Research article

Immunomodulatory effects of *Echinacea laevigata* ethanol tinctures produced from different organs

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*Echinacea* supplements may prevent or reduce symptoms of upper respiratory infections by immunomodulation, possibly by altering the cytokine profile. Compared with other species in the genus, the immunomodulatory properties of *Echinacea laevigata* are poorly characterized. The purposes of this study were to compare the diversity and quantity of known bioactive compounds from aboveground organs of *E. laevigata*, and to characterize the *in vitro* immunomodulatory properties of ethanol tinctures produced from those structures. High-performance liquid chromatography (HPLC) was used to determine the levels of alkamides and caffeic acid derivatives. Peripheral blood mononuclear cells (PBMCs) were obtained from 16 adults and challenged *in vitro* with extracts. PBMC proliferation and production of the cytokines interleukin-2 (IL-2), IL-10 and tumour necrosis factor-α (TNF-α) were measured. Fresh flower, leaf and root extracts were able to augment TNF and IL-10 and proliferation, whereas fresh stem extract was only able to augment TNF. Extracts produced from flowers contained the greatest bioactive compound quantities and diversity. Caftaric acid was the most abundant compound and correlated with some (but not all) observed immune effects. These results suggest that of all aboveground parts, flowers have the greatest abundance and diversity of known bioactive compounds, and both flower and leaf extracts were immunomodulators.

**Key words:** coneflower, cytokine, interleukin, peripheral blood mononuclear cell, proliferation, tumour necrosis factor

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**Introduction**

*Echinacea* (Asteraceae) is a genus of nine plants native to the United States (McGregor, 1968; Wu et al., 2009). These species have been used for medicinal purposes (Moerman, 1998; Barnes et al., 2005), with current interest in their use as immunomodulatory therapies for upper respiratory infections (URIs) such as colds and influenza. Clinical reports conflict regarding their utility, with some reporting efficacy and others not (Karsch-Völkl, Barrett and Linde, 2015; Schapowal, Klein and Johnston, 2015). Some of the discrepancies may be explained by the rampant adulteration and mislabeling common to many commercial preparations, or the lack of control for such variables (Gilroy et al., 2003; Krochmal et al., 2004; Wolsko et al., 2005).

Each of the nine species of *Echinacea* differs in its diversity and abundance of purported bioactive compounds (Wu et al., 2004; Pellati et al., 2005; Kraus et al., 2006). Within a single species, phytochemical profiles differ between different organs, such as aboveground (aerial) and belowground parts (Qu et al., 2005; Senchina et al., 2009a). Four classes of bioactive compounds have been identified from *Echinacea* extracts (alkamides, caffeic acid derivatives, ketones and polysaccharides). Only alkamides and caffeic acid derivatives are likely of physiological relevance (Mathias et al., 2005; Ye et al., 2011; Goey et al., 2012), because ketones readily oxidize (Qiang et al., 2013) and polysaccharides are likely modified in the gut (Woelkart et al., 2008).

Much research on the therapeutic potential of *Echinacea* extracts has concentrated on inflammatory pathways.
**Echinacea** bioactive compounds modulate transcription factors (Gertsch et al., 2004; Matthias et al., 2008) that lead to the modulation of cytokines associated with inflammation, such as tumour necrosis factor (TNF) (Lalone et al., 2010; Hou, Huang and Shyur, 2011; Senchina et al., 2011; Dapas et al., 2014) and interleukin-1β (IL-1β) (Senchina et al., 2009a, c; Zhang et al., 2012). Non-inflammatory cytokines, such as IL-10, may also be modulated (Zhai et al., 2007; Kapai et al., 2011; Ritchie et al., 2011; Senchina et al., 2011). Thus, contingent on a multitude of agricultural and experimental factors, a given **Echinacea** extract can influence Th1 responses, Th2 responses or both simultaneously. Consistent with their varying phytochemical profiles, different **Echinacea** species have different cytokine-modulating effects (Senchina et al., 2006a).

