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PHENETIC COMPARISON OF SEVEN *Echinacea* SPECIES BASED ON IMMUNOMODULATORY CHARACTERISTICS¹

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Abstract

The purpose of the present investigation was to compare similarities and differences in immune response among *Echinacea* species, which are commonly used to treat upper respiratory infections. The investigation involved two components: acquisition of immunomodulatory data reported here for the first time, and combined phenetic analysis of these data along with previous reports. Experimental data were obtained by stimulating human PBMC in vitro with extracts from *Echinacea* spp. and assaying production of three cytokines (interleukin-1 β [IL-1 β], interleukin-2 [IL-2], and tumor necrosis factor- α [TNF- α]). Phenetic analyses were employed to compare responses across the entire data set, including UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and neighbor-joining methods. In the immune experiments conducted for this investigation, *E. angustifolia*, *E. paradoxa*, *E. purpurea*, *E. simulata*, and *E. tennesseensis* extracts significantly augmented IL-1 β and TNF- α production, whereas no extracts significantly modulated IL-2. All phenetic methods produced similar dendrograms, revealing two species pairs (*E. angustifolia* + *E. simulata* and *E. pallida* + *E. sanguinea*) where both species cluster tightly and have similar immune-response profiles. These two species-pairs are maximally dissimilar from each other. The remaining species (*E. paradoxa*, *E. purpurea*, and *E. tennesseensis*) occupy intermediate positions in the dendrogram. Our results suggest that *Echinacea* spp. act heterogeneously on immune function. The utility of these data for science and industry is discussed.

Keywords

comparison; cytokine; *Echinacea*; PBMC; phenetic; taxonomy

Introduction

In modern herbal medicine, *Echinacea* is most commonly employed for treating upper respiratory infections, particularly viral infections such as colds (rhinoviruses) and influenza (Caruso and Gwaltney 2005). However, data are in conflict regarding its efficacy, with some studies supporting effectiveness (Goel et al. 2005; Lindenmuth and Lindenmuth 2000; Schulten

et al. 2001) and others discounting it (Barrett et al. 2002; Turner et al. 2005; Yale and Liu 2004).

The vast majority of immunomodulatory studies of *Echinacea* have centered on three of the nine traditionally recognized species: *E. angustifolia*, *E. pallida*, and *E. purpurea* (Barnes et al. 2005). By comparison, other *Echinacea* species have been neglected, presumably because of economic factors, as the aforementioned species are the ones most frequently employed in commercial phytomedicinal preparations. Considerable research has been aimed at elucidating the phytochemical profiles of *Echinacea* species, but the relationship between phytochemistry and medicinal function has yet to be demonstrated satisfactorily (Barnes et al. 2005). Studies of immune function, both *in vitro* and *in vivo*, remain the methods of choice for evaluating immunomodulatory efficacy. Our laboratory (Senchina et al. n.d.; Senchina et al. 2005; McCann et al. n.d.), as well as others (Binns et al. 2002b), have demonstrated that the remaining species also may harbor immunomodulatory properties.

A comparison of immunomodulatory properties across *Echinacea* species has yet to be conducted; thus, it is unclear at present how *Echinacea* species compare to one another regarding potential immunomodulatory characteristics. In the present investigation, we demonstrate how *in vitro* models of immune function may be used to systematically investigate the similarities and differences in such functions across seven species of the genus *Echinacea*. Our report encompasses full development of the investigation from novel experimental data acquisition through final analyses.

Methods

Experimental Data Collection

All procedures involving human subjects were pre-approved by the Iowa State University Internal Review Board for human subject research. Peripheral blood mononuclear cells were harvested from 15 healthy human young adult donors using methods previously described (Senchina et al. 2005). Cells were adjusted to a concentration of 1.0×10^6 cells/mL and cultured for 24 hours with extracts described previously from seven *Echinacea* species (*E. angustifolia* [ANG], *E. pallida* [PAL], *E. paradoxa* [PAR], *E. purpurea* [PUR], *E. sanguinea* [SAN], *E. simulata* [SIM], and *E. tennesseensis* [TEN]) and compared to control [CON]; however, in contrast to the study reported earlier, the extracts employed here had been stored at -20°C for one month before being used in this investigation. Supernatants were harvested and tested for production of interleukin- 1β (IL- 1β), IL-2, and tumor necrosis factor- α (TNF- α) via ELISA (BD Biosciences, Pharmingen, San Diego, CA). Statistical analyses employing a general linear model of repeated measures were performed in SPSS (Chicago, IL).

