection. In one experiment the supernatants were also evaluated by means of the Quantibody® cytokine array system (Sharma et al., 2009). *Leishmania* showed stimulation of IL-6 and IL-8, in agreement with the ELISA results, and again EP-1 abolished this stimulation (data not shown).

Discussion

All four *Echinacea* preparations exhibited dose-dependent antileishmanial and trypanocidal activities after 24, 48 and 72 h incubation. However, the relative potency of *Echinacea* varied depending on the preparations used. Of the *Echinacea* preparations tested on *L. donovani*, the 48% ethanol extract EP-1 (*E. angustifolia*) was the most effective. In contrast, the aqueous extract EP-3 (*E. purpurea*) proved to be the most potent against *T. brucei*.

Table 2. Effect of *Echinacea* extract EP-1 on induced secretion of pro-inflammatory cytokines.

Cells	Treatment*	IL-6 (pg/mL ± SD)	IL-8 (pg/mL ± SD)
BEAS-2B	None, control	19.8 ± 3.6	42.8 ± 10.4
	+ EP-1 (*Echinacea*)	29.2 ± 4.5	45.7 ± 12.8
	+ Ld (*Leishmania donovani*)	63.1 ± 36.6	147.53 ± 28
	+ Ld + EP-1	25.8 ± 9.6	18.1 ± 2.2
BEAS-2B	None, control	82.5 ± 3.2	131.1 ± 11.4
	+ EP-1	72.5 ± 3.5	118.5 ± 21.8
	+ RV 1A (rhinovirus)	760 ± 56.6	1326.9 ± 38
	+ RV 1A + EP-1	29.4 ± 10.5	69.9 ± 1.2
Human skin fibroblasts	None, control	29.8 ± 4.6	62.0 ± 12.5
	+ EP-1	38.2 ± 6.5	74.4 ± 10.8
	+ Ld	207.8 ± 26.6	320.0 ± 18
	+ Ld + EP-1	9.6 ± 1.5	42.8 ± 12.2

*Cells were treated with *Echinacea* EP-1, or *L. donovani*, or rhinovirus 1A, or combinations indicated, and after 48 h cell free supernatants were removed for ELISA determinations of IL-6 and IL-8.

It was anticipated that different *Echinacea* preparations might have different effects on the protozoans tested. Research has shown that the efficacy of *Echinacea* preparations depends on their chemical composition, wherein different concentrations of components either alone or together can result in different and even opposing immunomodulating effects (Matthias et al., 2008; Vimalanathan et al., 2009). Therefore, a next step to this study would be to further analyze the chemical compositions of the *Echinacea* extracts to determine which compounds in the preparations are responsible for the antileishmanial and/or trypanocidal activities. It should be noted that concentrations of ethanol alone showed some negative effect on the proliferation of *L. donovani* and *T. brucei*. Nevertheless, incubation with the ethanol *Echinacea* extracts resulted in enhanced inhibition of proliferation. It is possible that these compounds work synergistically, in which case differing relative amounts of the compounds in *Echinacea* preparations may result in an extract with high antileishmanial and/or trypanocidal activities.

Despite being classified as trypanosome protozoans, the different results observed between *L. donovani* and *T. brucei* suggest that *Echinacea* acts on or involves structures that are different between *Leishmania* and *Trypanosoma*. Research has shown that plant extracts can have different trypanocidal effects depending on the species tested (Atawodi et al., 2003). Further tests of the *Echinacea* preparations with other species of *Leishmania* or *Trypanosoma* may show whether the effects of *Echinacea* are also species-dependent.

The mode of action of *Echinacea* on these parasites is not known at present. However, morphological observations were made and these show that *Echinacea* slowed/eliminated their motility and caused rounding of both *L. donovani* and *T. brucei* at high concentrations. Observing the cellular structures of *Echinacea*-treated parasites by electron microscopy could provide valuable insights to its mode of action.

It is questionable whether *Echinacea* negatively affects proliferation by killing the parasites (which would then question whether it stimulates apoptosis or necrosis) or through growth inhibition. For example, 1/10 dilution of EP-3 treated *L. donovani* promastigotes showed that upon re-suspension in M199 media lacking *Echinacea* (after 5 days incubation with EP-3), the cells were able to recover over a period of four days (data not shown). More of these tests need to be carried out with the other *Echinacea* extracts and with *T. brucei*.

L. donovani also showed pro-inflammatory activity by stimulating the secretion of IL-6 and IL-8 (CXCL8)

in two different human cell lines, a bronchial epithelial line and a skin fibroblast line. Similar stimulation of these cytokines was shown in these cell lines by a variety of other viral and bacterial pathogens (Sharma et al., 2008, 2009). In both cell types, the selected *Echinacea* preparation (EP-1) inhibited these *Leishmania*-induced responses, as it did for the viral inducers.

Thus certain *Echinacea* preparations are capable of controlling growth of these parasites, and in at least one case can inhibit the inflammatory activity induced by them.

Declaration of interest

No funding was received for this study. All authors declare no conflict of interest

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