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Cytokine- and Interferon-Modulating Properties of *Echinacea* spp. Root Tinctures Stored at −20°C for 2 Years

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Abstract

Echinacea spp. phytomedicines are popular for treating upper respiratory infections. The purpose of this investigation was to examine the immunomodulatory properties of *Echinacea* tinctures from seven species after being stored at −20°C for 2 years. Two experimental techniques were employed using human peripheral blood mononuclear cells (PBMC). In the first set of experiments, PBMCs were stimulated *in vitro* with tinctures alone and assayed for proliferation and production of interleukin-10 (IL-10), IL-12, and tumor necrosis factor-α (TNF-α). In the second set of experiments, subjects were immunized with influenza vaccine. PBMCs from vaccinated individuals were stimulated *in vitro* with *Echinacea* tinctures and influenza virus; cytokine production (IL-2, IL-10, and interferon-γ [IFN-γ]) was compared prevaccination and postvaccination. In the first experiments, (1) tinctures from *E. angustifolia*, *E. pallida*, *E. paradoxa*, and *E. tennesseensis* stimulated proliferation and tended to increase IL-10, (2) *E. sanguinea* and *E. simulata* stimulated only proliferation, (3) *E. purpurea* stimulated only IL-10, and (4) none of the extracts influenced IL-12 or TNF-α. In the second experiments, (1) tinctures from *E. pallida*, *E. paradoxa*, *E. sanguinea*, and *E. simulata* diminished influenza-specific IL-2, and (2) none of the extracts influenced influenza-specific IL-10 or IFN-γ. For *in vitro* models using *Echinacea*, immune response may vary based on stimulus (*Echinacea* alone vs. *Echinacea* + recall stimulation with virus).

INTRODUCTION

Herbal remedies (phytomedicines) and supplements made from plants of the genus *Echinacea* are becoming increasingly popular.[1] Known in vernacular as coneflowers, this group of plants has been used to treat upper respiratory infections caused by rhinoviruses or other cold viruses or influenza viruses.[1,2] Other medicinal benefits have been attributed to *Echinacea*, including anti-inflammatory[3,4] and wound-healing[5] properties.

The scientific literature regarding the efficacy of *Echinacea* in the context of upper respiratory infection is conflicting, some supporting[6,7] and others refuting[8,9] its efficacy. Discrepancies among scientific reports may be attributable to many factors. Studies differ in the plant species used, type of extract made (including commercially prepared vs. laboratory prepared), and precise method of extraction. Three species (*E. angustifolia*, *E. pallida*, and *E. purpurea*) are used most widely[1]; however, it has been demonstrated that several other species in the genus also harbor medicinal activities.[10,11] Differences in subject demographics (e.g., age) and experimental protocols may also yield different results.

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Although variables, such as species used, type of extract, method of extraction, and subject demographics, are frequently detailed in scientific reports, other unreported factors may also explain inconsistencies between studies. One such factor may be extract storage conditions. For example, rarely do scientific reports state at what temperature or for how long their *Echinacea* extracts are stored before or during use. In a previous publication,[10] we prepared *Echinacea* root tinctures and infusions using methods similar to those employed by lay herbalists and demonstrated that these extracts, when stored for 4 days at 4°C, changed in their immunomodulatory properties.

In the present investigation, we tested aliquots from the same stock tinctures[10] after 2 years storage at −20°C for their abilities to modulate peripheral blood mononuclear cell (PBMC) proliferation and cytokine production. To our knowledge, no study has yet documented the effects of such storage on changes in *Echinacea* extract immunomodulatory activity, yet research laboratories typically freeze and reuse extracts over months or years. We expected that these extracts would show diminished immunomodulatory properties after storage compared with when they were tested fresh but that these properties might be altered when an antigen (influenza virus) was cocultured with the cells of immunized donors. Immune parameters were selected according to their potential importance in the immune response to viral infection. The proliferation of lymphocytes in response to pathogen is an important component of the adaptive immune response, as expansions of such lymphocytes as T cells is essential to combat infection. Tumor necrosis factor-α (TNF-α) is a cytokine that promotes an inflammatory response, which in the context of viral infection may be deleterious to the pathogen. However, excessive or prolonged levels of TNF-α may contribute to certain disease states, such as the pathology of influenza infection[12] or chronic inflammatory conditions.[13–15] In contrast to TNF-α, interleukin-10 (IL-10) is associated with anti-inflammatory activities. During viral infection, upregulation of IL-10 may lead to faster quelling of the inflammatory response, in turn leading to faster symptoms resolution.[16] IL-12 has the ability to stimulate $CD8^+$ T cell (cytotoxic T cell) responses; as $CD8^+$ T cells can directly attack virally infected cells, augmentation of IL-12 may lead to enhanced viral clearance. IL-2 is relevant in the context of viral infection, as it enhances T helper (Th) cell function and is thus a potential key player in the adaptive immune response. Finally, unique among the cytokines in this paper, interferon-γ (IFN-γ) has direct antiviral activity and is also important in activating the cytotoxic lymphocyte response.

MATERIALS AND METHODS

Plant material, extraction procedure, and extract storage conditions

Three-year-old plant specimens grown from seed in outdoor fields were obtained from the USDA North Central Regional Plant Introduction Station (Ames, IA) in October 2003. Seven species were harvested: *E. angustifolia* var. *angustifolia* Ames 24994 (ANG), *E. pallida* PI 631256 (PAL), *E. paradoxa* var. *paradoxa* PI 633664 (PAR), *E. purpurea* (unknown parentage) (PUR), *E. sanguinea* Ames 23879 (SAN), *E. simulata* PI 631251 (SIM), and *E. tennesseensis* PI 631250 (TEN). Voucher specimens for each plant used have been filed in the Ada Hayden Herbarium, Iowa State University (Ames, IA: ISC 435974–435980). Seven separate tinctures were made for each species by dicing root material and combining it with a 50% ethanol/50% water solvent at a ratio of 1 part plant/9 parts solvent within 2.5 h after harvest and incubating for 20 min at ambient temperature before filtering to remove particulate matter.[10] Tinctures were stored in scintillation vials containing ambient air and remained undisturbed at −20°C for 24 months. Phytochemical profiling of these species has been published elsewhere.[17] Briefly, ANG, PUR, and SAN had the highest concentration and greatest diversity of amides, PAL and TEN had few amides, and no amides were detected from PAR and SIM. Ketones were present in PAL, PAR, and SIM but were absent in others; and caffeic

acid derivatives were represented in all species, although molecules and quantities varied by species. Extracts were diluted 1:12.5 in AIM-V medium (GIBCO/Invitrogen, Carlsbad, CA) immediately prior to use.

Subject recruitment and immunization protocol

Human subject protocols were filed and approved by the Institutional Review Board for Human Subjects. Our inclusion criteria were that subjects had to be healthy and aged between 19 and 36 years. Our exclusion criteria were such that subjects could not be allergic to eggs (i.e., they would be capable of receiving the standard influenza vaccine), be on immunosuppressive medications or other medical or dietary supplements that might alter immune response, have had surgery in the past 12 months, or had cancer in the previous 5 years. After screening for inclusion and exclusion criteria, 20 subjects participated in the first experiments not involving immunization, and 21 subjects participated in the second experiments using influenza antigen as the immune stimulus. Twelve subjects participated in both studies.

In fall 2005, 21 subjects between the ages of 19 and 36 were immunized with the 2005/2006 trivalent influenza Fluzone vaccine (A/New Caledonia/20/99 H1N1 [**A1**]; A/New York/55/04 H3N2 [**A2**]; B/Jiangsu/10/03) (Aventis Pasteur, Swiftwater, PA) within 8 h after a preimmunization blood draw. A second blood draw was performed at 4 weeks postimmunization. One subject withdrew from the study, and one subject had insufficient PBMC counts, resulting in a count of 19 subjects.