Far less is known about **Echinacea laevigata** compared with other species in the genus (**E. augustifolia**, **E. pallida**, **E. purpurea**), for which the biochemical and immunomodulatory properties are relatively well-characterized. Compared with other **Echinacea** species, **E. laevigata** extracts had high caffeic acid content (Pellati et al., 2004, 2005), and contained alkamides 2a, 4–6, 8a, 10a, and 11 (Wu et al., 2004; Senchina et al., 2011). Therefore, **E. laevigata** may harbour relevant phytomedical capacity and deserves consideration. To our knowledge there is only one report of the immunomodulatory properties of **E. laevigata** (Senchina et al., 2011). It demonstrated that **E. laevigata** tinctures exhibit immunomodulatory capacities, but only roots were examined. It is unknown whether aerial parts of **E. laevigata** (flowers, leaves, stems) harbour similar activities.

The purpose of this study was to compare the diversity and quantity of alkamides and caffeic acid derivatives from different aboveground organs of **E. laevigata**, and to characterize the *in vitro* immunomodulatory properties of ethanol tinctures produced from these structures. The research question was: do ethanol tinctures produced from **E. laevigata** aerial organs demonstrate immunomodulatory activity in an *in vitro* primary cell culture model? It was hypothesized that (a) of the aboveground organs, flower extracts would exhibit greater abundance and diversity of alkamides and caffeic acid derivatives than leaf or stem extracts, and consequently greater immunomodulatory activity; (b) of all plant organs, theelowground roots would have the greater abundance and diversity of alkamides and caffeic acid derivatives compared with any of the aboveground organs.

**Methods**

**Plant harvesting and extraction**

**Echinacea laevigata** (Ames 25 161) plants were harvested as whole plants with root bundles intact from a common garden in September 2007 at the United States Department of Agriculture (USDA) North Central Regional Plant Introduction Station in Ames, Iowa. Plants were identified and provided courtesy of Dr. Joe-Ann McCoy, were 3 years old at harvest and had been enclosed in pollination cages to prevent cross-pollinating. A voucher specimen was deposited in the Ada Hayden Herbarium at Iowa State University (ISC#447184). Specimens were extracted immediately post-harvest. Plants were divided by organ (flower, leaf, stem, root), and each organ was processed separately. After separation, organs were diced manually using a surgical scalpel. The diced material was extracted at a ratio of 1:9 plant material/solvent in 50% ethanol–50% cell culture water using methods described elsewhere (which simulate lay herbalist preparations) (Senchina et al., 2006b). Extracts were allowed to steep for 1 h at room temperature on a horizontal shaker before being passed through sterilized tulle and stored at −80°C and tested within 1 week of production. Extracts were vortexed prior to use in any phytochemical or immunomodulatory assays.

**Phytochemical profiling**

Phytochemical composition of the extracts was determined via high-performance liquid chromatography (HPLC) with UV detector for alkamides, ketones and caffeic acid derivatives using previous methods (Senchina et al., 2011). Briefly, HPLC analysis was performed with ZMC-Pack ODS-AM RP C18 (250 × 4.6 mm, 5 μm) analytical column (Waters; Bedford, MA, USA). The solvent system was acetonitrile/H2O with 0.01% formic acid for lipophilic constituents as well as hydrophilic constituents but with varying gradients. The flow rate was maintained at 1.0 ml/min. Alkamides were separated at a linear gradient of 40–80% acetonitrile over 45 min. Hydrophilic constituents, e.g. caffeic acid, were determined on a linear gradient of 10–35% acetonitrile over 25 min. The column temperature was 30°C. UV spectra recorded were in the range of 200–400 nm, while 330 nm was used for quantification of caffeic acid derivatives and 254 nm for alkamides. Bystander endotoxin levels were quantified from all extracts using a colorimetric method. The cell culture model employed here is insensitive to endotoxin levels of 10 EU/mL or less (Senchina et al., 2006b).

