Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: informahealthcare.com/journals/iphb20

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To cite this article: S. Vimalanathan, J.T. Arnason & J.B. Hudson (2009) Anti-inflammatory activities of *Echinacea* extracts do not correlate with traditional marker components, Pharmaceutical Biology, 47:5, 430-435, DOI: 10.1080/13880200902800204

To link to this article: https://doi.org/10.1080/13880200902800204

Pharmaceutical Biology, 2009; 47(5): 430–435

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RESEARCH ARTICLE

Anti-inflammatory activities of *Echinacea* extracts do not correlate with traditional marker components

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Abstract

Numerous preparations of *Echinacea* (Asteraceae), mainly derived from three species, *Echinacea purpurea* (L.) Moench, *Echinacea angustifolia* (D.C.) and *Echinacea pallida* (Nutt.) Nutt., are available for the treatment of symptoms of colds and influenza which, along with a number of other respiratory conditions, have been ascribed to the induction of pro-inflammatory cytokines. We evaluated various chemically characterized extracts and fractions, derived from the three species of *Echinacea*, for their possible inhibitory effects on the secretion of pro-inflammatory cytokines IL-6 and IL-8 (CXCL-8) by human bronchial epithelial cells infected with rhinovirus type 14. All of the *E. purpurea* fractions, comprising aqueous or ethanol extracts of roots, leaves and stems, but to a lesser degree flowers, strongly inhibited the secretion of both cytokines. In contrast, corresponding fractions derived from *E. angustifolia* and *E. pallida* showed relatively weak cytokine-inhibitory activity, whereas their aqueous fractions significantly enhanced cytokine secretion, both in virus-infected and in uninfected cells. These properties did not correlate with the presence or absence of alkylamides or specific caffeic acid derivatives, although the alkylamide rich fraction of *E. angustifolia* showed a significant inhibitory effect. However, there was some correlation between anti-cytokine effects and our previously reported anti-viral activities. Thus, none of the groups of compounds traditionally used as "markers" for *Echinacea* are responsible for anti-inflammatory activity. Consequently, we believe that other constituents of *Echinacea* should be evaluated.

Keywords: *Bronchial epithelial cells; cytokines;* Echinacea purpurea*;* Echinacea angustifolia*;* Echinacea pallida*; IL-6; IL-8 (CXCL-8); rhinovirus*

Introduction

Different species and parts of *Echinacea* (Asteraceae) have been used traditionally in North America for the treatment of symptoms of upper respiratory infections such as colds and influenza, as well as inflammatory conditions (Barrett, 2003; Barnes et al., 2005). A number of *Echinacea* marker compounds have been characterized, including polysaccharides, caffeic acid derivatives (CADs) and alkylamides (Bauer, 1998; Binns et al., 2002), and these have all been shown to possess infection-related bio-activities in various tests *in vitro* and *in vivo*, including immune-modulating activities, anti-viral activities, and antibiotic activities (Burger et al., 1997; Bauer, 1998; Rininger et al., 2000; Goel et al., 2002; Merali et al., 2003; Gertsch et al., 2004; Vimalanathan et al., 2005; Hudson et al., 2005; Raduner et al., 2006; Sharma et al., 2006; LaLone et al., 2007).

Rhinoviruses have been implicated as major players in common colds and various types of allergic rhinitis and bronchial syndromes (Message & Johnston, 2004; Schaller et al., 2006). However, numerous studies have shown that rhinovirus infection in cultured epithelial cells, and in nasal epithelial tissues *in vivo*, results in relatively low levels of virus replication and cytopathology, apparently due to the small number of cells supporting virus replication (Mosser et al., 2005). In contrast, there is substantial induction of secretion of certain

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(Received 09 June 2008; revised 22 August 2008; accepted 09 September 2008)

ISSN 1388-0209 print/ISSN 1744-5116 online
DOI: 10.1080/13880200902800204

pro-inflammatory cytokines and chemokines, including the important multifunctional cytokines implicated in various inflammatory conditions, interleukin-6 (IL-6) and interleukin-8, CXCL-8 (IL-8) (Message & Johnston, 2004; Sharma et al., 2006; Schaller et al., 2006; Edwards et al., 2007). Thus, the typical symptoms of a common cold, such as sneezing, coughing, runny nose, stuffed nasal passages and others are not the direct result of viral pathology, but rather the indirect stimulation of pro-inflammatory cytokines and chemokines. Consequently, successful treatment of colds and influenza might be obtained by appropriate use of an anti-inflammatory material.