PBMC isolation

PBMCs were isolated using Ficoll-Paque plus (Amersham Pharmacopia Biotech, Piscataway, NJ) gradient centrifugation. Cell counts were performed with a hemacytometer using try-pan blue to assess viability; all cell suspensions were adjusted to 2.0×10^6 cells/mL in AIM-V medium.

Cell culture for cell proliferation

In the *Echinacea* alone model, 100 μL AIM-V medium containing 2.0×10^6 cells were plated per well in 96-well flat-bottomed Costar plates (Cambridge, MA). Cells were stimulated with 5 μL *Echinacea* preparations diluted as described or AIM-V medium (control, CON). All trials were conducted in triplicate. Cells were incubated for 5 days at 37°C, 5.0% CO_2 in a humidified atmosphere, and proliferation was assessed via CellTiter (Promega, Madison, WI) and read in a Bio-Rad plate reader (Hercules, CA). Previous data from our laboratory indicated that cells incubated for 5 days did not show reduced viability compared with cells incubated for 3 or 4 days. Our sample size for proliferation was $n = 15$.

Cell culture for cytokine production

In the *Echinacea* alone model, 1 mL AIM-V medium containing 2.0×10^6 cells was plated per well in 24-well Costar tissue culture plates. Fifty microliters of one of the *Echinacea* preparations diluted 1:12.5 was added per treatment well; the control well received 50 μL AIM-V medium (CON). This extract concentration was determined from preliminary studies of human PBMC ethanol tolerability (data not shown). Cell cultures were incubated for 24 h at 37°C, 5.0% CO_2 in a humidified atmosphere. Supernatants were harvested and stored at −20° C until used in cytokine quantification assays for IL-10, TNF-α, and IL-12 via ELISA (BD Biosciences Pharmingen, San Diego, CA). Because of low PBMC counts, our sample size for IL-10 and TNF-α was n = 14 and for IL-12 was $n = 17$.

In the *Echinacea* + virus model, 1 mL AIM-V medium containing 2.0×10^6 cells was plated per well in 24-well Costar tissue culture plates. Fifty microliters of one of the *Echinacea*

preparations diluted 1:12.5 was added per treatment well. All treatment wells received 50 μL of a 10 hemagglutination units (HAU)/mL solution containing the same type A H1N1 virus (A/New Caledonia/20/99) as in the vaccine. Two control wells were designated, one received 100 μL AIM-v medium (CON), and the other received 50 μL AIM-V medium and 50 μL virus (VIR). Cell cultures for IL-2 and IFN-γ were incubated for 48 h at 37°C, 5.0% CO_2 in a humidified atmosphere. Cell cultures for IL-10 were incubated at both 24 h and 48 h under the same conditions. Supernatants were harvested and stored at −20°C until used in cytokine quantification assays for IL-2, IL-10, and IFN-γ via ELISA (BD Biosciences Pharmingen). Due to experimental complications during testing of the extracts, our sample size for IFN-γ was reduced to $n = 8$; for all other cytokines, the full $n = 19$ was used. Control experiments indicated that for all assays, wells receiving no virus produced less cytokine than wells receiving virus alone (all $p \leq 0.01$).

Statistical analyses

For the *Echinacea* alone model, a one-way ANOVA was used to compare the effects of the different species of *Echinacea* on cytokine production and cell proliferation. *Post hoc* tests were performed when a significant effect of treatment was found. For the *Echinacea* + virus model, a general linear model of repeated measures was used to test for main effects of time, treatment, and treatment X time interactions (SPSS, Chicago, IL). When significant effects were discovered, follow-up *post hoc* tests (LSD) were performed.

Endotoxin determination

Glassware used in the extract preparation was baked at 180°C for 16 h prior to use to minimize endotoxin contamination. Sterile water was obtained from Hospira, Inc. (Lake Forest, IL) and used for all tinctures. Endotoxin levels were determined from both sterile water (0 experimental units [EU]/mL), and all stock tinctures were determined using Bio-Whitaker QCL 1000 kits (Cambridge, MA).

Extract physiochemical profiling

We wished to document changes in biochemical composition of extracts over a similar period to see if changes in immune results may be attributable to changes in composition. However, our original report[10] did not include phytochemical profiling, and we, therefore, had to use a second set of extracts by proxy. For each *Echinacea* species except *E. paradoxa*, 6 g was extracted with 95% ethanol for 6 h using a Soxhlet apparatus. After extraction, the ethanol was roto-evaporated at 30°C to obtain the dry residue. The residues were redissolved in 10 mL DMSO and stored at −20°C until HPLC analysis. Extracts were profiled shortly after generation and again after 20 months of uninterrupted storage at −20°C.

Regent-grade ethanol (100%) was purchased from Chemistry Stores, Iowa State University. HPLC-grade acetonitrile and certificated-grade DMSO were purchased from Fisher Scientific (Fair Lawn, NJ). Milli-Q water prepared by Milli-Q system (Millipore Co., Bedford, MA) was used to prepare all the mobile phase for HPLC analysis.

Four *Echinacea* alkamides, undeca-2*Z*, 4*E*-diene-8,10-diynoic acid isobutylamide (alkamide 2), dodeca-2*E*, 4*E*, 8*Z*-trienoic acid isobutylamide (alkamide 10), dodeca-2*E*-ene-8,10-diynoic acid isobutylamide (alkamide 14), and 9-hydroxytrideca-2*E*-ene-10, 12-diynoic acid isobutylamide, synthesized by Dr. Kraus' group (Department of Chemistry, Iowa State University), were used as the external standards for *Echinacea* alkamide HPLC gradient.[18–20] The stock solutions of all the standards were stored under nitrogen at −80°C.

The *Echinacea* extracts were analyzed by HPLC, which consisted of a Beckman System Gold 126 solvent module, a Beckman model 508 autosampler, a Beckman model 168 detector

(Beckman Coulter, Inc., Fullerton, CA) and an RP-C_{18}, 5 μm, 250 × 10 mm i.d. YMC-ODC-AM-303 column (YMC, Inc., Wilmington, NC). All *Echinacea* extracts were filtered through 0.45-μm polytetrafluoroethylene filters (Alltech Associates Inc., Deerfield, IL) before being injected into the HPLC.

A modification of the Bauer HPLC method[21] of alkamide analyses formed the *Echinacea* alkamide gradient. The two mobile phases for the alkamide gradient were (A) degassed Milli-Q water and (B) acetonitrile. A linear gradient of increasing 40% B to 80% B was developed within 45 min at a flow rate of 1.0 mL/min with UV detection from 200 to 600 nm with a Beckman 32 Karat software (version 5.0) and UV profiles at 210 nm and 260 nm for alkamides. The injection volume was 15 μL. Alkamides were identified by comparison of relative retention times and UV absorbance patterns using the procedures in references 21 and 22 and authentic standards.

RESULTS

Extracts from seven *Echinacea* species were tested in two different *in vitro* models using PBMCs from young adults. The first set of experiments used the tinctures themselves as the sole cell stimulus (*Echinacea* alone model). The second set of experiments compared the effects of *Echinacea* tinctures when used in conjunction with influenza virus (antigen) in vaccinated individuals (*Echinacea* + virus model). We chose to use two different experimental designs, as some researchers have demonstrated *in vitro* immune outcomes may yield contrasting results contingent on mitogen/antigen differences.[23]

Endotoxin levels

Endotoxin levels were determined from all extracts previously and are as follows (in EU/mL): ANG (0.088), PAL (0.031), PAR (0.116), PUR (0.395), SAN (0.03), SIM (0), and TEN (0.108). All extracts were diluted following a procedure outlined in Materials and Methods, with final endotoxin concentrations in the wells (expressed as EU/mL) being ANG (0.0003), PAL (0.0001), PAR (0.0004), PUR (0.0014), SAN (0.0001), SIM (0), and TEN (0.0004). We have shown experimentally[17] that these low levels of endotoxin do not influence our immune outcomes. These results suggest that the effects observed in our assays are caused by *Echinacea* extracts themselves rather than possible contaminating bacterial endotoxin.