Data Matrix Construction

A data matrix was constructed for use in the phenetic analysis. This matrix was derived from previous investigations (Senchina et al. n.d.; Senchina et al. 2005; McCann et al. n.d.) on PBMC proliferation and cytokine production as well as unpublished data on cytokine production by *Echinacea* preparations presented here. Day-four data for interleukin-10 and tumor necrosis factor- α from study (Senchina et al. 2005) were excluded due to possible effects of contaminating endotoxin. We elected to use only studies from our own laboratory as this eliminated variability due to differences in experimental methodologies.

Individual immune outcomes from all studies were treated as distinct characters. In total, 25 characters were employed in the matrix. Characters 1–22 represent previously published data, whereas characters 23–25 represent previously unpublished data. For each character, the effect

of the *Echinacea* species was compared against a control; statistical methods for this comparison varied by study and can be found in the individual papers. The control was designated as having a character state of “0.” Within a given immune parameter, species that statistically augmented the outcome as compared to the control were assigned a state of “1”; likewise, species that statistically diminished the outcome were assigned a state of “2.” Thus, for each immune outcome, there were three possible unordered and equidistant character states.

Phenetic Analysis

The 25 individual immune responses from the data matrix were converted to the NEXUS data format (Maddison et al. 1997) and imported into the software program Phylogenetic Analysis Using Parsimony* version 4.0b10 (Swofford 2003). Data were treated as unordered and because the mechanisms underpinning the immune responses are unknown, all immune response categories were given equal weight. A distance matrix was constructed using the mean pairwise character differences adjusted for missing data. This distance matrix was then subjected to both neighbor-joining and clustering analyses, the latter using the un-weighted pair group method with arithmetic mean (UPGMA) method, and final dendrograms were produced in R (Team 2005).

Results

Experimental Data

We tested *Echinacea* extracts from seven species that had been stored for one month at -20°C for their ability to modulate interleukin- 1β (IL- 1β), IL-2, and tumor necrosis factor- α (TNF- α) from peripheral blood mononuclear cells (PBMCs) of young adults. Results are presented in Table 1. Extracts from *E. angustifolia*, *E. paradoxa*, *E. purpurea*, *E. simulata*, and *E. tenesseeensis* were all able to significantly augment IL- 1β and TNF- α production. None of the extracts significantly altered IL-2 production, compared to the control.

Phenetic Analysis

The data matrix used in the phenetic analysis is given in Table 2. While some immune parameters appear multiple times on the table, each is distinct due to differences in extract preparation and storage methods, subject demographics, or cell culture protocols between studies; these differences also may explain apparent contradictions in results. For example, differences in IL-2 outcomes may be due to the human subjects used: extracts had no effect on IL-2 production in young subjects, however they appeared to diminish IL-2 production in older adults and diminished capacity to produce IL-2 is known to be a consequence of aging (Kohut and Senchina 2004). Similarly, ethanol tinctures from *E. pallida* suppressed IL-10 production in older adults, whereas similar tinctures had no effect or augmented IL-10 production in young adults. TNF- α production was enhanced only when extracts had not undergone long-term storage.

The data matrix was subjected to multivariate analysis as described (see Methods), yielding the dendrogram shown in Figure 1.

Discussion

In this report, we validate that data from immunological investigations of seven *Echinacea* species may be analyzed phenetically to compare similarities and differences among species. Our demonstration included the acquisition of original data. We consider each of these aspects separately below, as well as possible applications. While we used *Echinacea* in this report, the system outlined here might be applicable to other medicinal plant taxa.

Consideration of Original Data

Echinacea extracts used in this study either had no effect on or stimulated production of the cytokines surveyed (Table 1). Five species (*E. angustifolia*, *E. paradoxa*, *E. purpurea*, *E. simulata*, and *E. tenesseeensis*) stimulated production of IL-1 β and TNF- α , but did not augment IL-2 production. Two other species, *E. pallida* and *E. sanguinea*, did not influence levels of any of these three cytokines. Though the extracts tested here are the same as those reported elsewhere (Senchina et al. 2005), a direct comparison between the two studies is difficult due to numerous differences in experimental design (mode of stimulation, extract time of storage).

IL-1 β and TNF- α are cytokines produced during the inflammatory response by cells of the innate immune system such as macrophages (Durum et al. 1985; Strieter et al. 1993). Consequently, it is not surprising that the effects of the different *Echinacea* species on IL-1 β and TNF- α were similar. Our findings that *Echinacea* tinctures augmented production of these cytokines are consistent with reports from both human (Barak et al. 2002; Gertsch et al. 2004; Randolph et al. 2003) and rodent (Bodinet et al. 2002; Luettig et al. 1989; Rininger et al. 2000; Stimpel et al. 1984) model systems; however, two teams found no effect of *Echinacea* extracts on these cytokines (Elsasser-Beile et al. 1996; Schwarz et al. 2002). These differences may be due to species selection, plant organ selection, extraction method, or other experimental choices as discussed above.