Certain formulations of *Echinacea* could fulfill this role. However, there are hundreds of *Echinacea* products in the market, mostly prepared from aerial parts of *E. purpurea* or from roots of *E. purpurea* (L) Moench, *E. angustifolia* (D.C.) and *E. pallida* (Nutt.) Nutt (Barrett, 2003; Barnes et al., 2005). They are also available as tinctures, teas, and powders in capsules. However, their chemical composition is significantly different (Bauer, 1998; Binns et al., 2002).

We previously evaluated aqueous and ethanol fractions, and various solvent sub-fractions, derived from the three species, for antiviral activities, which we found in many of the fractions, although there was no apparent correlation with the presence or composition of the principal marker compounds, the alkylamides, polysaccharides and caffeic acid derivatives (Vimalanathan et al., 2005; Hudson et al., 2005).

In this study, we investigated the same kinds of extract for their possible immune modulation effects, by measuring their effects on rhinovirus-induced secretion of the key pro-inflammatory cytokines IL-6 and IL-8, in human bronchial epithelial cells.

Materials and methods

Echinacea *sources*

Echinacea purpurea (L.) Moench, *E. angustifolia* (D.C.) and *E. pallida* (Nutt.) Nutt. (voucher numbers, UO #010502-18; UO #010410-12; UO #010410-11, respectively) were grown by a commercial grower in North America. Species were identified by Shannon Binns, University of Ottawa.

Echinacea *extracts and fractions*

Complete extraction details were described previously (Vimalanathan et al., 2005; Hudson et al., 2005). Briefly, 10 g of the root materials were extracted exhaustively at 40°C with an accelerated solvent extractor (Dionex, ASE100) with either distilled water or 70% ethanol and extracts brought to a final volume of 50 mL. The 70% ethanol fractions were sequentially extracted three times in a separatory funnel with equal volumes of water and hexane to produce a combined hexane extract. The hydroalcoholic extract was extracted twice with ethyl acetate to produce an ethyl acetate extract, and an aqueous residue as described in Hudson et al. (2005) (all adjusted to 5 mL/g root). Analysis of the extracts was performed by reverse phase HPLC/DAD on a 7. 5 cm reverse phase C18 column (3 μm particle size) using acetonile:water (6:1) eluted at 1 mL/min (Agilent 1100 HPLC) with detection at 210 and 260 nm (Binns et al., 2002).

Cells and viruses

BEAS-2B human epithelial cells, originally obtained from ATCC, were grown in Dulbecco MEM (DMEM) in 10% fetal bovine serum. For the experiments, cells were sub-cultured in 6-well trays, and when confluent the medium was changed to DMEM without serum. The H-1 sub-clone of HeLa cells American Type Culture Collection (ATCC) was cultivated in the same medium. No antibiotics or antimycotic agents were used.

Rhinovirus type 14 (RV 14, from ATCC), was propagated and assayed, by plaque assay (Hudson et al., 1982), in H-1 cells. The stock virus had a titer of 2×10^7 pfu/mL.

Experimental system

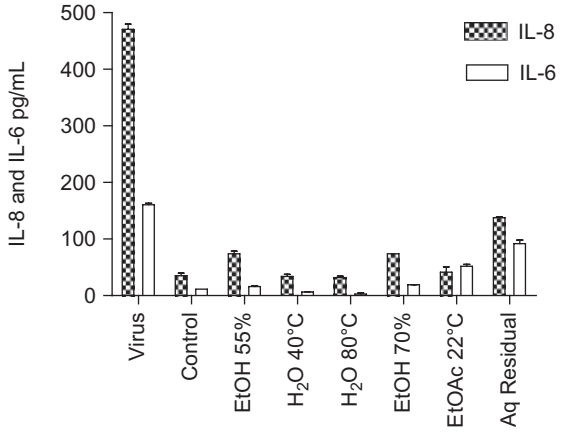
Details of the test system were described previously (Sharma et al., 2006). BEAS-2B cells were grown in complete medium, in 6-well trays, to produce confluent monolayers. The medium was then replaced with serum free DMEM for the experiments. Virus was added to the cells at 1 infectious virus per cell (1 pfu/cell), for one hour at 35°C, followed by a dilution of the *Echinacea* fraction to yield a final concentration of 250 μg/mL. Culture supernatants were harvested after 48 h, which we determined to be the optimum time of cytokine secretion, and assayed for IL-6 and IL-8. Controls included cells with no virus and cells (+/- virus) with equivalent amounts of solvent only.