PBMC proliferation at 5 days in the *Echinacea* alone model

Results for PBMC proliferation following 5 days of incubation are shown in Figure 1. A main effect of treatment was observed ($p = 0.003$). Wells treated with ANG ($p < 0.001$), PAL ($p < 0.001$), PAR ($p < 0.001$), SAN ($p = 0.036$), SIM ($p < 0.001$), and TEN ($p < 0.001$) showed a significant increase in PBMC proliferation compared with control (CON).

Cytokine production in *Echinacea* alone model

Table 1 displays our results for TNF-α and IL-12. No main effect of treatment was observed ($p = 0.164$ and $p = 0.142$, respectively). IL-10 production following 24 h incubation is shown in Figure 2. Results indicate a trend toward a significant effect of treatment ($p = 0.085$) such that *Echinacea* treatment may enhance IL-10 production.

Cytokine production in *Echinacea* + virus model

Results for IL-2 production after 48 h of culture are presented in Figure 3. There was a main effect of treatment ($p = 0.029$), such that *Echinacea* tended to cause a decrease in IL-2 production. Wells treated with PAR ($p = 0.012$) and SAN ($p = 0.044$) demonstrated significantly decreased IL-2 production compared with wells receiving virus alone, whereas

wells treated with PAL ($p = 0.084$) and SIM ($p = 0.08$) showed a trend in this direction. No time or treatment X time interaction was found. It is possible that IL-2 production peaked at an earlier time point; hence, by 48 h, secreted IL-2 may have already been taken up by target cells so that the overall amount detected in supernatants appears to be lower.

Production of IL-10 after 24 h and 48 h of culture is shown in Table 2. There was a main effect of time ($p < 0.001$) such that subjects produced more IL-10 at the postvaccination time point compared with prevaccination time point in both the 24-h and 48-h cultures. There were no main effects of treatment or any treatment X time interactions for either incubation time.

Results for IFN-γ production after 24 h culture are shown in Table 2. Subjects produced more IFN-γ postvaccination than prevaccination indicating a main effect of time ($p = 0.005$). There was no main effect of treatment or treatment X time interaction.

Phytochemical profiling

Results of our alkamide analysis are shown in Figure 4, Figure 5, Figure 6, Figure 7, Figure 8, and Figure 9. The HPLC chromatograms for *E. pallida* (Fig. 5) and *E. sanguinea* (Fig. 7) show almost no change in peak heights for the major alkamide constituents. The chromatograms for *E. angustifolia* (Fig. 4), *E. purpurea* (Fig. 6), *E. simulata* (Fig. 8), and *E. tennesseensis* (Fig. 9) show peaks slightly higher in the 2-year-old extracts compared with the fresh extracts, although the pattern of peaks remains unchanged.

DISCUSSION

PBMC proliferation in *Echinacea* alone model

All *Echinacea* tinctures except those from *E. purpurea* enhanced PBMC proliferation at 5 days in the *Echinacea* alone model (Fig. 1). The tincture from *E. purpurea* produced a similar mean level of proliferation as *E. angustifolia* and *E. sanguinea*, but the standard deviation was larger, leading to a lack of significance. Data on the effects of *Echinacea* on PBMC proliferation or proliferation of specific lymphocyte subpopulations are conflicting. Some studies have demonstrated minimal impact by *Echinacea* supplementation,[24,25] whereas others have shown proliferation-enhancing effects by *Echinacea* polysaccharides.[26,27] It is possible that *Echinacea* may impact PBMC proliferation indirectly; for example, *Echinacea* may stimulate macrophages, which in turn drive proliferation of lymphocytes, such as T cells.[28]

Cytokine production in *Echinacea* alone model

None of the 2-year-old extracts modulated TNF-α production (Table 1). The majority of studies examining the effects of *Echinacea* on TNF-α production have shown a stimulatory effect in both human and rodent models.[10,26,29–31] Transiently elevated TNF-α levels, which may be achieved by short-term *Echinacea* supplementation, may be beneficial in combating viral infections but may be deleterious if a chronic inflammatory disease is present. *Echinacea* alkylamides[31,32] and polysaccharides[26,33] are known to influence TNF-α production.

Regarding IL-12, none of the 2-year-old extracts modulated production (Table 1). Few reports have documented a stimulatory effect of *Echinacea* preparations on IL-12,[10,30] and one study showed it had little effect.[34] The biochemical fraction responsible for this activity is unknown. The limited data thus suggest that *Echinacea* holds only modest abilities to influence IL-12 production.

The augmentation of IL-10 observed here (Fig. 2) is so low that it may not be of physiologic relevance. Using these same extracts tested fresh, we previously found that only *E. angustifolia* and *E. purpurea* augmented IL-10.[10] Several publications have reported a

stimulatory effect of *Echinacea* spp. extracts on IL-10 production.[10,29,30] The accumulated data imply that most *Echinacea* extracts harbor the ability to augment IL-10 production, which may lead to a faster resolution of viral infection symptoms. It is unknown which *Echinacea* constituents may be responsible for modulating IL-10 production.

Cytokine production in *Echinacea* + virus model

Extracts from *E. paradoxa* and *E. sanguinea* and, to a lesser extent, *E. pallida* and *E. simulata*, diminished IL-2 production (Fig. 3). In our prior investigation using the same tinctures fresh,[10] only *E. angustifolia* influenced IL-2. *Echinacea* products have been shown to increase[35,36] or have no effect[26,37] on IL-2 levels in a variety of human and rodent models; however, our laboratory is the only one that has used an antigen component. Thus, the effect of *Echinacea* on IL-2 is currently ambiguous, possibly attributable to differences in experimental methods between studies.

Interestingly, when virus was not employed during the 24-h culture (Table 2), *E. angustifolia*, *E. pallida*, *E. paradoxa*, *E. purpurea*, and *E. tennesseensis* all slightly increased IL-10 synthesis, but again these increases are of dubious physiologic relevance. Therefore, a comparison of Figure 2 and Table 2 suggests that *Echinacea*'s effects are not contingent on the presence of virus. In the context of viral infection, our results seem to suggest that the effects of *Echinacea* on IL-10 may be unrelated to the presence of the virus in the host.

Echinacea did not seem to increase production of IFN-γ in this study (Table 2). Extracts from *Echinacea* have been shown to increase IFN-γ synthesis[27,36,38,39] in most models, although some teams found no effect of *Echinacea* on IFN-γ.[26,37] In a separate investigation, *Echinacea* stimulation increased gene expression of IFN-α, also antiviral.[40] One *in vivo* model showed oral supplementation with *E. purpurea* increased circulating IFN-γ titers.[34] Taken together, these results suggest that *Echinacea* extracts may be able to augment IFN-γ production, which may directly reduce viral load in the host during infection, although the enhancement is general rather than specific to antigen.

Effects of 2 years' storage at −20°C

We observed that alkamide concentrations of *Echinacea* extracts followed one of two patterns after 2 years' storage at −20°C. Alkamide concentrations appeared to increase with storage in *E. purpurea*, *E. simulata*, and *E. tennesseensis*, whereas no changes were seen in *E. angustifolia*, *E. pallida*, or *E. sanguinea*. All extracts were tightly sealed and stored similarly, but solvent evaporation may explain these results. The pattern of peaks remains almost the same. Other researchers also have observed changes in alkamide concentrations of *Echinacea* extracts stored at freezing temperatures. One study found that alkamide concentrations did not change with 7 months of storage at −20°C.[41] Another team found that alkamide levels declined after approximately 1 year with storage at −18°C.[42] These three studies thus all report different effects of cold storage on *Echinacea* alkamide concentrations.

The results presented here (Fig. 1 and Fig. 2 and Table 1) allow for a comparison between the same extracts tested under the same assays fresh[10] and after 2 years' storage at −20°C. Using the original data from this study and reference 10, we performed a GLM analysis in SAS with a least squares means analysis as a follow-up to statistically compare results between the two time points. It should be noted that we had a larger pool of subjects in the present study than in the previous investigation. Because different subjects and different sample sizes were used between the two studies, it is most accurate to compare mean fold changes in immune outcomes (relative to control) across time points instead of absolute values.

