Assessing the Effect of Orally Administered *Echinacea purpurea* Extracts vs. Placebo on Pro-Inflammatory Cytokine Responses *Ex Vivo*

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ABSTRACT

Objective: To determine if aqueous, polysaccharide-containing *Echinacea purpurea* extracts taken orally increase pro-inflammatory cytokine responses *ex vivo.*

Design: In two separate studies, the levels of TNF-alpha (TNF), interleukins 2 and 6 (IL-2 and IL-6) and interferon gamma (IFN-γ) secreted by phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from healthy adults randomized to take one of three *E. purpurea* formulations or placebo orally for 10 consecutive days were measured. Blood was obtained from participants at baseline and on days 2, 3, 7, and 10 while on study medication. PBMC were isolated and stimulated with PHA for 24 h, and supernatants collected for measurement of pro-inflammatory cytokine levels.

Outcome Measures: Primary outcomes were peak concentrations of PHA-induced TNF, IL-2, IL-6, and IFN-γ from PBMC isolates collected while on study medication. Cytokine responses of PBMC from participants randomized to one of the *Echinacea* formulations were compared with those of placebo recipients by regression analysis.

Results: Cytokine levels were obtained from mitogen-activated PBMC from 86 participants, collected while on study medication. No significant differences in the peak levels of PBMC-secreted TNF, IL-2, IL-6 and IFN-γ were observed between PBMC from those taking active *Echinacea* preparation vs. a placebo. After adjusting for age, a trend toward increased IL-6 secreted by PHA-stimulated PBMC isolated on day 3 of oral administration was observed for the group taking one of the *E. purpurea* formulations compared with placebo (P=0.064).

Conclusions: Oral administration of *E. purpurea* did not significantly enhance peak pro-inflammatory cytokine responses in mitogen-stimulated PBMC.

Keywords: *Echinacea*; Cytokines; Immunity; Immunomodulation; Interleukin-6; Phytotherapy

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INTRODUCTION

Although *Echinacea* is frequently used to prevent and/or treat upper respiratory tract infections (URIs), the results of randomized controlled trials (RCTs) of its efficacy are decidedly mixed.[1–8] One meta-analysis of *Echinacea* RCTs found that *Echinacea* products significantly reduced incidence and duration of the common cold in treated groups compared with controls.[6] However, several studies showed no significant effect of the *Echinacea* product studied on duration or severity of URIs.[2,4,8] A Cochrane systematic review found that although several RCTs report positive results of *Echinacea* treatment, the comparability of the *Echinacea* preparations is limited due to variations in the chemical composition of the different extracts tested.[9] *Echinacea* preparations vary considerably in their phytochemical profiles based on differences in extraction methods, including extraction of different species (most commonly, *E. purpurea* or *E. angustifolia*), plant parts (aerial or root), and use of fresh or dried plants, as well as different extraction solvents.[10] Given this variation in extract composition, a major challenge exists in identifying an *Echinacea* product with biological activity to test in trials evaluating the efficacy of *Echinacea* for preventing or treating URIs.

With regard to its biological activity, *Echinacea* has long been reported to have immune-modulating actions.[11] However, a lack of consensus in the field regarding which constituents are active in enhancing antiviral immune responses complicates the process of selecting an active *Echinacea* formulation for a clinical trial.[12] The two main *Echinacea* constituent groups with reported immune modulating actions are polysaccharides[13] and alkylamides,[14–16] the levels of which vary based on different species, plant parts, and extraction methods. Polysaccharides are extracted at high levels in water-based aqueous solutions, but are not extracted in less polar alcohol extracts, while the lipophilic alkylamides are present at high levels in alcoholic extracts, but only minimally extracted in aqueous solutions.[17] Although an earlier study reported that *Echinacea*-derived alkylamides have immune-stimulating effects in rats,[14] other studies, both *in vitro* and in humans, report evidence of anti-inflammatory actions of *Echinacea*-derived alkylamides[18] and cytokine-inhibitory effects of alkylamides and alkylamide-containing *E. purpurea* alcoholic extracts.[15,16,19] In contrast, *in vitro* and *in vivo* evidence shows that *Echinacea*-derived polysaccharides stimulate production of pro-inflammatory cytokines.[11,20–23] In a study assessing biological activity of different *Echinacea* extracts, one polysaccharide-containing *E. purpurea* extract significantly enhanced interleukin 6 (IL-6) production in interferon gamma (IFN-γ)-stimulated macrophages *in vitro*.[17] *E. purpurea* polysaccharides are reported to trigger both toll-like receptor (TLR)-4 dependent and independent signaling pathways that stimulate pro-inflammatory cytokine production.[22] Pro-inflammatory cytokines released during innate immune responses are required to prime the adaptive lymphocyte responses that result in effective antiviral immunity.[24] This evidence suggests that the URI-preventative properties of *Echinacea* may be due to enhanced pro-inflammatory cytokine production by polysaccharides in aqueous extracts of *E. purpurea*. Because polysaccharide content and biological activity varies in the different commercially-available *E. purpurea* extracts,[17] studies are needed to identify which of the commercially available polysaccharide-containing *E. purpurea* extracts have detectable biological activity before testing them in clinical trials.