All cultures were in duplicate and each supernatant was assayed in duplicate. All data presented are from individual experiments, but all experiments were repeated at least once, with consistent results.

ELISA assays were carried out with commercial kits, according to the instructions supplied by the companies (Immunotools, Friesoythe, Germany, and e-Bioscience, San Diego, USA).

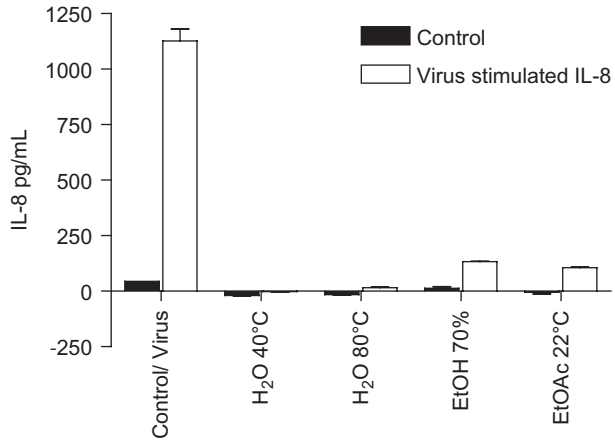
Results

Figure 1 shows the anti-inflammatory effects of the different *Echinacea purpurea* (L.) Moench root fractions.



Compound	Ethanol 55%	Water 40°C	Water 80°C	Ethanol 70%	Ethyl acetate	Aqueous residue
Caffeic	27	7	14	28	23	5
Caftaric	781	0	109	895	890	125
Cichoric	4774	0	23	4861	4499	210
Chlorogenic	55	0	0	61	42	5
Cynarin	0	0	0	0	0	0
Echinacoside	0	0	0	0	0	0
Tet 8/9	0	0	0	0	0	0

Figure 1. Anti-cytokine effects of *E. purpurea* root extracts. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 μg/mL of the indicated fraction of *E. purpurea* root extract. Controls received no virus. Solvent controls showed no effect. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Composition of marker compounds is shown below the figure.



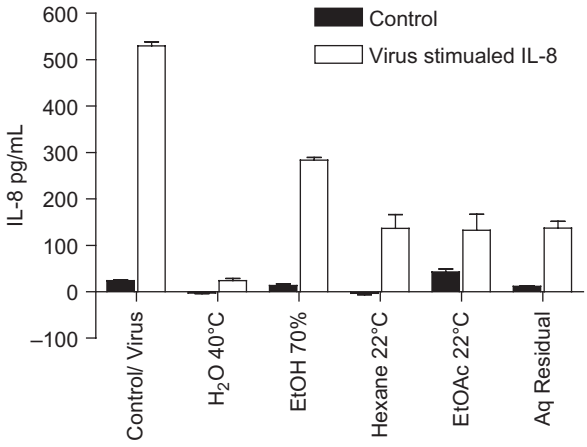
Compound	Water 40°C	Water 80°C	Ethanol 70%	Ethyl acetate
Caffeic	13	181	421	259
Caftaric	105	762	913	1174
Cichoric	12	489	6001	3993
Chlorogenic	63	0	45	0
Cynarin	0	0	5	0
Echinacoside	0	0	0	0
Tet 8/9	0	0	0	0

Figure 2. Anti-cytokine effects of *E. purpurea* leaf plus stem extracts. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 μg/mL of the indicated fraction of *E. purpurea* extract. Controls received no virus. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Not all solvent fractions were available for these tests. Only IL-8 is shown in this graph. Similar results were obtained for IL-6. Composition of marker compounds is shown below the figure.