We observed changes in both proliferation and cytokine production capacities between time points. When tested fresh, none of the extracts enhanced proliferation[10]; in this study, all but *E. purpurea* extract enhanced proliferation (Fig. 1). There were significant time, treatment, and treatment X time interactions between the two studies (all $p \leq 0.029$), suggesting that proliferation was enhanced by the 2-year-old extracts moreso than the fresh extracts. Mean fold changes in proliferation were not much different for extracts from *E. angustifolia*, *E. purpurea*, and *E. sanguinea* across studies; however, extracts from both *E. pallida* and *E. tennesseensis* exhibited a greater capacity to increase proliferation after 2 years' storage (change in proliferation was 1.1-fold increase fresh vs. 1.7-fold increase after 2 years for *E. pallida* extract, $p < 0.001$; 1.1-fold increase fresh vs. 1.6-fold increase after 2 years for *E. tennesseensis* extract, $p = 0.014$).

In contrast, cytokine production was stimulated to a greater extent with fresh extracts than with extracts stored for 2 years. When tested fresh, *E. angustifolia* and *E. purpurea* extracts were able to increase TNF-α synthesis[10]; in this study, none of the extracts increased production of this cytokine (Table 1). Significant time, treatment, and treatment X time interactions were found for TNF (all $p < 0.001$). Mean fold stimulation of TNF by the *E. sanguinea* and *E. tennesseensis* extracts was not much different between the two studies, but it was much greater when the extracts were tested fresh vs. after 2 years' storage for *E. angustifolia* (10.5-fold increase fresh vs. 1.3-fold increase stored), *E. pallida* (10.5-fold increase fresh vs. 1.1-fold increase stored), and *E. purpurea* (20-fold increase fresh vs. 1.2-fold increase stored; all comparisons $p < 0.001$).

E. angustifolia extract was the only extract able to augment IL-12 production when tested fresh[10]; none of the extracts significantly enhanced IL-12 in this study (Table 1). Significant time, treatment, and treatment X time interactions were found for TNF (all $p < 0.03$). *E. angustifolia* extract demonstrated the most notable difference in IL-12 production across studies (3.5-fold increase fresh vs. 1-fold increase stored, $p < 0.001$), but *E. sanguinea* demonstrated a similar pattern (1.5-fold increase fresh vs. 0.9-fold increase stored, $p = 0.048$).

When tested fresh, only *E. angustifolia* and *E. purpurea* enhanced IL-10 production[10]; in this study, five of the seven species statistically increased IL-10 production (Fig. 2). Significant time and treatment X time interactions were found (both $p \leq 0.009$). Most interesting were differences in mean fold IL-10 production for *E. angustifolia* (4.9-fold increase fresh vs. 1.5-fold increase stored, $p < 0.001$), *E. purpurea* (2.5-fold increase fresh vs. 1.3-fold increase stored, $p = 0.0596$), and *E. sanguinea* (2.8-fold increase fresh vs. 1.5-fold increase stored, $p = 0.0582$).

Taken together, our findings indicate that 2 years' storage at −20°C alters the immunomodulatory properties of *Echinacea* tinctures. Time seems to augment the abilities of the extracts to stimulate proliferation but diminish their abilities to enhance cytokine production. We could not discern any patterns between results seen in our HPLC chromatograms and our immunomodulatory results.

Effects by species

We have focused on the effects of *Echinacea* extracts overall on the immune parameters studied here, but it is important to clarify that these effects vary by the species selected. This phenomenon is highlighted in Table 3. Four species (*E. pallida*, *E. paradoxa*, *E. sanguinea*, and *E. simulata*) behaved similarly in all eight immune assays, increasing PBMC proliferation in the nonviral model and decreasing IL-2 production in the viral model. *E. angustifolia* and *E. tennesseensis* also behaved similarly, as they both increased PBMC proliferation and IL-10 production in the nonviral model and had no effect on IL-2 in the viral model. Additionally, *E. tennesseensis* was able to augment IL-10 production at 24 h in the viral model. By contrast,

the only statistically significant activity seen from *E. purpurea* was an enhancement of IL-10 production in the nonviral model. However, results variability was greater for *E. purpurea* in some instances, and this may explain why *E. purpurea* appeared to be the least active.

In conclusion, a summary of our results is shown in Table 3. These findings contribute to our understanding of *Echinacea* in the context of viral infection. We found that after 2 years' storage, *Echinacea* tinctures had no effect on TNF-α (a proinflammatory cytokine produced by macrophages) in the nonviral model but did alter response to several T cell-associated parameters (such as IL-2 and IL-10) in both the nonviral and viral models and proliferation in the nonviral model. Reductions in IL-2 (a Th1 cytokine) may inhibit T cell proliferation, whereas increases in IL-10 (a Th2 cytokine) may minimize an inflammatory response such as that produced in the presence of TNF-α. Thus, in the context of viral infection, the tinctures in this investigation might exert their effects by downregulating an immune response. This behavior may be physiologically beneficial, as it may expedite the healing process after infection. In the context of viral infection, *Echinacea* tinctures may induce a shift in the Th1/Th2 balance. If this is true, the benefit of consuming *Echinacea* preparations may not be to fight the viral infection *per se* but moreso to alleviate the symptoms wrought by the immune response to infection, as other investigators have also suggested.[7,43]

Our findings may have important ramifications for scientists examining *Echinacea* extracts. Length of time under storage is likely an important factor in determining how *Echinacea* extracts may behave under laboratory conditions. Also, immune response may vary based on stimulus (*Echinacea* alone vs. *Echinacea* + recall stimulation with virus) in *in vitro* models. It is likely that these factors may also lead to variable results in the studies of other medicinal plant species.

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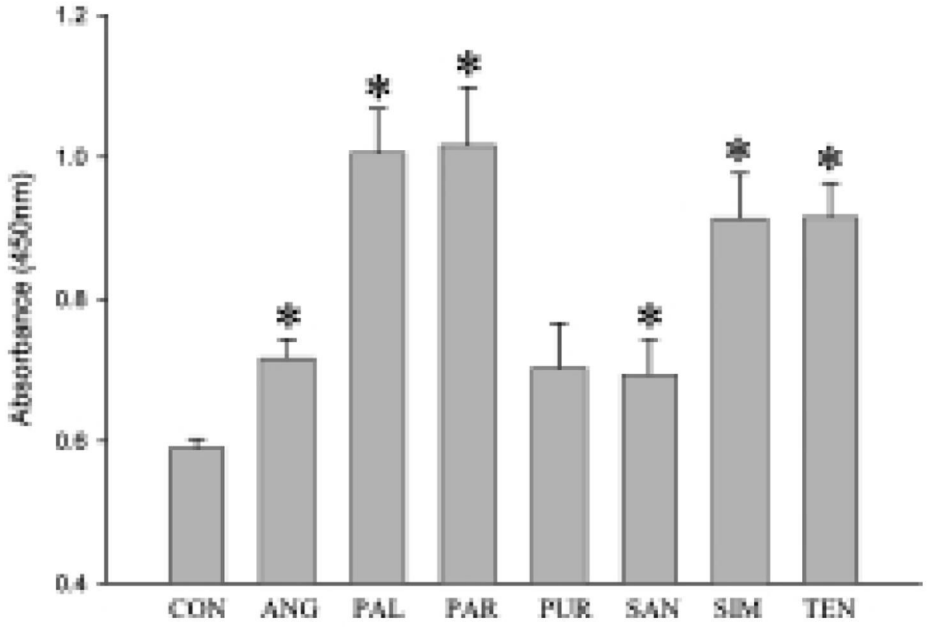

FIG. 1.

PBMC proliferation in the *Echinacea* alone model. Species abbreviations are described in Materials and Methods. *Statistically significant ($p \leq 0.05$) increases in wells receiving *Echinacea* tincture compared with control (medium alone) wells. Bars = standard errors.