In a previous RCT within a pediatric population, a fresh-pressed juice of *E. purpurea* aerial parts did not decrease URI severity or duration compared with placebo when taken orally at the time of a reported cold.[2] However, a secondary analysis of data from this study showed that children in the *Echinacea* group had fewer colds than those in the placebo group over a 4-month observation period,[3] supporting the hypothesis that certain polysaccharide-containing aqueous *E. purpurea* extracts may prevent incidence of URIs. Prior to conducting a phase II RCT in children to test for efficacy of an orally-administered *E. purpurea* extract on URI prevention, two clinical trials were conducted to identify an *E. purpurea* product with detectable biological activity beyond that of placebo. *Ex vivo* cytokine responses were measured to test the hypothesis that a 10-day course of orally administered polysaccharide-containing *E. purpurea*

extracts would lead to detectable increases in pro-inflammatory cytokines compared with placebo, consistent with the proposed mechanism of action by which URIs could be prevented by *Echinacea*.

In these studies, three different aqueous formulations of *E. purpurea* extracts were evaluated for pro-inflammatory cytokine-enhancing effects. We specifically chose these *Echinacea* extracts because they were reported by their manufacturers to contain relatively high polysaccharide levels, one of which was a new lot of the formulation that prevented URIs in a previous RCT.[2, 3] Furthermore, because the ultimate goal of this project was to conduct an RCT assessing the efficacy of *E. purpurea* in preventing URIs in children, only liquid, ethanol-free preparations were selected. The primary outcome measure was determination of peak levels of pro-inflammatory cytokines secreted by peripheral blood mononuclear cell (PBMC) isolates from adult volunteers randomized to an *Echinacea* formulation or to a taste- and color-matched placebo. We postulated that peak levels of one or more of the cytokines secreted by mitogen-stimulated PBMC isolated from individuals receiving an active *Echinacea* preparation would be significantly higher than those from placebo recipients.

METHODS

Three aqueous *E. purpurea* formulations were assessed among 87 adult participants enrolled in two separate randomized, placebo-controlled clinical trials. Cytokines assayed included TNF, IFN-γ, IL-2, and IL-6. Study data were collected between March 2009 and March 2011. Both studies were registered on clinicaltrials.gov and approved by Bastyr University's institutional review board. Written informed consent was obtained from all participants.

STUDY PREPARATIONS

The three *E. purpurea* formulations chosen for study were polysaccharide-containing aqueous formulations of *E. purpurea* herb (above-ground parts). Because the objective was to identify a formulation with biological activity significantly higher than a placebo and not to directly compare the biological activity of one product to another, the manufacturer's recommended daily dosages were used for each preparation. One formulation was Echinacin Saft (Madaus AG, Cologne, Germany), shown to have URI-preventative effects in a previous study.[3] This product was a dried, expressed juice from fresh, flowering *E. purpurea* herb (ratio of fresh herb/dried pressed juice 31.5–53.6:1), reconstituted in a 100 g water solution containing 0.2 g potassium sorbate and 15 g xylitol, citric acid, and orange flavor. The polysaccharide content was 27 mg/mL (assayed by the University of Georgia Complex Carbohydrate Research Center). As recommended by the manufacturer, study participants received 15 mL of Echinacin Saft per day in three divided doses.

The second *E. purpurea* formulation was prepared specifically for this study by Gaia, Inc. (Brevard, NC). The product was a water extract of the above-ground parts of *E. purpurea* harvested at budding, mixed with glycerin and water to provide a preparation with a concentration of 100 mg/mL *E. purpurea*. The measured polysaccharide content was 3.2 mg/mL. Study participants randomized to the formulation received 25 mL/day in divided doses.

The final *Echinacea* preparation assessed was Echinamax Alcohol-free, an aqueous extract of *E. purpurea* herb (aerial parts) manufactured by Webber Naturals (Coquitlam, Canada). This product had previously been reported to have a high polysaccharide content and to significantly increase the amount of IL-6 secreted by stimulated macrophages *in vitro*.[17] According to the manufacturer, this formulation had a polysaccharide content of 2.5%, which, given the concentration of 1000 mg/mL *Echinacea* plant material reported for this extract by Vohra *et al.*[17] is approximately 25 mg/mL polysaccharides. As recommended by the manufacturer, study participants randomized to Echinamax Alcohol-free received 3 mL per day in three divided doses.