**Human subjects and cell isolation**

Approval to work with human subjects was granted by the Drake University Institutional Review Board (ID 2007-08015). Sixteen young adults (7 females and 9 males; 23.5 ± 3.8 years) gave written informed consent prior to participation and donated blood. Blood was drawn from the antecubital vein following Universal Precautions and peripheral blood mononuclear cells (PBMCs) were separated as described elsewhere (Senchina et al., 2009a, c). Blood was collected in heparinized tubes, and then diluted 1:1 with phosphate-buffered saline before being layered on top of Ficoll-Paque and being centrifuged at 1800 rpm and 4°C for 15 min; the centrifuge was allowed to stop without any braking. Leucocytes were then extracted from the Ficoll layer using pipettes and washed twice with Hank’s buffered saline solution (HBSS) before being manually counted via hemocytometer and standardized to 1.0 × 10⁶ cells/ml in AIM-V media. Extracts were diluted 1:12.5 in AIM-V media before being added to cell
culture wells by using 50 μl per 1 ml of cell culture fluids; more details may be found elsewhere (Perera et al., 2014). A negative control (solvent vehicle, just AIM-V media) was run with all subjects to serve as a baseline. A positive control (phytohemagluttin, PHA; stock 100 μg/ml) was run with a subset of subjects (n = 6) to ensure the experimental techniques worked. For proliferation, cells were cultured for 72 h and then tested via a 3-hour tetrazolium salt assay (Cell Titer, Catalogue #G3580, Promega). For cytokine assays, culture time varied by assay, being 24 h (TNF), 48 h (IL-2), or 72 h (IL-10); culture supernatants were collected at their respective time points, stored at −80°C, and assayed by ELISA (BD Biosciences, Catalogue #555212, #555190, #555157).

Statistics

Paired samples t-tests were used to determine whether proliferative or cytokine production differences were seen between extract- or PHA-treated cells, with a corrected α-level of 0.0125 owing to multiple comparisons for each of the four plant organs (0.05/4 = 0.0125). Significance was defined as p ≤ α and trends towards significance were defined as α < p < 2α. Pearson correlations were run to examine whether phytochemical composition or bystander endotoxin levels correlated with immune outcomes.

Results

Phytochemistry and correlations

Several different alkamides and caffeic acid derivatives were detected in the extracts (Table 1). Caftaric acid was the most widely-distributed compound; some alkamides, caftaric acid and other caffeic acid derivatives were observed only in individual extracts.

Correlations between alkamide/caffeic acid derivative content and immune outcomes are provided in Table 2. All detectable compounds except alkamide 8a correlated positively and significantly with IL-10 production. Only caftaric acid correlated positively with TNF production. None of the compounds correlated with either IL-2 or PBMC proliferation. Endotoxin levels from the extracts were (in EU/ml): flower = 2.2, leaf = 0.1, stem < 0.1, root = 1.1. Endotoxin levels never correlated with immune outcomes (all p ≥ 0.148).

Immunomodulatory properties

Table 1. HPLC analysis of the extracts

<table>
<thead>
<tr>
<th></th>
<th>Flower</th>
<th>Leaf</th>
<th>Stem</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkamide 2a</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.001</td>
</tr>
<tr>
<td>Alkamide 8a</td>
<td>0.029</td>
<td>0.001</td>
<td>0.023</td>
<td>ND</td>
</tr>
<tr>
<td>Alkamide 14</td>
<td>0.012</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Caftaric acid</td>
<td>0.004</td>
<td>0.007</td>
<td>0.004</td>
<td>0.007</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.014</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cichoric acid</td>
<td>0.007</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Echinacoside</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are expressed as mg/mL. Although tested for, none of the following compounds were detected: alkamide 2b, alkamide 8b, alkamide 10a, alkamide 10b, alkamide 11, alkamide 12, alkamide 13, and ketone 23. ND, not detected.

Table 2. Correlations between bioactive compounds and immunomodulatory activity

<table>
<thead>
<tr>
<th></th>
<th>IL-2</th>
<th>IL-10</th>
<th>TNF</th>
<th>Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkamide 2a</td>
<td>0.014 (0.901)</td>
<td>0.293 (0.018)*</td>
<td>0.179 (0.111)</td>
<td>0.134 (0.238)</td>
</tr>
<tr>
<td>Alkamide 8a</td>
<td>0.006 (0.958)</td>
<td>0.135 (0.283)</td>
<td>0.009 (0.94)</td>
<td>0.018 (0.872)</td>
</tr>
<tr>
<td>Alkamide 14</td>
<td>0.145 (0.200)</td>
<td>0.211 (0.091)*</td>
<td>−0.075 (0.507)</td>
<td>0.320 (0.777)</td>
</tr>
<tr>
<td>Caftaric Acid</td>
<td>−0.111 (0.326)</td>
<td>0.263 (0.034)*</td>
<td>0.290 (0.009)*</td>
<td>0.164 (0.145)</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>0.153 (0.176)</td>
<td>0.283 (0.022)*</td>
<td>−0.038 (0.738)</td>
<td>0.063 (0.580)</td>
</tr>
<tr>
<td>Cichoric Acid</td>
<td>0.145 (0.200)</td>
<td>0.211 (0.091)*</td>
<td>−0.075 (0.507)</td>
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</tr>
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Values are Pearson correlation coefficients and their associated p-values in parentheses. Asterisks indicate significant relationships whereas daggers indicate trends towards significant relationships.
expected by increasing cytokine production or PBMC proliferation compared with solvent control in all instances (all $p \leq 0.037$; data not shown).