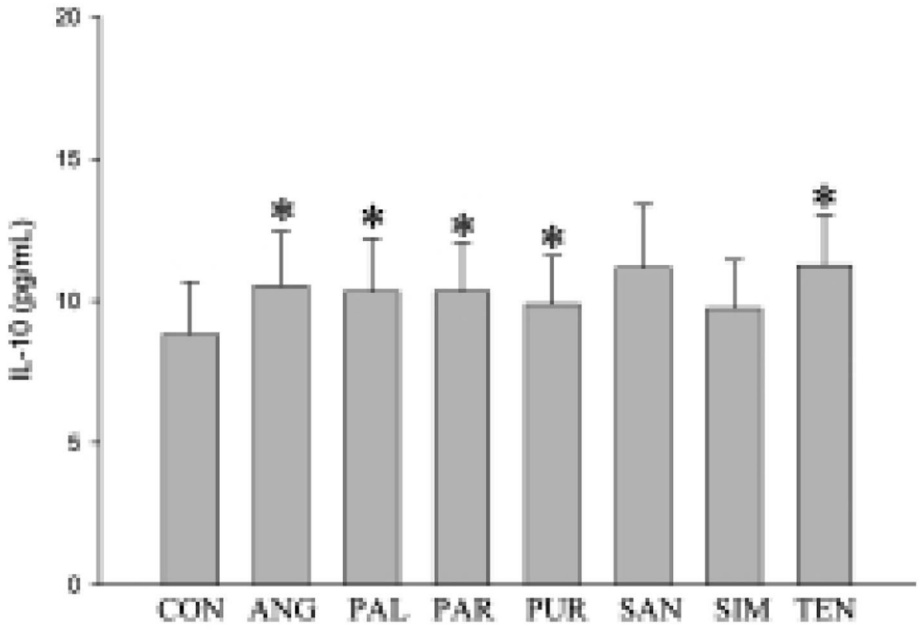

FIG. 2.

IL-10 production in the *Echinacea* alone model. Species abbreviations are described in Materials and Methods. *Statistically significant ($p \leq 0.05$) increases in wells receiving *Echinacea* tincture compared with control (medium alone) wells. Bars = standard errors.

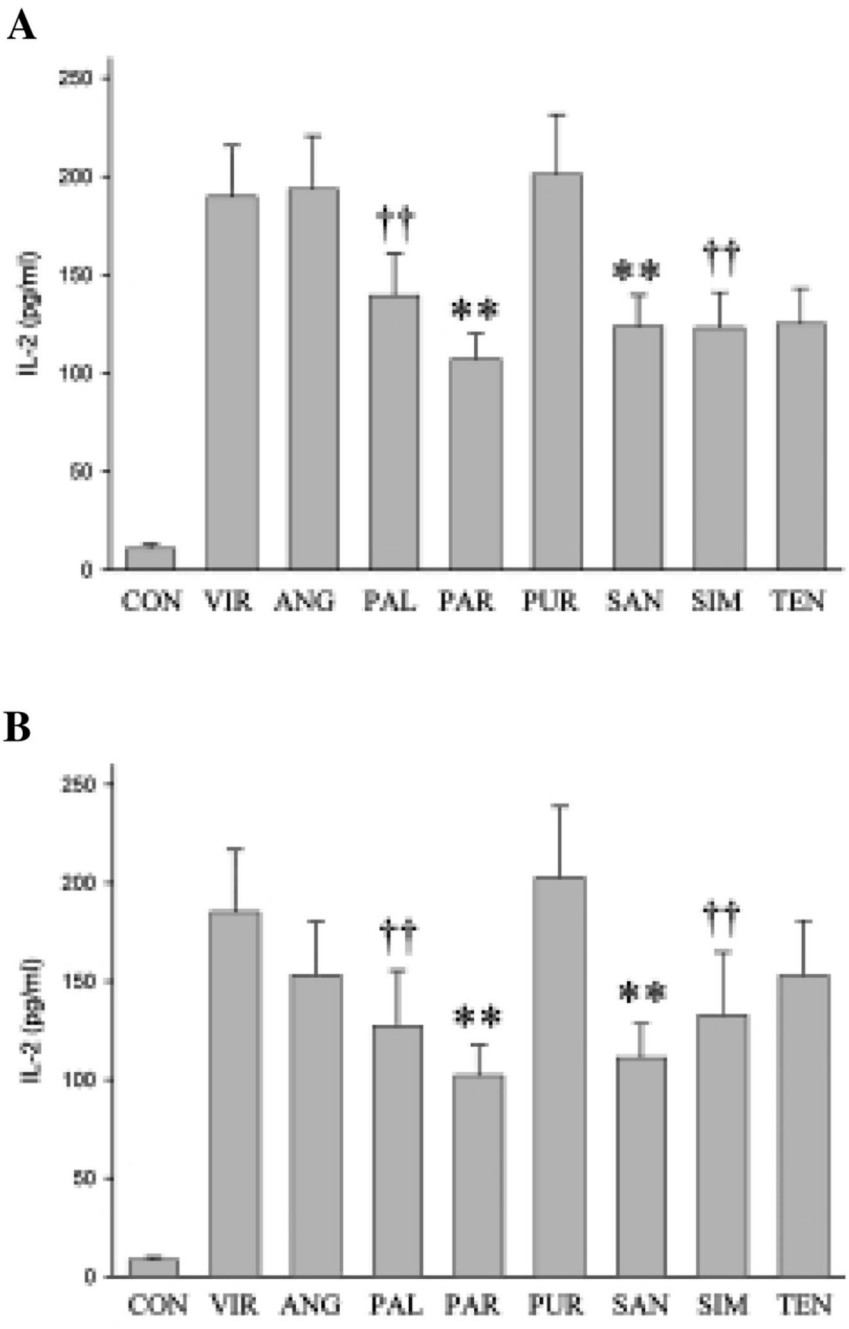

FIG. 3.

IL-2 production in the *Echinacea* + virus model. (**A**) Preimmunization. (**B**) Postimmunization. Species abbreviations are described in Materials and Methods. **Statistically significant ($p \leq 0.05$) decreases in wells receiving *Echinacea* tincture compared with control (medium alone) wells. ††Statistical trend ($0.1 \leq p \leq 0.05$) toward a decrease in wells receiving *Echinacea* tincture compared with control. Bars = standard errors.

