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Boswellia carterii Extract Inhibits TH1 Cytokines and Promotes TH2 Cytokines In Vitro

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Traditional herbal formulas used to treat inflammatory arthritis in China and India include *Boswellia carterii* or *Boswellia serrata*. They both contain boswellic acids (BAs) which have been shown to exhibit anti-inflammatory and antiarthritic properties. This study tests the hypothesis that mixtures of BAs derived from *B. carterii* have immunomodulatory properties. *B. carterii* plant resin obtained from China was prepared as an ethanol extract, and the presence of seven BAs was confirmed by column chromatography, high-performance liquid chromatography, and UV laser desorption/ionization tandem mass spectroscopy. The extract was then tested for its ability to alter in vitro production of TH1 cytokines (interleukin-2 [IL-2] and gamma interferon) and TH2 cytokines (IL-4 and IL-10) by murine splenocytes. Delivery of the resin extract using ethanol as a solvent resulted in significant cellular toxicity not seen with the addition of ethanol alone. By contrast, delivery of the resin extract using a sesame oil solvent resulted in a dose-dependent inhibition of TH1 cytokines coupled with a dose-dependent potentiation of TH2 cytokines. These results indicate that a purified mixture of BAs from *B. carterii* plant resin exhibits carrier-dependent immunomodulatory properties in vitro.

Complementary and alternative treatments of arthritis have gained popularity because they are purported to show clinical efficacy with minimal side effects compared to mainstream treatments. Frankincense, or olibanum, is obtained from the genus *Boswellia*, family *Burseraceae*, tree. Its extracted resin, salai guggal, is produced predominantly by four species, including *Boswellia serrata* in India, *Boswellia carterii* in East Africa and China, *Boswellia frereana* in Somalia, and *Boswellia sacra* in Arabia (4, 7, 21). The term guggals collectively refers to gum resins. The *Boswellia* gum oleoresin is comprised of essential oils, gum, and terpenoids. The *Boswellia* resin's chemical structure is similar to that of other pentacyclic triterpenes, which closely resembles that of anti-inflammatory steroids. Salai guggal has been shown to exhibit strong immunostimulant activity (17), and in the Ayurvedic Indian tradition, inflammatory polyarthritis and other forms of rheumatism have been successfully treated with herbal mixtures containing *Boswellia* resins or extracts (16).

The active constituents are contained in the extracted *Boswellia* terpenoid portion and are composed of boswellic acids (BAs) (20). The herbal formulas used for treatment vary, with BAs constituting from 37.5% to 65% of the total formulation (1). The plant resin from *B. carterii* is a frequent constituent of traditional Chinese herbal therapy, whereas BAs from *B. serrata* are key ingredients in Ayurvedic treatments for similar disease states (1). Both species contain BAs reported to have anti-inflammatory properties (2).

Human rheumatoid arthritis has a strong immune-mediated component and is marked by increased activated levels of TH1 cells and their cytokines, interleukin-2 (IL-2) and gamma interferon (IFN-γ), with fewer immunomodulatory TH2 cells and their cytokines, IL-4 and IL-10 (11, 18). It is possible, then, that the beneficial effect of boswellins in inflammatory arthritis may derive in part from immunomodulatory activity in addition to anti-inflammatory properties.

In this study, we address the in vitro immunomodulatory capacity of BAs derived from *B. carterii*. Moreover, we applied both quantitative chromatographic techniques and laser desorption/ionization tandem mass spectroscopy to ensure proper characterization and quantification of BAs present in the preparation and, thereby, proper standardization. Our results support the hypothesis that a portion of the therapeutic

TABLE 1. Observed versus expected masses of boswellic acids present in the initial acid extract (BCE) as determined by MS/MS analysis

BA species[a]	Formula	MH^+ predicted avg mass (AMU)	MN^+ predicted mass (AMU)	MNa^+ observed mass (AMU)
α/β-BA	$C_{30}H_{48}O_3$	456.7	478.4	478.6
acetyl-α/β-BA	$C_{32}H_{50}O_4$	498.7	520.4	520.8
11-keto-β-BA	$C_{30}H_{46}O_4$	470.7	492.3	492.9
acetyl-1-keto-β-BA	$C_{32}H_{48}O_5$	512.7	534.4	534.6
acetyl-11-dien-β-BA	$C_{32}H_{49}O_4$	497.7	519.4	519.5

[a] Experimental details are as described in the legend of Figure 3 and in Materials and Methods.