**Discussion**

**Phytochemical differences by plant organ**

The first part of the hypothesis was that, of the aboveground organs, flower extracts would exhibit greater abundance and diversity of alkamides and caffeic acid derivatives than leaf or stem extracts. Flower extract had greater abundance and diversity of bioactive compounds compared with leaf and stem extracts (Table 1), supporting the hypothesis. The present work is the first report of alkamide and caffeic acid derivative composition of *E. laevigata* leaves and stems. In a study of *E. laevigata* inflorescences, it was reported that the most abundant compounds in were (in order from greatest to least detected quantities): alkamides 8/9; cichoric acid, caftaric acid and alkamide 16 (Binns et al., 2002). Findings from the present study (Table 1) are largely consistent (Binns et al., 2002), with the exception of alkamide 16 which was not analysed. A study of fresh *E. tennesseensis* showed both flower and stem extracts had greater (though differing) alkamide levels compared with leaf extracts (Senchina et al., 2009a), again similar to the present study (Table 1). Compositional differences between *Echinacea* species are well-documented (Pellati et al., 2004; Wu et al., 2004; Kraus et al., 2006). Other reports on the phytochemistry of *Echinacea* aboveground parts are difficult to compare with this study because they assayed different compounds (Mazza and Cottrell, 1999) or dried material from a different species (Mølgaard et al., 2003) or because they analysed all aboveground parts together (Brown, Chan and Betz, 2010; Brown et al., 2011; Ramasahayam et al., 2011).

Though not the primary focus of this investigation, root extracts were also produced from fresh material as a comparison point with previous research. Alkamides previously reported from *Echinacea laevigata* root extracts include 2a, 4, 5, 6, 8a, 10a, and 11 (Wu et al., 2004; Senchina et al., 2011). In the present study, only alkamide 2a was found (Table 1), but alkamides 4–6 were not assayed due to a lack of available standards. Caffeic acid derivatives previously reported from *E. laevigata* root extracts include caftaric acid, cichoric acid and echinacoside (Pellati et al., 2005; Senchina et al., 2011). In the present study, caftaric acid, chlorogenic acid and echinacoside (but not cichoric acid) were detected (Table 1). The data on caffeic acid derivatives obtained in this study confirm and extend previous work, whereas levels of alkamides were lower in the present study than that seen in previous reports.

**Immunomodulatory differences by plant organ**

The second part of the hypothesis was that, of the aboveground organs, flower extracts would exhibit greater immunomodulatory activity. When the immunomodulatory capacities of all three extracts produced from aboveground fresh material were compared (Fig. 1), flower extracts influenced three of the four immune parameters, whereas leaf extracts influenced two and stem extracts influenced only one; thus, the immunomodulatory data also support the first

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**Figure 1.** In vitro immunomodulatory effects of *Echinacea laevigata* extracts on human PBMCs: (A) proliferation, (B) TNF, (C) IL-10. Values are means ± standard errors. Asterisks (*) indicate statistically significant differences ($p < 0.017$) between treatment and control, whereas daggers (†) indicate a trends (0.017 < $p < 0.034$) towards a statistically significant difference between treatment and control.
hypothesis. Though not the primary focus of this investigation, root extracts had the strongest immunomodulatory activity of all extracts significantly modulating three of the four immune parameters.