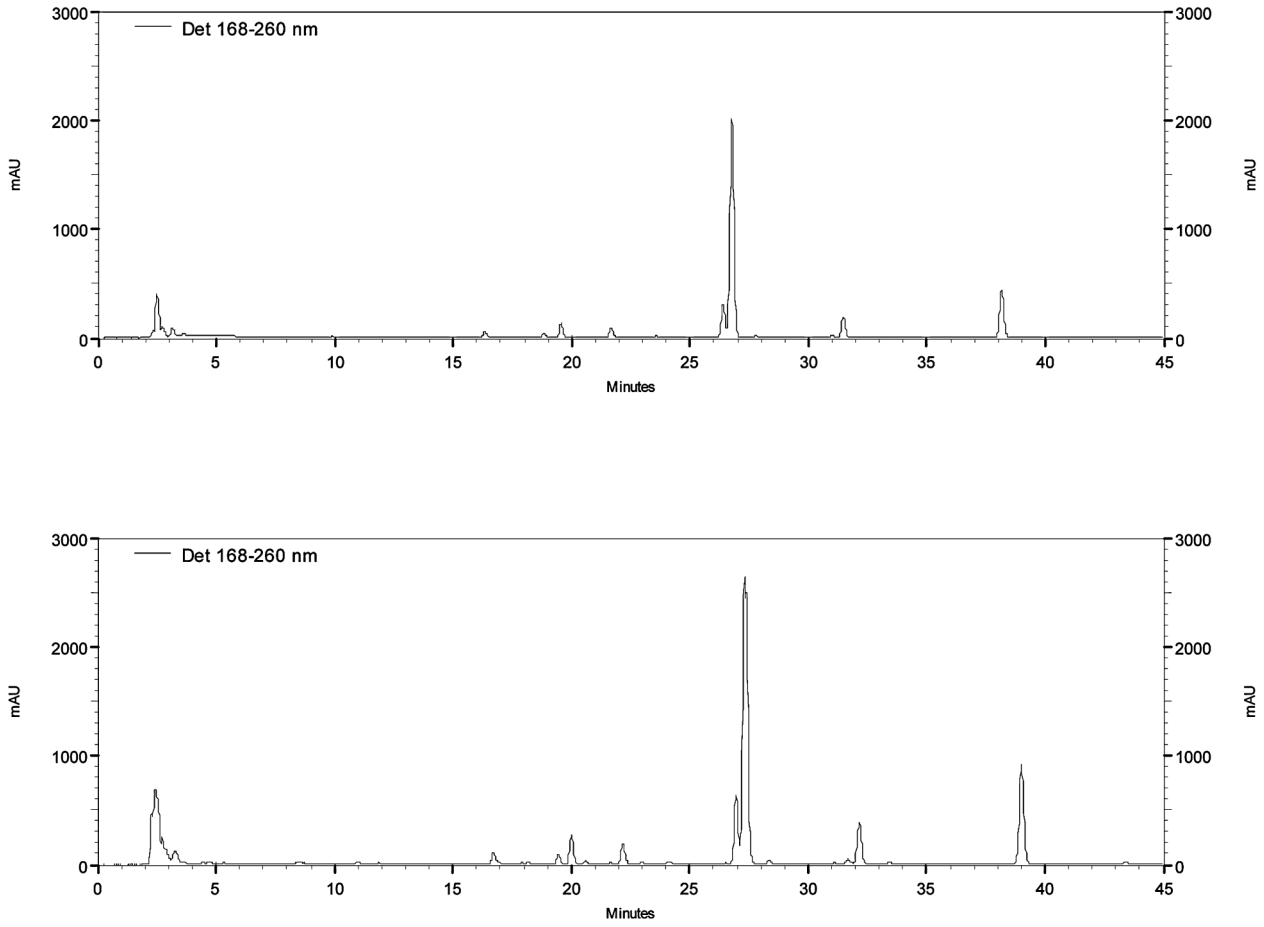

FIG. 4.
HPLC chromatography of *Echinacea angustifolia* extract both before (top) and after (bottom) 20 months storage at −20°C.

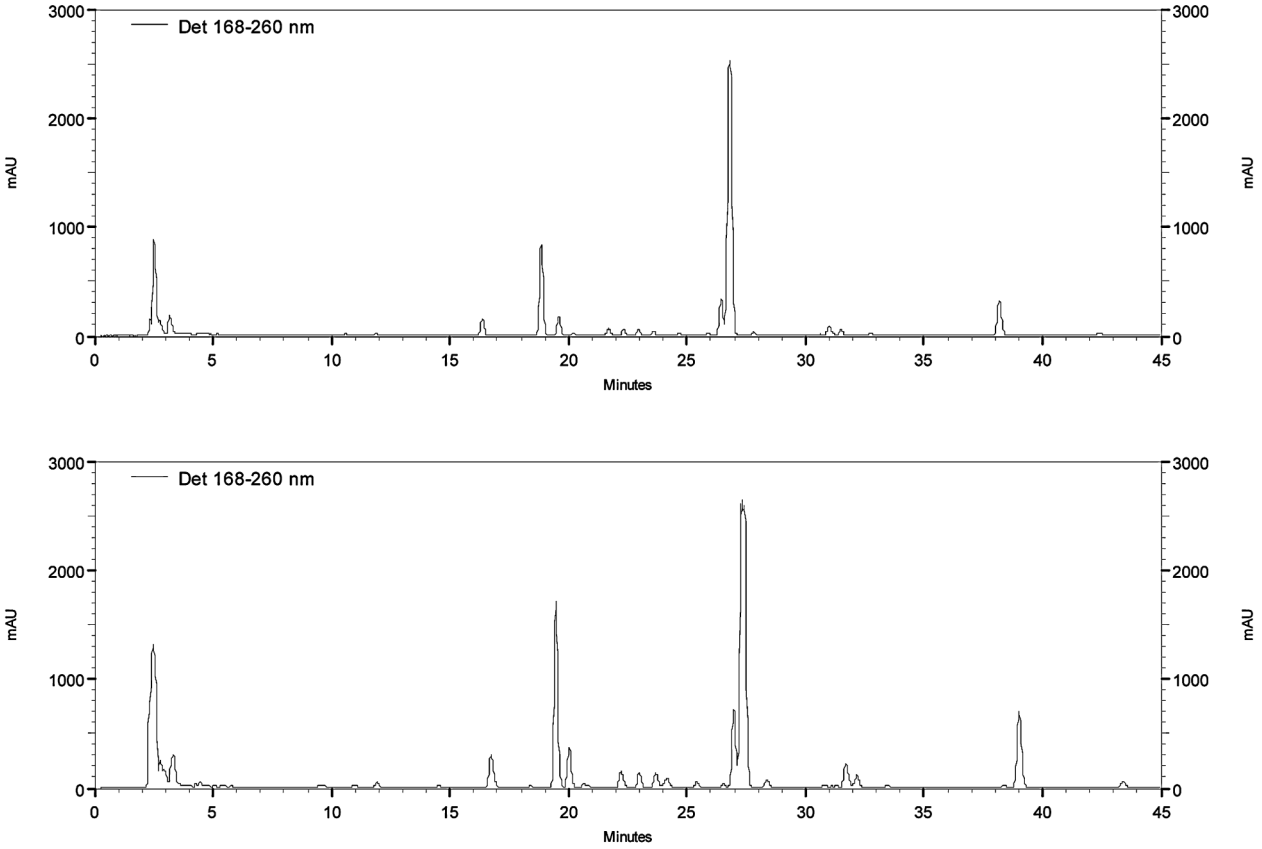

FIG. 5.
HPLC chromatography of *Echinacea pallida* extract both before (top) and after (bottom) 20 months storage at −20°C.