These three preparations were randomly designated Product A, B, or C, and were not assigned to correspond to products 1, 2, and 3, since the study objective was not to compare products to each other but each to their corresponding placebo. Placebo had a similar appearance and taste to each of the three *Echinacea* formulations and was provided in the same dose and frequency as the corresponding *Echinacea* medication.

STUDY PARTICIPANTS

Healthy adults, 21–65 years old, with no known immune-related diseases or inflammatory conditions were recruited. Individuals were excluded if they were taking medications other than contraceptives, were pregnant, or breast-feeding. Because of the possibility of an allergic reaction to the study medication, adults who were allergic to *Echinacea* or related species, or had asthma, atopic dermatitis, or allergic rhinitis were also excluded.[25]

STUDY PROCEDURES

Two separate, double-blind RCTs were conducted. In the first trial (study 1), 20 study participants were randomized (in blocks of 4) on a 1:1 basis to receive either Product C or corresponding placebo for 10 days. A second trial was conducted to test the two additional *E. purpurea* formulations chosen for study. In the second trial (study 2), study participants were randomized on a 1:1:1 basis, in blocks of 6, to receive, Product A, B, or placebo for 10 days. Initially a sample size of 60 participants was planned.

SAMPLE SIZE DETERMINATION

Sample size calculations were based on differences in peak TNF concentrations among participants randomized to an active *Echinacea* preparation and those receiving placebo. In study 1, additional assessments of cytokines using unstimulated PBMC (with no phytohemagglutinin [PHA] added) were conducted. The levels of cytokines were below the limit of detection in the majority of assays of unstimulated PBMC, so these data were not further analyzed. Power calculations were based on preliminary data collected prior to study 1 to determine baseline TNF concentration in unstimulated PBMC, which was 60±10 pg/mL. If peak levels were unchanged in placebo recipients and increased by 50% to 90 pg/mL in those receiving Product C, 10 participants in each group would be needed to have a power of 0.9 to detect a significant difference between treatment groups (2-sided alpha level=0.05). The sample size calculation was performed using Stata 9.2 (College Station, TX) and based on changes from baseline to peak of 0 pg/mL in placebo recipients and log 30 pg/mL in those receiving an *Echinacea* preparation, with a standard deviation of log 10 in both groups.

For study 2, power calculations to determine sample size were performed using data from a preliminary analysis of data in study 1. Based on an increase of TNF levels of 250 pg/mL from baseline to peak values among those receiving placebo, a sample of 20 participants receiving an active *Echinacea* formulation and 20 randomized to placebo would be needed to have a power of 0.9 to detect a difference in peak TNF levels if the increase in those receiving active medication was 1000 pg/mL or more, with a standard deviation of 700 pg/mL.

RANDOMIZATION AND BLOOD DRAWS

After eligibility was verified and written informed consent obtained, participants were consecutively assigned a study identification number. Each number corresponded to a specific study medication that was randomly determined with an electronic random number generator prior to beginning the study. Blood specimens were obtained from participants at approximately the same time of day at baseline (day 0), and on days 2, 3, 7, and 10 while on treatment. Follow up blood draws for *ex vivo* cytokine response testing occurred on approximately days 17 and 30.

ADVERSE EFFECTS MONITORING AND ANALYSIS

Adverse effects were systematically assessed at each blood draw using the Monitoring of Side Effects System (MOSES).[26] This scale lists 76 possible adverse events assessed on a 6-point Likert Scale (with scores ranging from 0 for "not present," to 5 for "FDA serious adverse effect"). To report adverse events (AE) data, participants were classified as having an AE if reported at least once. The rate of each AE in an active *Echinacea* group was compared with the rate of that AE in corresponding placebo groups using chi square tests.

EX VIVO CYTOKINE RESPONSE ASSAYS

After blood samples were obtained in sodium heparin, PBMC were isolated by ficoll-hypaque centrifugation, aliquoted in triplicate into culture plates at 1.0×10^6 cells/mL in 0.5 mL and stimulated for 24 h with PHA (1 μg/mL), at 37°C, 5% CO_2, 95% humidity. After 24 h, cell-free supernatants were collected and frozen at −80°C until time of

analysis to detect concentrations of cytokines TNF, IL-6, IFN-γ and IL-2.

For study 1, cytokine assays were conducted using a cytometric bead array technology using flow cytometry, which is reported to be a precise and consistent technique for multiple cytokine analysis.[27] Supernatants were added to test tubes containing multiplex bead mixtures. A blank tube (containing only beads) was also included. A biotin-conjugate was added and tubes incubated at room temperature for 2 h. Beads were washed, Streptavidin-PE added, and tubes incubated for 1 h. Sample tubes were washed and analyzed using a Beckman Coulter flow cytometer (Beckman Coulter, Inc., Miami, FL). Test concentrations were calculated relative to a series of standard curves generated for all cytokines tested.