In contrast, IL-2 is a marker of T-cell activation associated with adaptive immunity (Smith 1984). Our results are consistent with those from previous reports (Luettig et al. 1989) but in contrast with other reports showing application of *Echinacea* extracts to cell cultures induces production of IL-2 (Bodinet et al. 2002; Cundell et al. 2003). Again, these differences are likely due to differences in experimental design, such as choices of model organism, extract type, and species selected.

These results also suggest that *Echinacea* may influence innate immunity to a greater extent than adaptive immunity based on the limited number of parameters that were evaluated. This observation is consistent with previous findings from our lab, as well as findings from other investigators (Goel et al. 2005; Randolph et al. 2003).

Phenetic Analysis

The result of the phenetic analysis is shown in Figure 1. *Echinacea pallida* and *E. sanguinea* clustered tightly together and separate from all other taxa. Among the remaining five species, *E. angustifolia* and *E. simulata* were more similar to one another than to the remaining three species *E. paradoxa*, *E. purpurea*, and *E. tenesseeensis*. Finally, the immune response profiles of *E. paradoxa*, *E. purpurea*, and *E. tenesseeensis* were approximately equidistant from each other and from the *E. angustifolia* + *E. simulata* species-pair. One key observation is that the three species employed commercially (*E. angustifolia*, *E. pallida*, and *E. purpurea*) were widely separated from one another on the dendrogram.

Possible Applications for Phytomedicine

Multivariate relationships among species-specific immune responses may help inform phytomedicinal investigations. For example, it can be seen from Table 2 that *Echinacea pallida* tends to exhibit no or suppressive immune modulation, whereas *E. angustifolia* tends to exhibit mostly enhancing effects on immune function. Our dendrogram suggests that *E. angustifolia* and *E. pallida* have very different immunomodulatory capabilities from one another. It also suggests that if an immunosuppressive effect is desirable, species such as *E. pallida* and *E. sanguinea* should be favored; likewise, if enhanced immune responses are desired, species such as *E. angustifolia* and *E. simulata* should be selected. It is important to

stress that these data are based on the testing of only ethanol decoctions and water infusions from a representative sampling of species and accessions; the interpretation is thus limited to these extract types. Different immunomodulatory properties may be obtained by different extraction methods from different accessions.

Our results suggest that different *Echinacea* spp. act heterogeneously on immune function. Scientists studying *Echinacea* frequently report findings from only one species. The data presented here indicate that there is not one “representative” species of *Echinacea*, and that immune effects are highly contingent on species selection, extraction technique, and extract storage conditions. This has important ramifications for further investigations of the immunomodulatory activity. As reviewed above, studies disagree as to *Echinacea*’s effectiveness in the treatment of respiratory infections. Perhaps, as our results suggest, these discrepancies may arise in part due to investigators employing different species.

Possible Applications for Botanical Supplement Industries

Our findings of heterogeneous immune activity among species of *Echinacea* may be important to the botanical supplements industry. Currently, *E. angustifolia*, *E. pallida*, and *E. purpurea* are used in most *Echinacea* preparations, either alone or in combination. Species selection may be a potentially critical factor in the manufacture of *Echinacea* supplements and should be determined based on desired immune outcome. Further, this analysis suggests that many species of the genus (in particular, *E. sanguinea* and *E. simulata*) might be worthy of cultivation for phytomedicinal preparations, again based on desired immune outcome.

Immune responses and Evolutionary History of the Genus

While our phenetic analysis do not make any statements regarding evolutionary relationships between the seven taxa studied here, it is instructive to compare our results against two currently competing taxonomic interpretations of the genus. The traditional treatment constructed by McGregor (1968), and the treatment employed in this study, identifies nine species in the genus. However, one investigator (Binns et al. 2002a) more recently has proposed the genus be considered as only four species. Both treatments are based on morphological characters.

Binns’ treatment groups *E. angustifolia*, *E. pallida*, *E. sanguinea*, *E. simulata*, and *E. tennesseensis sensu* McGregor as five varieties within the species *E. pallida*. However, if these five taxa are compared on our dendrogram, we see that they do not cluster as a monophyletic group on the tree; rather, they are interspersed throughout the dendrogram. Additionally, in our phenetic analysis, *E. purpurea*, which Binns separated distinct from all other taxa, *E. purpurea* is distant from all other taxa with respect to overall levels of dissimilarity on the dendrogram. Taken together, this phenetic analysis appears more congruent with the taxonomic treatment proposed by McGregor.