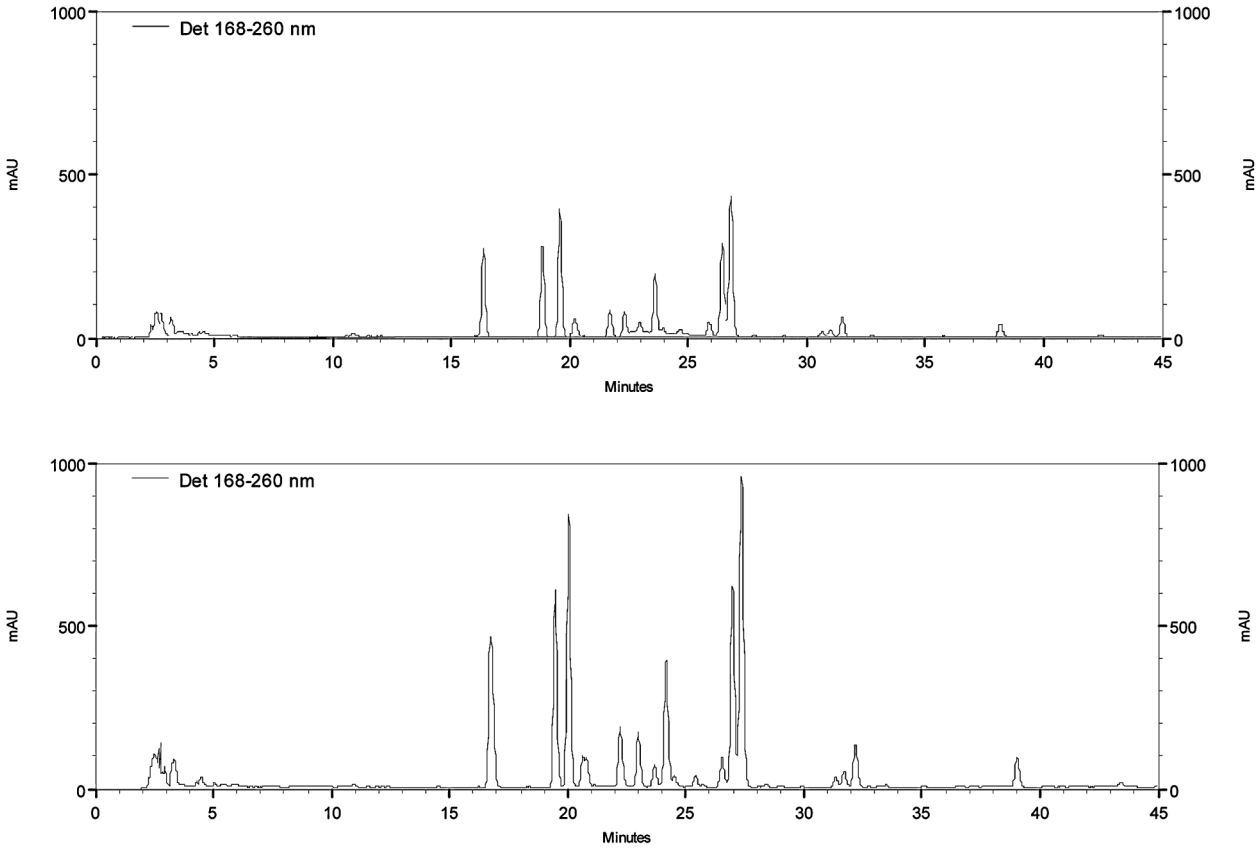

FIG. 6.

HPLC chromatography of *Echinacea purpurea* extract both before (top) and after (bottom) 20 months storage at −20°C.

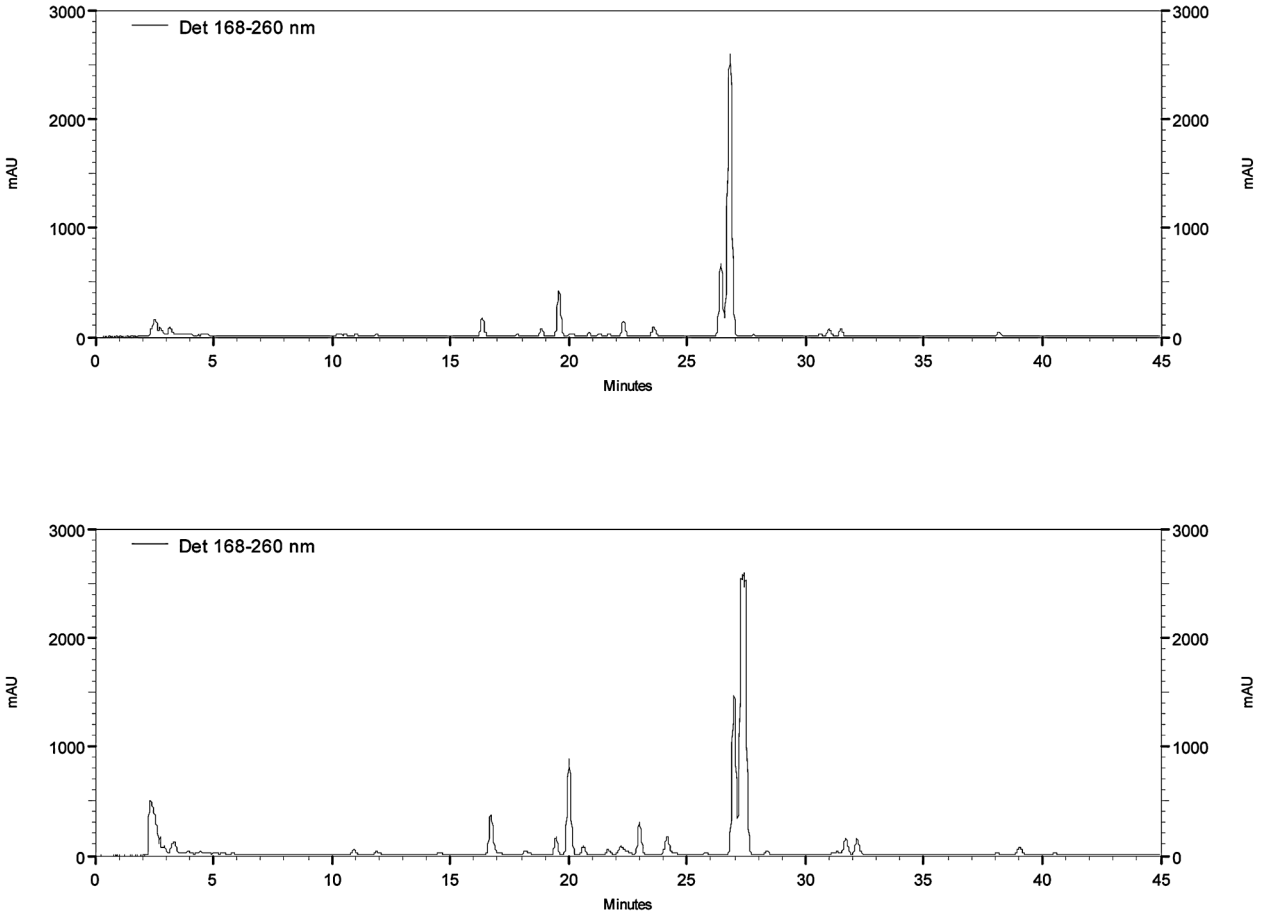

FIG. 7.
HPLC chromatography of *Echinacea sanguinea* extract both before (top) and after (bottom) 20 months storage at −20°C.