Due to variability in cytokine levels detected by the multiplex technology in the first study, a different method was used to assay cytokine levels in study 2. For this trial, cytokines were detected by the Luminex sandwich immunoassay (Luminex Corporation, Austin, TX), which has also been shown to be a valid and high precision method for multiple cytokine assessment[28] that correlates well with the enzyme-linked immunosorbent assay (ELISA) method of cytokine determination.[29] Samples were incubated with Luminex microbeads coated with cytokine-specific antibodies. Beads were incubated with biotinylated cytokine antibodies and then a fluorescently-tagged streptavidin conjugate (washing beads after each step). Samples were read on a Luminex 200 instrument, classifying each bead as to its cytokine-specificity and fluorescence intensity, the latter being directly proportional to the cytokine concentration in the first incubation step. Cytokine standards were run with each assay and the sample concentrations calculated from the standard curve, correcting for any sample dilution. For study 2, two supernatants were prepared from each blood sample and analyzed twice.

EX VIVO CYTOKINE RESPONSE ANALYSIS

Mean concentrations and standard deviations of TNF, IL-6, IFN-γ and IL-2 cytokine concentrations secreted by 24-h cultures of PHA-stimulated PBMC isolates from blood collected at each study time point were calculated. The primary study outcome was the maximal (peak) concentration of each cytokine secreted by stimulated PBMC isolates from each study participant during the 10-day treatment period. Regression analysis was performed with the peak concentration of a specific cytokine as the dependent variable. Baseline levels of each cytokine and treatment group (*Echinacea* or placebo) were included as predictor variables. In addition, since assays were completed in multiple batches, and run-to-run variation in cytokine concentrations was observed, a "run" variable was included in all models. Secondary analyses included comparison of concentrations of specific cytokines secreted by stimulated PBMC isolates from placebo and *Echinacea* recipients on individual days using a similar regression model. In addition, data from all days on which participants were receiving treatment were included in a single regression model to assess the overall effect of each *Echinacea* preparation on individual cytokines; generalized estimating equation techniques were used to account for multiple measurements on the same individual for these analyses. Baseline levels of the corresponding cytokine were also included, as was a "run" variable in all secondary analyses.

A total of 552 blood samples was obtained for the two studies. Cytokine levels were below the limit of detection of the assay for 3.1% of TNF measurements, 0.9% of IL-6 measurements, 1.8% of IFN-γ assessments, and 20% of IL-2 assessments. When calculating descriptive statistics, these measurements were assigned a value of 0. Because of the skewed nature of the results, a log transformation was performed on all the data on levels of TNF, IL-6 and IFN-γ for the regression analyses. For levels of cytokines below the limit of detection, a value of 0.9× the next lowest level in the data being analyzed in a particular regression model was imputed for the log transformation. Because of the high number of values of IL-2 below the limit of detection, log transformation was not done for IL-2 values and a value of 0 was used for the regression analyses. For study 1, data from participants receiving Product C were compared with results from those randomized to the corresponding placebo, while in study 2, cytokine levels from participants receiving Product A and Product B were separately compared with the cytokine data from those receiving placebo.

Nonparametric analyses were also conducted to determine if significant differences in percent change in peak cytokine concentrations from baseline could be detected by the Mann-Whitney test. In study 2, the Kruskal-Wallis test was used to determine

if significant differences were observed in percent change from baseline between all three groups.

RESULTS

CHARACTERISTICS OF STUDY PARTICIPANTS

Characteristics of the 20 participants in study 1 are summarized in Table 1, and the disposition of the participants is shown in Figure 1. All 20 enrolled individuals contributed data while receiving treatment. One participant dropped out of the study on day 3 because of issues regarding transportation, and 19 of the 20 participants completed study 1.

Sixty-seven individuals were enrolled in study 2. The characteristics of those randomized to Product A, Product B or placebo are summarized in Table 2. The mean age of those receiving placebo was significantly less than those who were randomized to either Product A or Product B (P=0.04 for both comparisons). There were no other significant differences between groups. The disposition of study participants in study 2 is shown in Figure 2. One individual who had been randomized to placebo discontinued the

Table 1: Characteristics of participants in study 1 randomized to Product C or placebo.

Characteristic	Product C (*n*=10)	Placebo (*n*=10)
Mean age, years (standard deviation)	35.5 (12.6)	37.4 (14.3)
Female	8	7
White race*	10	6
Hispanic ethnicity	0	1

*Additional race information for participants in the placebo group: Asian, 2; other race, 2.

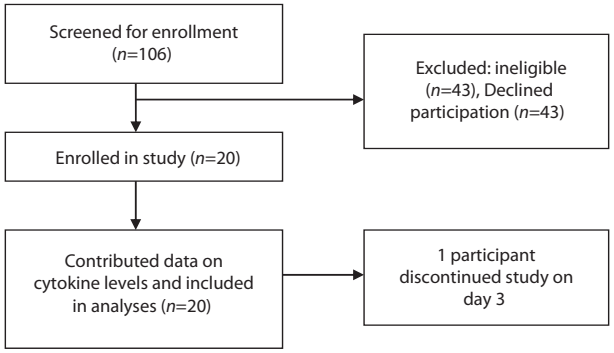

Figure 1: Disposition of participants recruited and enrolled in study 1.