In this culture system the levels of IL-8 secreted were usually several-fold more than IL-6. The virus itself stimulated the secretion of both pro-inflammatory cytokines substantially, but all the fractions significantly reduced these levels, particularly the aqueous extracts. They had little effect, however, on the low control levels (in uninfected cells) of cytokine secretion. This anti-inflammatory effect was clearly not influenced by the concentrations of caffeic acid derivatives, which varied widely between the fractions (see tabulated data beneath Figure 1), and furthermore did not require alkylamides, which were absent in these fractions.

Figure 2 shows the IL-8 results for cultures treated with *E. purpurea* leaf and stem combined extracts (essentially the same results were obtained for IL-6, not shown). In this case also, there was a consistent and marked inhibition in cytokine release, including the control levels. Thus the results were very similar to the root extracts shown in Figure 1, although the leaf plus stem fractions showed greater inhibitory activity than root fractions, since all fractions were evaluated at the same concentration. Again, these impressive anti-inflammatory effects did not correlate with specific CADs, and occurred in the absence of alkylamides.

E. purpurea flower extracts showed the same pattern (Figure 3 for IL-8; similar results were obtained for IL-6, not shown), except that in this case the degree of inhibition was uniformly much less. To determine if polysaccharides could play a role in these effects, we tested polysaccharide enriched and purified AGP (arabinogalactan fraction) for effects on cytokine secretion (Figure 4). The starting crude extract, the *E. purpurea* pressed juice extract, showed the usual inhibitory effect, whereas the polysaccharide enriched fraction (>100 kDa mol. wt. polysaccharides derived from the juice; Vimalanathan et al., 2005) was much less inhibitory. Furthermore the arabinoglactan fraction (AGP), derived by SEC column chromatography of the previous polysaccharide fraction, showed no inhibition. In addition none of these fractions had a significant effect on the control level of cytokine secretion.



Compound	Water 40°C	Ethanol 70%	Hexane	Ethyl acetate	Aqueous Residue
Caffeic	16	160	179	130	11
Caftaric	19	919	969	1212	355
Cichoric	9	7340	2470	6291	296
Chlorogenic	0	208	25	126	35
Cynarin	0	0	0	0	0
Echinacoside	0	0	0	0	0
Tet 8/9	0	39	0	8	0

Figure 3. Anti-cytokine effects of *E. purpurea* flower extracts. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 μg/mL of the indicated fraction of *E. purpurea* flower extract. Controls received no virus. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Only IL-8 is shown in this graph; similar results were obtained for IL-6. Composition of marker compounds is shown below the figure.

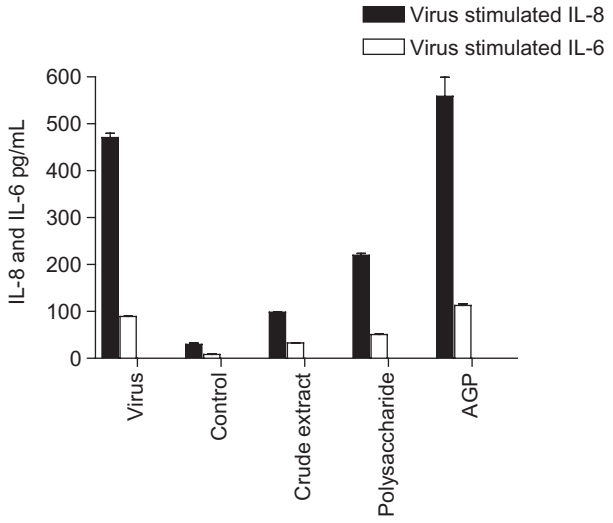
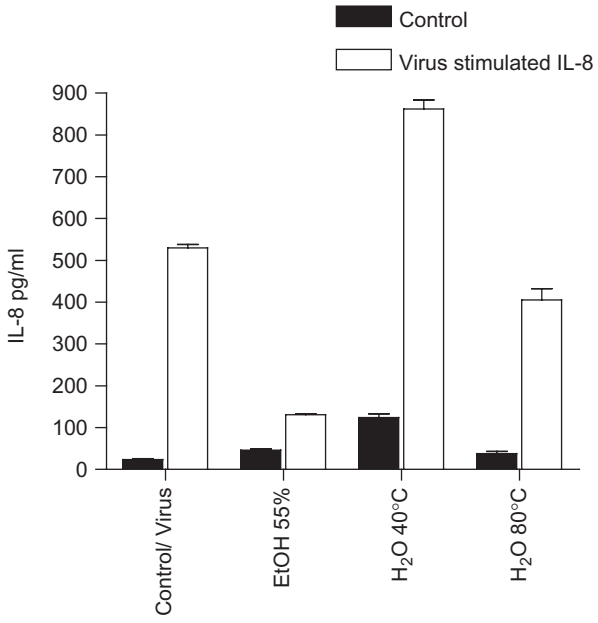