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Fig. 1. An UPGMA dendrogram showing results of phenetic analysis of the immunomodulatory properties of seven species of *Echinacea*. Species abbreviations are given in methods. Taxa linked at nodes with lower levels of dissimilarity have greater overall similarity in their ability to modulate the immune properties studied here.

Table 1

Results from immune assays. CON represents control values (no extract added); species abbreviations are given in the Methods. Values represent mean cytokine production in pg/ml $\pm$ standard errors. Asterisks indicate values statistically significantly different from the control.

	IL-1 β	IL-2	TNF- α
CON	10.3 $\pm$ 0.68	2.91 $\pm$ 0.62	8.56 $\pm$ 0.35
ANG	242.78 $\pm$ 36.84*	2.54 $\pm$ 0.42	697.29 $\pm$ 65.81*
PAL	10.59 $\pm$ 0.7	2.61 $\pm$ 0.79	47.92 $\pm$ 16.69
PAR	41.34 $\pm$ 7.97*	2.92 $\pm$ 0.64	324.23 $\pm$ 78.86*
PUR	215.60 $\pm$ 32.61*	2.48 $\pm$ 0.45	697.32 $\pm$ 64.27*
SAN	16.96 $\pm$ 2.65	2.35 $\pm$ 0.51	143.6 $\pm$ 45.9
SIM	49.08 $\pm$ 11.91*	2.79 $\pm$ 0.46	482.62 $\pm$ 75.76*
TEN	44.95 $\pm$ 11.65*	3.22 $\pm$ 0.85	248.47 $\pm$ 57.12*

Data matrix used in phenetic analysis, using data generated previously (characters 1–22) as well as unpublished data (characters 23–25). Extract type abbreviations are as follows: OH = 50% ethanol/50% water tincture, CW = cold water infusion, HW = hot water infusion. Species abbreviations are given in Methods. For species outcomes, the symbol “↑” indicates significant enhancement of cytokine production compared to the control, whereas the symbol “↓” indicates significance decrease in cytokine production compared to the control; “N” indicates “no change” compared to control. Blanks represent unavailable data.

Table 2

Source	Immune Parameter (Extract Type)	ANG	PAL	PAR	PUR	SAN	SIM	TEN
Senchina et al. (2005)	1. Tumor necrosis factor- α , Day 1 (OH)	↑	N		↑	N		N
	2. Tumor necrosis factor- α , Day 1 (CW)		↑		↑	N		N
	3. Tumor necrosis factor- α , Day 1 (HW)	N	N		↑	N		N
	4. Interleukin-10, Day 1 (OH)	↑	N		N	N		N
	5. Interleukin-10, Day 1 (CW)	↑	N		N	N		N
	6. Interleukin-10, Day 1 (HW)	↑	N		↑	N		N
	7. Interleukin-12, Day 1 (OH)	↑	N		N	N		N
	8. Interleukin-12, Day 1 (CW)	↑	↑		N	N		N
	9. Interleukin-12, Day 1 (HW)	N	N		N	N		N
	10. Interleukin-12, Day 4 (OH)	N	N		↓	N		↓
	11. Interleukin-12, Day 4 (CW)	N	N		↑	N		↑
	12. Interleukin-12, Day 4 (HW)	N	N		N	N		↑
	13. PBMC Proliferation, Day 1 (OH)	N	N		N	N		N
	14. PBMC Proliferation, Day 1 (CW)	N	N		N	N		N
	15. PBMC Proliferation, Day 1 (HW)	N	N	↓	N	N	↓	N
	16. Interleukin-2 (OH)	↓	↓		↓	↓		↓
(McCann et al. n.d.)	17. Interleukin-10 (OH)	↑	↓	N	↑	↑	N	↑
	18. Interferon- γ (OH)	N	↓	↑	N	↓	N	↑
	19. PBMC proliferation (OH)	↑	↑	↑	N	↑		
Present study	20. Tumor necrosis factor- α (OH)	N	N	N	N	N	N	N
	21. Interleukin-10 (OH)	↑	↑	↑	↑	N	N	↑
	22. Interleukin-12 (OH)	N	N	N	↑	N	N	↑
	23. Interleukin-1 β (OH)	↑	N	↑	↑	N	↑	↑
	24. Interleukin-2 (OH)	N	N	N	N	N	N	N
	25. Tumor necrosis factor- α (OH)	↑	N	↑	↑	N	↑	↑