Numbers of people screened, excluded and enrolled are noted, and the number of study participants who contributed to data on PHA-stimulated PBMC cytokine levels shown, as well as the number of participants who discontinued the study.

Table 2: Characteristics of participants in study 2 randomized to Product B, Product A or placebo.

Characteristic	Product A (*n*=22)	Product B (*n*=22)	Placebo (*n*=23)
Mean age, years (standard deviation)	34.1 (10.6)	35.1 (13.5)	28.2 (8.0)
Female	16	14	17
White race*	21	19	20
Hispanic ethnicity	0	1	0

*Additional race information: Product A: Asian, 1. Product B: Asian, 1; other race, 2. Placebo: African American, 2; Asian, 1.

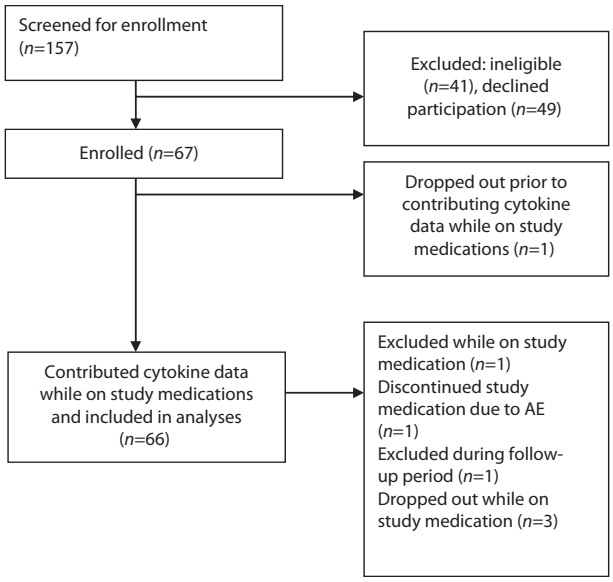

Figure 2: Disposition of participants recruited and enrolled in study 2.

Numbers of people screened, excluded and enrolled are noted, and the number of study participants who contributed to data on PHA-stimulated PBMC cytokine levels shown, as well as the number of participants who discontinued, were excluded or dropped out from the study while on study medication.

study prior to contributing data while on treatment because of discomfort related to the baseline blood draw. Three participants (one from each of the three treatment groups) dropped out during treatment: one was lost to follow-up, one dropped out because of stressful life circumstances and one discontinued the study because of discomfort from the blood draws. One participant, randomized to Product B, was excluded while on study medication for taking a commercial *Echinacea* product for cold symptoms, and another who received placebo was excluded for taking *Echinacea* after completing the treatment, but prior to the follow-up blood draws. A final individual was asked to discontinue study medication on day 2 because of a potential allergic reaction to *Echinacea* or an ingredient in the placebo formulation. The participant reported throat tingling and pruritus shortly after taking a dose of the medication. The randomization code was broken and it was determined that the patient was receiving Product A. In all participants who had at least one blood sample obtained while on study medication, but who did not complete the study, data were analyzed up until the individual dropped out or was excluded from further participation. Of the 67 participants enrolled, 60 participants completed study 2.

RESULTS OF STUDY 1

The main results for study 1 are summarized in Figure 3. There were no significant differences in baseline values between participants randomized to Product C and those receiving placebo for any of the four cytokines secreted by PHA-stimulated PBMC isolates, as determined by regression analysis. There were also no significant differences in peak levels of any measured cytokine while the participants were on the assigned study medication. As can be inferred from the data presented, there was wide variation in both baseline and peak levels of each cytokine among participants in each of the two treatment groups, with standard deviations approximately 0.5–1.6-fold higher than mean values.

Several secondary analyses were conducted on study 1 data. These included the comparison of *Echinacea* and placebo groups for each cytokine assayed – first at each individual study time point, and then over all time points in a single analysis. In a total of 32 different regression models (eight

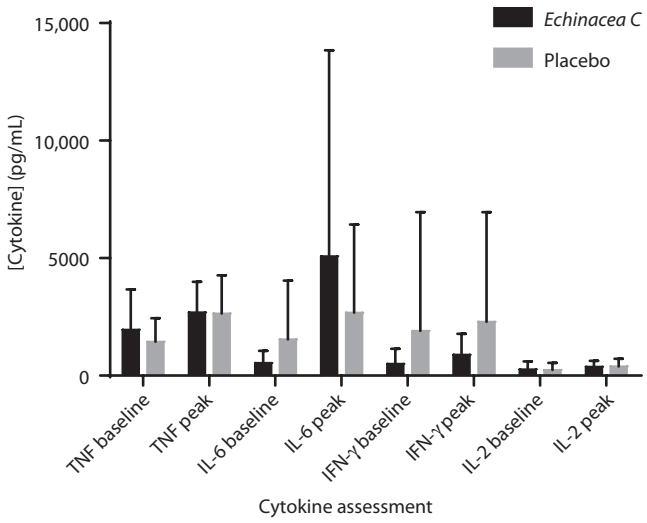

Figure 3: Baseline and peak cytokine concentrations from PHA mitogen-stimulated PBMC isolates for *Echinacea* C and placebo groups.