Figure 4. Anti-cytokine effects of polysaccharide-enriched fractions. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 μg/mL of the indicated fraction derived from *E. purpurea* aqueous pressed juice. Controls received no virus. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Polysaccharide-enriched fraction, P50 fraction (polysaccharides > 100 kDa) isolated from pressed juice; AGP fraction, arabinogalactan isolated by SEC 1 column from the polysaccharide fraction.

The corresponding results for *E. angustifolia* and *E. pallida* root fractions were quite different from *E. purpurea* (Figure 5 for *E. angustifolia* and Figure 6 for *E. pallida*). Data are shown for IL-8 only, although IL-6 gave similar results. Only the ethanol fraction of *E. angustifolia* was inhibitory (anti-inflammatory), although to a much smaller degree than the corresponding *E. purpurea* root fractions. However, the aqueous fractions had no inhibitory activity, and in fact some of them induced even higher levels of cytokine secretion, in the case of *E. pallida* by 2-3-fold. In other words, *E. pallida* aqueous fractions enhanced the pro-inflammatory response, in spite of the complete absence of CADs and alkylamides.

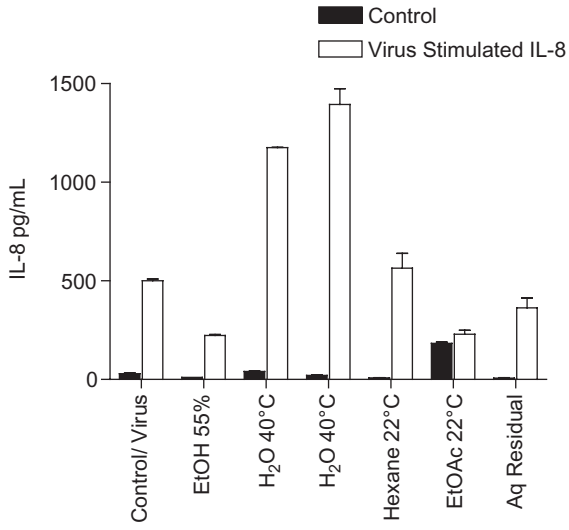
Discussion

Several conclusions can be derived from these results. Probably the most important is that we cannot correlate cytokine inhibitory effects, i.e., anti-inflammatory properties, with specific individual compounds or groups of marker compounds, namely, alkylamides, polysaccharides, and CADs. In the case of *E. purpurea*, all the fractions derived from roots, leaves plus stems, and flowers, were anti-inflammatory according to IL-6 and IL-8 measurements. The cytokine IL-6 is one of the principal pro-inflammatory cytokines, and is considered to be an excellent indicator of inflammatory status (Message & Johnston, 2004; Schaller et al., 2006). The cytokine IL-8 is one of the most important chemokines which attracts neutrophils and other inflammatory cells to sites of infection (Schaller et al., 2006). Numerous viral and bacterial infections, as well as wounds, result in substantial stimulation in levels of these two cytokines, which are consequently considered to represent markers of an inflammatory condition. Thus, any compound or herbal extract which can inhibit or reverse this elevation in IL-6/8 may be considered as a potential anti-inflammatory agent. Consequently, any preparation derived from roots or aerial parts of *E. purpurea* would be expected to possess anti-inflammatory properties, although flowers would be a considerably less potent source. This suggests that, if the same components were responsible for the inhibition, then substantially lower concentrations must be present in flowers. Some fractions contained small amounts of alkylamides, but these clearly did not enhance the anti-inflammatory effect. The fact that such preparations contain few if any alkylamides (Binns et al., 2002; Vimalanathan