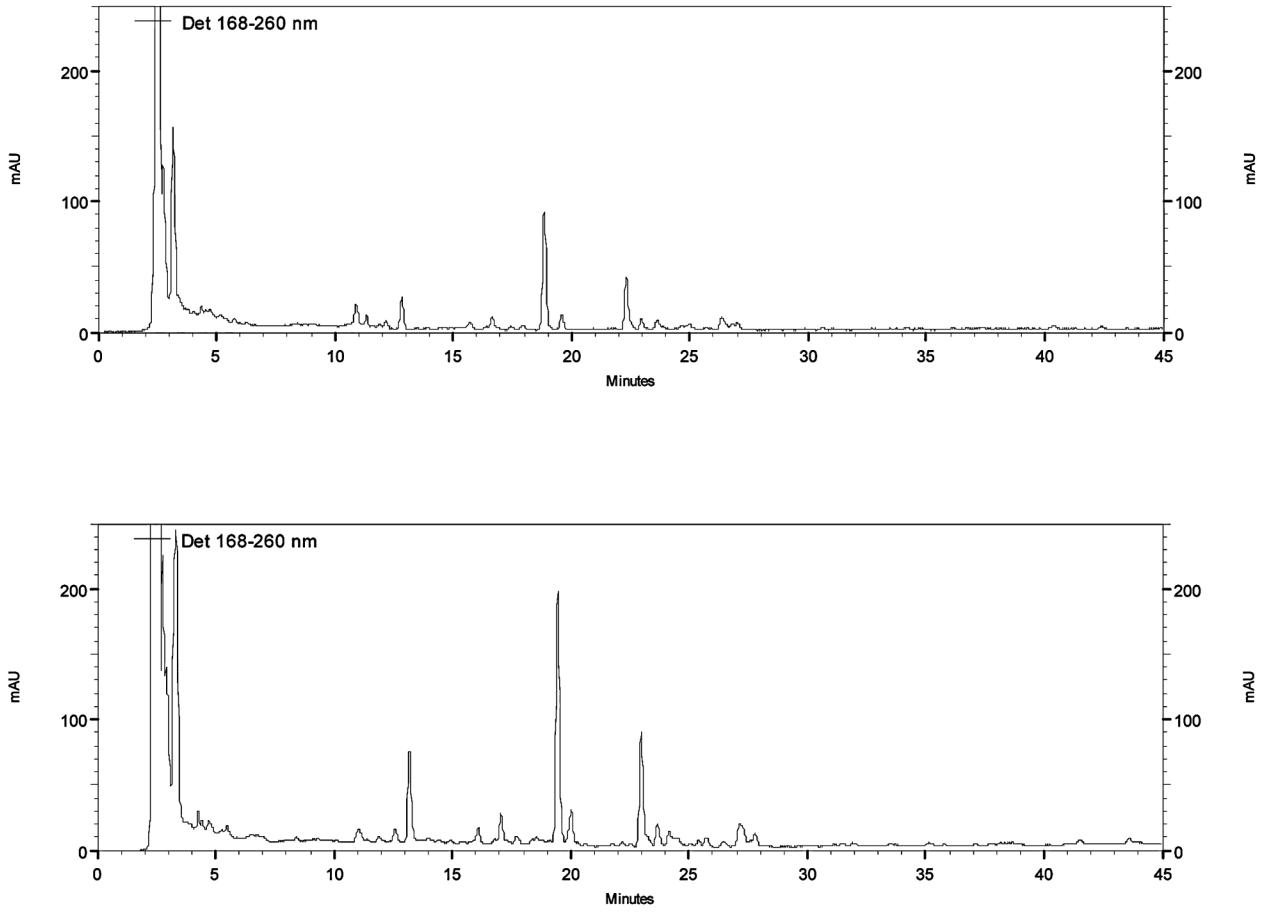

FIG. 8.
HPLC chromatography of *Echinacea simulata* extract both before (top) and after (bottom) 20 months storage at −20°C.

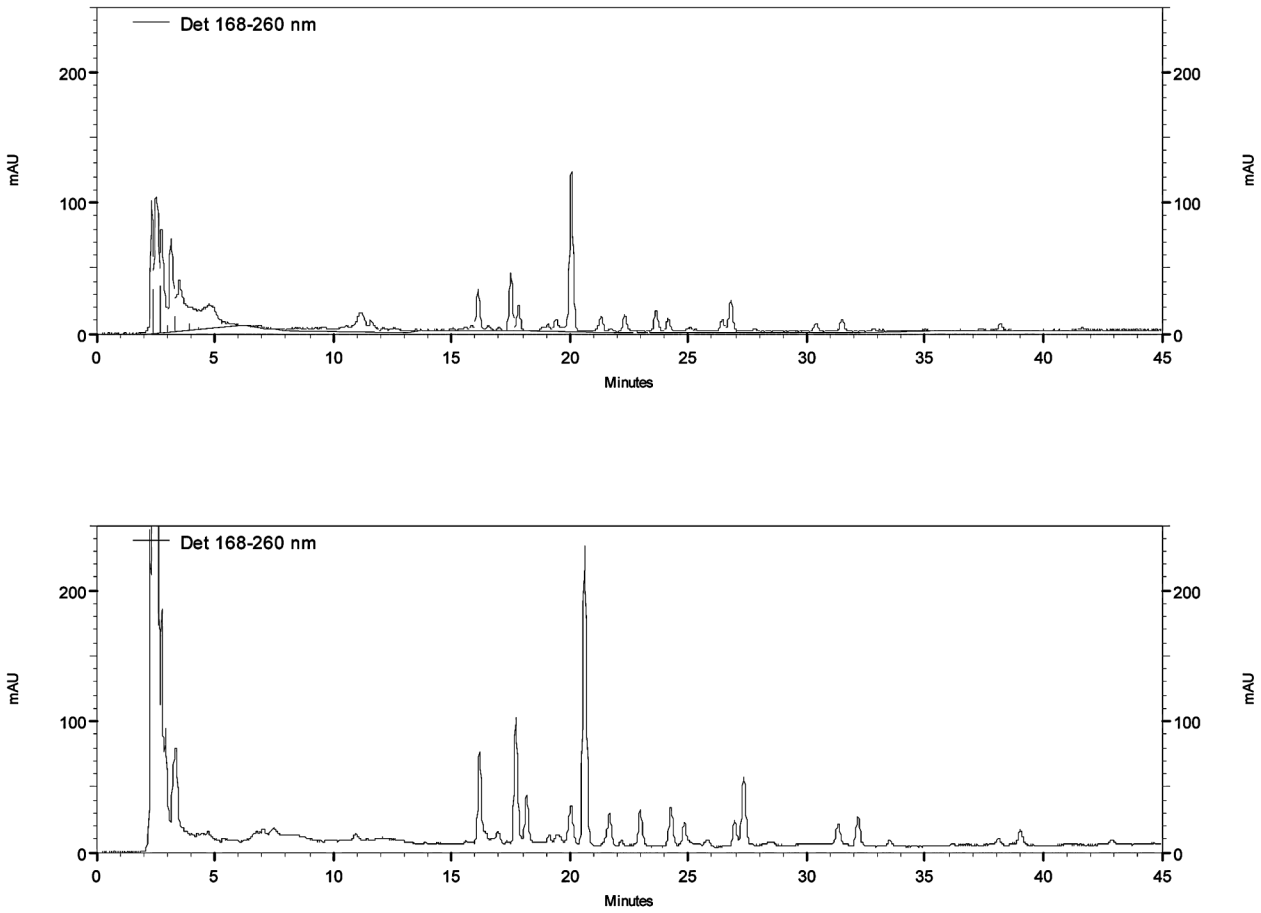

FIG. 9.
HPLC chromatography of *Echinacea tennesseensis* extract both before (top) and after (bottom) 20 months storage at −20°C.

Table 1

Selected results from *Echinacea* alone model[a]

Parameter	CON	ANG	PAL	PAR	PUR	SAN	SIM	TEN
TNF-α	14.08 ± 1.95[b]	16.33 ± 2.17	14.5 ± 1.87	14.92 ± 1.86	15.66 ± 1.79	22.18 ± 5.89	13.4 ± 1.77	20.11 ± 2.65
IL-12	11.88 ± 1.36	12.27 ± 1.31	11.71 ± 1.41	11.9 ± 1.38	12.16 ± 1.35	10.98 ± 1.59	10.93 ± 1.54	10.43 ± 1.4

[a] Species abbreviations are described in Materials and Methods.

[b] Means ± standard error in pg/mL.

Table 2

Selected results from *Echinacea* + virus model[a]

Parameter	CON	VIR	ANG	PAL	PAR	PUR	SAN	SIM	TEN
IL-10 at 24 h	12.72 ± 1.9[b]	19.75 ± 1.97	27.68 ± 3.48	30.98 ± 7.56	41.58 ± 22.75	23.17 ± 2.65	25.25 ± 8.01	22.91 ± 4.24	55.04 ± 19.37
IL-10 at 48 h	14.42 ± 3.33	105.91 ± 17.65	123.58 ± 16.38	103.09 ± 15.11	135.49 ± 57.47	108.89 ± 17.22	95.16 ± 13.69	80.46 ± 15.07	100.19 ± 16.25
IFN-γ	3.01 ± 1.05	934.21 ± 168.82	960.73 ± 166.01	693.92 ± 103.92	650.31 ± 118.27	771.31 ± 170.93	693.18 ± 117.99	546.1 ± 73.7	853.08 ± 123.93

[a] Species abbreviations are described in Materials and Methods.

[b] Means ± standard error in pg/mL.

Table 3

Summary of results[a]

Model	Immune parameter	ANG	PAL	PAR	PUR	SAN	SIM	TEN
Echinacea alone	PBMC proliferation	↑	↑	↑	N	↑	↑	↑
	TNF-α	N	N	N	N	N	N	N
	IL-10	↑	↑	↑	↑	N	N	↑
	IL-12	N	N	N	N	N	N	N
Echinacea + virus	IL-2	N	↓	↓	N	↓	↓	N
	IL-10 at 24 h	N	N	N	N	N	N	N
	IL-10 at 48 h	N	N	N	N	N	N	N
	IFN-γ	N	N	N	N	N	N	N

[a]Species abbreviations are described in Materials and Methods. For species outcomes, ↑ indicates significant enhancement of cytokine production compared with control; ↓ indicates significant decrease in cytokine production compared with control (or a trend in this direction); N indicates no change compared with control.