Blood was drawn from study participants at baseline, day 2, 3, 7, and 10 while on study treatment and PBMC samples isolated, stimulated with PHA for 24 h, and supernatants harvested and frozen at −80°C until time of cytokine concentration determination by cytometric bead array technology using flow cytometric analysis. Mean cytokine concentrations at baseline and at the time point of highest level (peak)+standard deviation in pg/mL are shown. *P*-values for differences between the *Echinacea* C group and placebo group were computed using regression analysis with log transformation of cytokine concentrations and were all above 0.05, ranging from 0.18 (for [IL-6] at baseline) to 0.87.

analyses for each of the four cytokines), no statistically significant differences in cytokine levels between participants randomized to Product C vs. placebo were observed; all *P*-values were >0.20.

RESULTS OF STUDY 2

Analysis of results for baseline and peak levels of each cytokine from PHA-stimulated PBMC isolates in each of the three treatment groups is shown in Figure 4. No significant differences in the baseline levels of any cytokine between those in the placebo group and either of the active medication groups were observed. The peak levels of each cytokine were not significantly different between those who received either Product A or Product B and those who received placebo. As with the data in study 1, the measured levels of the cytokines varied widely among study participants, with standard deviations that were approximately 0.71–3 fold higher than the corresponding mean value.

The same secondary analyses were conducted using study 2 data as were done in study 1. The levels of IL-2 secreted by PHA-stimulated PBMC were significantly lower on day 7 in participants receiving Product A than in placebo recipients (31±32 pg/mL and 46±70 pg/mL, respectively, *P*=0.039). For this analysis, levels of IL-2 were below the level of detection in stimulated PBMC isolates from 14/60 participants (23%). There were no other significant differences found in any of these analyses in the levels of PBMC-secreted cytokines in participants randomized to either Product A or Product B vs. those who received placebo; the *P*-value for all other comparisons was >0.10. Because of the differences in the age of participants randomized to the three treatment groups, all of the analyses, both on the primary and secondary outcomes, were repeated after including age as a variable in the regression models. There were no statistically significant differences found in any analysis. However, after controlling for age, the difference in mean levels of IL-6 secreted by PHA-stimulated PBMC isolates from day 3 of the study tended to be higher in those receiving Product A than in placebo recipients (14,296±37,861 pg/mL and 6188±26,265 pg/mL, respectively, *P*=0.064). In addition, after adjusting for age, levels of PHA-stimulated PBMC-secreted IL-2 from day 2 isolates tended to be higher in participants randomized to Product A than in those receiving placebo (63±82 pg/mL and 44±39 pg/mL, respectively, *P*=0.063) and lower in those receiving

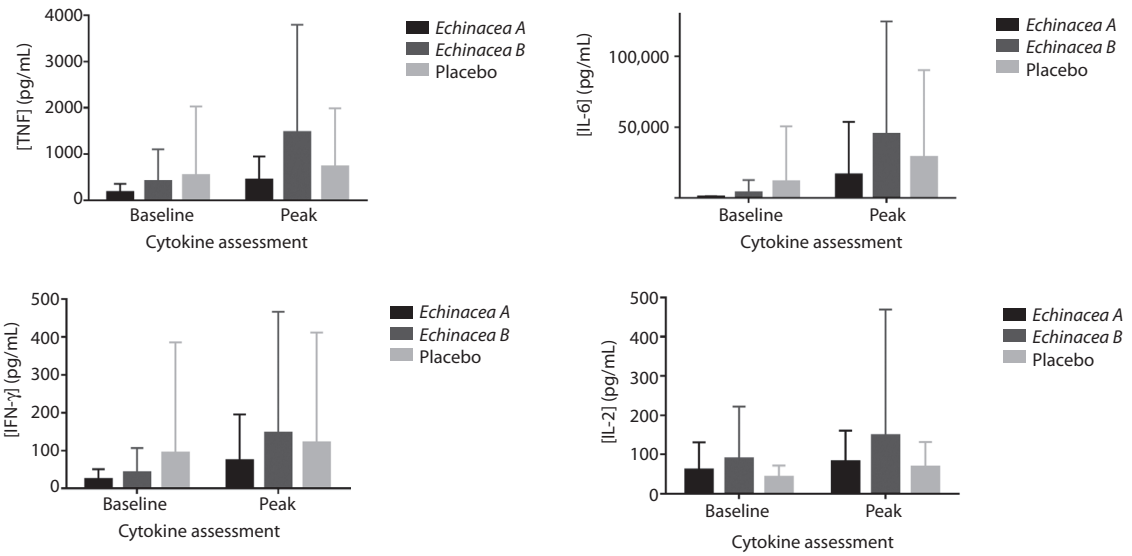

Figure 4: Baseline and peak cytokine concentrations from PHA mitogen-stimulated PBMC isolates for *Echinacea* A, *Echinacea* B and placebo groups.