Modulation of cytokine production or cell proliferation may relate to the potential utility of Echinacea in the context of URIs. For example, in the context of pathogen-induced inflammation, some immunology is beneficial as it makes the environment less hospitable for the pathogen; however, inflammation also causes cell and tissue damage, and is responsible for many of the symptoms associated with infection. As reviewed previously elsewhere (Senchina, Hallam and Cheney, 2013), several studies of Echinacea extracts and leucocytes have shown a general up-regulation of cytokine production, regardless of whether those cytokines are pro- or anti-inflammatory. However, other studies (LaLone et al., 2009; Cech et al., Lalone et al., 2010; Ritchie et al., 2011; Dapas et al., 2014) have shown that Echinacea phytochemicals may selectively inhibit pro-inflammatory cytokines like TNF while promoting cytokines such as IL-10; this may lead to a reduction of symptom severity in the context of upper respiratory tract infection. Discrepancies between the two groups of studies may be explained by phytochemical differences in the compounds or supplements tested, specifically the alkamides, but composition did not always correlate with observed effects (Cech et al., 2010). Findings from this study echo the former pattern of a generalized up-regulation of cytokines (Fig. 1). Since the extracts tested in this study contained alkamides alongside other compounds such as caffeic acid derivatives (Table 1), the most parsimonious explanation is that TNF and IL-10 are being modulated by different compound classes (discussed in the Limitations and future directions section). Thus, present results do not resolve the current conflicts among different reports.

The present work is the first report of the immunomodulatory properties of E. laevigata flowers, leaves and stems. In the lone previous report of E. laevigata immunomodulatory activities (Senchina et al., 2011), root extracts augmented IL-10 but did not have a significant effect on IL-2, TNF or proliferation; flower, leaf and stem were not tested. Given all the pre-clinical and laboratory factors that can influence extract immunomodulatory activity (Senchina et al., 2009b) and differences in methods and subjects between the two studies, it is likely premature to make any comparisons between the immunomodulatory capacities of aboveground vs. belowground parts of E. laevigata. The finding that E. laevigata extracts widely influenced proliferation, TNF and IL-10 (but not IL-2) is consistent with the studies regarding the in vitro immunomodulatory properties of other Echinacea species (Rininger et al., 2000; Randolph et al., 2003; Gertsch et al., 2004; Hwang, Dasgupta and Actor, 2004; Mishima et al., 2004; Senchina et al., 2006a, 2009a).

Phytochemical composition and immunomodulatory activity

Statistical correlations between extract composition and in vitro immunomodulatory activity (Table 2) yielded a few significant relationships. Given the idiosyncratic nature of the significant relationships, the correlations may not be physiologically relevant. It may be more likely that other compounds are responsible for the effects shown in Fig. 1. A similar lack of correlation between known bioactive compounds and extract immunomodulatory activity has been reported previously for Echinacea (Vimalanathan, Arnason and Hudson, 2009), and has also been observed in studies of other herbal supplements including Pueraaria (Cherdhewasart and Sutjit, 2008) and Sanguinaria (Perera et al., 2014). The lack of any robust, consistent correlations is unsurprising given the complex phytochemical milieu of plants and possible unaccounted pre-experimental factors, yet it may suggest that the alkamides and caffeic acid derivatives do not account for all immunomodulatory activity from Echinacea extracts.

Limitations and future directions

Some limitations may be identified from the research conducted here. All work was performed in vitro, so it is unclear whether these results would translate directly to clinical scenarios. Although all plants grew in the same common garden and were the same age, they were grown under outdoor conditions that are not precisely replicable, and it is unknown what role environmental variables may have played in these results.

Data obtained in this study point naturally to several potential future directions. Additional data on the effects of pre-harvest conditions (sunlight, hydration, soil conditions, etc.) post-harvest conditions (such as extraction methods or drying) are needed to better understand heterogeneous findings between studies that use similar species or extraction techniques, as these variables would quite likely explain disparities between studies. More cross-genus comparisons (controlling for the pre-experimental conditions highlighted previously) would illuminate the interplay between species selection and environmental conditions, helping parse out which immunomodulatory effects are consistent across the genus vs. peculiar to individual species or species groups. The immunomodulatory properties of one species, E. atrorubens, have not yet been reported.

Conclusions

Echinacea laevigata appears to have phytochemical and immunomodulatory properties similar to other members of its genus. Extracts generated from fresh aboveground material demonstrated immunomodulatory effects. Similar to other Echinacea species, E. laevigata extracts appear able to stimulate both Th1 and Th2 aspects of immunity.

Author biography

Ekta Haria obtained her bachelor’s degree from Drake University, Des Moines, Iowa in 2014. She is currently a Physician Assistant student at the University of Nebraska Medical Center, Omaha, Nebraska.
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References