Compound	Ethanol 55%	Water 40°C	Water 80°C
Caffeic	15	0	0
Caftaric	20	0	17
Cichoric	46	9	28
Chlorogenic	282	0	35
Cynarin	238	7	37
Echinacoside	1859	33	158
Tet 8/9	483	0	0

Figure 5. Anti-cytokine effects of *E. angustifolia* root extracts. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 µg/mL of the indicated fraction of *E. angustifolia* root extract. Controls received no virus. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Only IL-8 is shown; the results for IL-6 were similar. Not all solvent fractions were available for this experiment. Composition of marker compounds is shown below the figure.



Compound	Ethanol 55%	Water 40°C	Water 80°C	Hexane	Ethyl acetate	Aqueous Residue
Caffeic	7	6	7	0	6	0
Caftaric	48	83	86	0	61	22
Cichoric	32	7	17	0	35	0
Chlorogenic	3	3	4	0	0	0
Cynarin	0	6	0	0	0	0
Echinacoside	618	106	431	0	0	561
Tet 8/9	0	0	0	0	0	0

Figure 6. Anti-cytokine effects of *E. pallida* root extracts. BEAS-2B cells were infected with rhinovirus type 14 (1 pfu/cell) for 60 min at 35°C, followed by 250 µg/mL of the indicated fraction of *E. pallida* root extract. Controls received no virus. After 48 h, cell supernatants were removed for assay of IL-6 and IL-8 by standard ELISA tests. Standard curves were constructed for each experiment and the absorbance readings at 450 nm were converted into pg/mL. Only IL-8 data are shown; the results for IL-6 were similar. Composition of marker compounds is shown below the figure.

et al., 2005) argues against the concept that alkylamides could be largely responsible for the immune modulation properties of *Echinacea*. Furthermore these extracts were all devoid of cynarin and echinacoside, while the flowers, with significantly less anti-inflammatory effect, were particularly rich in cichoric acid, all of which strongly suggests that CADs are also not important individually for anti-inflammatory activity.

In contrast, *E. angustifolia* and *E. pallida* fractions showed little or no such anti-inflammatory effects, and in fact some fractions from these extracts stimulated the already elevated levels of IL-6 and -8, especially those from *E. pallida*. Nevertheless, it is possible that *E. angustifolia* ethanol fraction possesses significant anti-inflammatory activity by virtue of its high concentration of alkylamides. In general, however, these observations support the conclusion that the inflammatory modulation ascribed to certain *Echinacea* extracts cannot be explained in terms of alkylamides, polysaccharides, or CADs individually.

It is also interesting to compare the anti-inflammatory activities described here with the previous antiviral results. *E. purpurea* root extracts contained substantial antiviral activity in their aqueous fractions, although much less in the ethanol fractions (Hudson et al., 2005). In contrast, roots of *E. angustifolia* did contain ethanol soluble but no water soluble antiviral activity, while *E. pallida* contained no antiviral activity at all. All aerial parts of *E. purpurea* contained antiviral activities, and these were found in most of the aqueous and ethanol fractions tested (Vimalanathan et al., 2005), but we

were not able to correlate these activities with marker compounds.

Thus, it appears that the antiviral and anti-inflammatory activities do correspond, in that those fractions containing significant antiviral activity also have anti-inflammatory activity, but it is not clear which components of the *Echinacea* are responsible for them. Further studies need to be done with individual pure compounds and mixtures.

Although we cannot ascribe immune modulation properties to specific classes of marker compounds in the *Echinacea* preparations, on the basis of these results we could predict that most authentic preparations of *E. purpurea* roots, and aqueous preparations of *E. purpurea* aerial parts, should be effective anti-inflammatory mediators, at least in rhinovirus infected tissues, and therefore can be recommended for the alleviation of cold symptoms.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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