Blood was drawn from study participants at baseline, day 2, 3, 7, and 10 while on study treatment and PBMC samples isolated, stimulated with PHA for 24 h and supernatants harvested and frozen at −80°C until time of cytokine concentration determination using the Luminex immunoassay (Luminex Corporation, Austin, TX). Mean cytokine concentrations at baseline and at the time point of highest level (peak)+standard deviation in pg/mL are shown. *P*-values for differences between the *Echinacea* A group and placebo, and between the *Echinacea* B group and placebo, were computed separately using regression analysis with log transformation of cytokine concentrations and were above 0.05, ranging from 0.23 to 0.98.

Product A than in those randomized to placebo on day 7 (*P*=0.070).

Ad hoc analyses conducted on both study 1 and 2 data sets also revealed no significant differences in percent change in peak cytokine concentrations from baseline between the two groups in study 1, nor between the three groups in study 2.

ADVERSE EVENTS

With the use of the 76-item MOSES checklist as a surveillance method to detect AE, side effects were frequently reported by study participants. In study 1, 65% of participants reported at least one minor side effect during the 30-day study period, including 7/10 of those receiving Product C and 6/10 placebo recipients (*P*=0.64).

At least one side effect was reported by 63.6% of participants randomized to Product A in study 2, by 81.8% of those receiving Product B, and by 72.7% of the study 2 placebo recipients (*P*-values comparing each active drug to placebo=0.52 and 0.47, respectively). Reported AEs ranged from minor to moderate and self-resolved, and no serious adverse events occurred. There were no differences in the rates of any side effect among those in any of the treatment groups, so none of the *Echinacea* treatments increased AEs compared with placebo.

DISCUSSION

No significant effect of *E. purpurea* on the primary outcome measure of peak levels of pro-inflammatory cytokines induced in PHA-stimulated PBMC isolates *ex vivo* were observed in the studies conducted. There are several possible explanations for the lack of detection of an enhancing effect of the *Echinacea* extracts tested on mitogen-induced PBMC cytokine responses. First, the *Echinacea* formulations tested at the dosages given may have no significant effect on mitogen-induced PBMC cytokine responses. Each of the products tested contained measurable levels of the polysaccharides that are thought to be important in stimulating cytokine production.[11,17,21,22] However, other *Echinacea* products extracted differently to give different combinations or amounts of reportedly active polysaccharide and alkylamide constituents, or these

same non-alcoholic extracts given at higher doses, may have had detectable enhancing effects on pro-inflammatory cytokine responses.

Secondly, because of the wide variation in measured cytokine levels, larger sample sizes might be required to detect differences in the outcome measured between participants receiving an *Echinacea* formulation or placebo. For example, based on the standard deviations in peak levels of TNF we found in study 2, a sample size of approximately 150 participants would be required to have a power of 0.9 to detect a 1000 pg/mL difference in peak levels of TNF among those receiving *Echinacea* versus placebo recipients. In addition to high variability in cytokine levels, a potentially confounding effect was that the placebo group in study 2 was a younger cohort than those in the *Echinacea* treatment groups. When data for specific collection days were age-adjusted, a trend toward significant increases in IL-6 and IL-2 concentration from day 3 PBMC isolates from the *Echinacea* A treatment group was observed. This may have simply been a multiple test artifact. Alternatively, it is possible that the younger placebo recipients in study 2 had more robust general immune responses and higher pro-inflammatory cytokine levels that could have been a confounding factor that was corrected for by adjusting for age.

Another plausible explanation for the lack of detectable effect on cytokine responses in adults taking *Echinacea* is that the exact mechanism of action of the botanical medicine in preventing URIs is unknown. The cytokines we assayed were chosen because they are important in the activation of the innate immune response and thus, theoretically, important in preventing infection.[30] However, the effect of *Echinacea* in preventing URIs may be mediated through a different pathway. Finally, all of our testing was conducted in healthy volunteers; the results may have been different if the participants had been "challenged" by a respiratory virus.

The *ex vivo* cytokine response results of this study contrast with findings of *in vitro* assessments of the pro-inflammatory effects of *Echinacea*, which are suggested to be mediated, at least in part, by plant polysaccharides.[12,17] As early as 1989, Luettig *et al.* demonstrated that polysaccharides from *E. purpurea* stimulated production of TNF in mouse macrophages. Subsequent studies, testing a variety of *Echinacea* constituents or whole preparations, have reported that *Echinacea* extract or *Echinacea*-derived polysaccharides induced stimulation of multiple pro-inflammatory cytokines *in vitro*.[17,23,31,32] Published evidence suggests the possibility that liver-mediated metabolic actions on *Echinacea* alkylamide constituents weakens their immune modulating effects.[19] Thus, *in vitro* actions of *Echinacea* extracts may not occur, or are weaker and more difficult to detect *in vivo*.

Even so, evidence in animal models of *in vivo* macrophage-stimulating activity of orally administered *E. purpurea* extract, as well as both alkylamide and polysaccharide isolates, does exist.[14,22,33] Further, in a study in human volunteers, Roseler *et al.*, reported that *Echinacea* polysaccharides given intravenously led to *in vivo* evidence of phagocyte activation and increased serum CRP levels.[23] Given these positive *in vivo* study results, our findings of no significant *ex vivo* cytokine response enhancing effects of orally administered *Echinacea* extracts suggest the possibility that enhancing effects of *Echinacea* on pro-inflammatory cytokines may be inactivated with oral administration in humans. Alternatively, the formulations we assessed may have stimulated *in vivo* production of one or more of the cytokines measured, but this effect may have been modified or not detectable by *ex vivo* measurement of cytokine responses. More sensitive and less variable biomarkers of inflammatory response may be needed to detect *Echinacea*-induced immune activity upon oral administration. These could include serum measurements of acute phase proteins such as C-reactive protein (CRP), or gene expression analysis to assess changes in cytokine expression patterns at the RNA level in different immune cell subsets.

With regard to analyzing levels of acute phase proteins in serum, we conducted a *post hoc* analysis in which levels of the inflammatory marker highly sensitive C-reactive protein (hsCRP) were assessed in serum samples collected during study 2. Change in hsCRP levels from baseline to

each treatment time point was compared among individuals receiving placebo or either Product A or Product B. With product B, no significant differences in change from baseline compared with placebo for any study day were observed. However, on day 10, the change from baseline was significantly greater (P=0.02) in study participants randomized to Product A than in placebo recipients. This preliminary result indicates that certain *Echinacea* extracts may induce an inflammatory response that is detectable using a more sensitive and less variable marker of inflammation such as hsCRP in serum samples as compared with the *ex vivo* analysis of cytokines from mitogen-stimulated PBMC isolates.

The main findings of this study are consistent with other recent clinical trials in which no efficacy of *Echinacea* in preventing or treating URIs has been found.[1,2,4,8] However, given its previously demonstrated *in vitro* and *in vivo* immune-modulating activity, further research on *Echinacea* using more sensitive and less variable outcome measures may better address the question of whether specific *Echinacea* extracts taken orally have bioactivity that could help to prevent or resolve URIs. This trial aimed at detecting evidence of biologic activity by assessing a hypothesized mechanism of action resulting from the pharmacologic properties of polysaccharides, which constitute one type of many different constituents in *E. purpurea.* Whether activity occurs in another immunologic pathway due to effects of a different constituent or group of constituents, or using higher doses of the preparations than assessed, is unknown.

Research on the bioactivity of *Echinacea* constituents is now known to be more complicated than earlier realized. Notably, recent evidence suggests that endophytic bacteria are associated with *Echinacea* plant material and that these bacterially-derived lipoproteins and lipopolysaccharides contribute to the immune-enhancing activity of *Echinacea* extracts *in vitro* and may account for variability in *Echinacea*-induced immunological effects.[34] Extracts from other plant species also contain macrophage-activating polysaccharides and lipoproteins from endophytic bacteria,[35] and recent studies report the presence of fungal endophytes with biological activity, that were isolated from two different plant species.[36,37] These results suggest that variability in macrophage-activating actions of natural product formulations may be due, at least in part, to the presence of varying levels of endophytic bacterial- and/or fungal-derived constituents. Determining whether variability in endophytic bacterial and fungal content in botanical extracts influences biological activity *in vivo* has become an important focus of study in the botanical medicine field.

Given these complexities, future research on *Echinacea* might best be initially directed toward identifying *in vivo* activity of a specific, adequately characterized, formulation. The results of this study suggest that the non-alcoholic, polysaccharide-containing *Echinacea* extracts tested do not significantly enhance pro-inflammatory cytokines secreted by mitogen-stimulated PBMC isolates from healthy individuals. The use of newer techniques, such as more sensitive and less variable hsCRP serum testing or potentially more informative gene expression analysis to detect the "biologic signature" of *Echinacea* should be explored.

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DISCLOSURE OF INTERESTS

The authors have no other interests to disclose.

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