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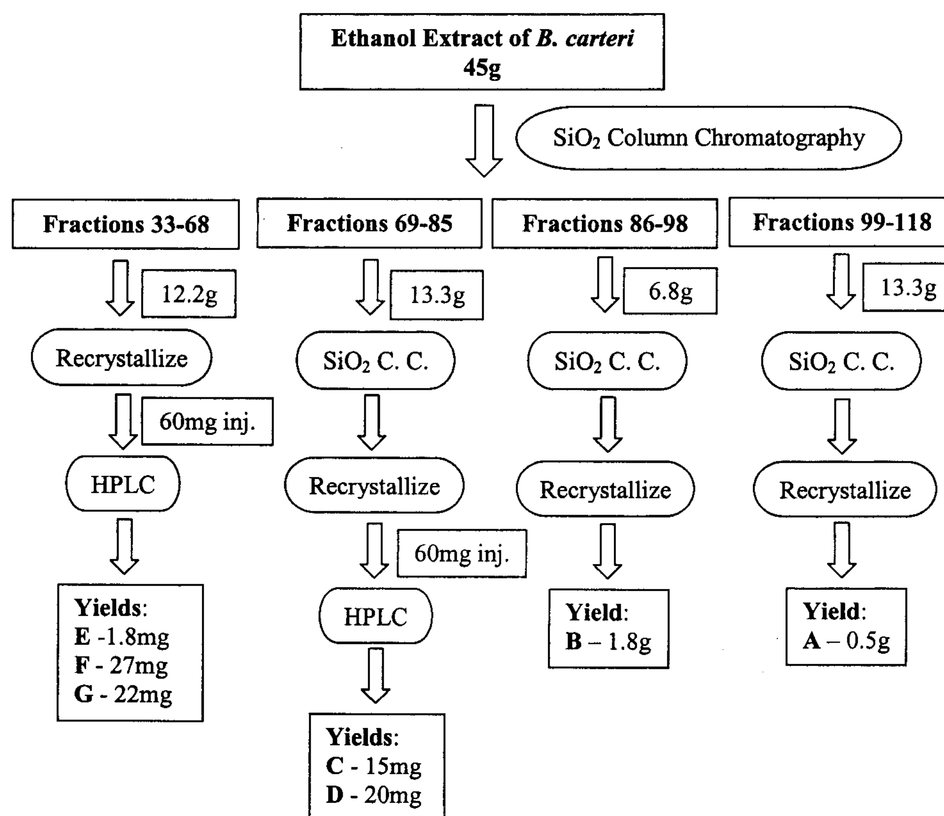

FIG. 1. Fractionation scheme of BCE. The initial ethanol extract was subjected to SiO_2 column chromatography, and fractions 33 through 68 and 69 through 85 were subpurified by HPLC. Yields shown are derived from a 60-mg column injection. Yields from fractions 86 through 98 and 99 through 118 are shown following recrystallization without subpurification. A, 11-keto-β-BA; B, acetyl-11-keto-β-BA; C, α-BA; D, β-BA; E, acetyl-11-dien-β-BA; F, acetyl-α-BA; G, acetyl-β-BA.

effects of *B. carterii*-derived BAs could reflect immunomodulation.

MATERIALS AND METHODS

Procurement and preparation of crude *B. carterii* extract. The *B. carterii* plant resin was procured and identified by Q. C. Fang at the Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College in Beijing, China. Ten kilograms of resin was manually ground into powder and passed through a mesh screen (#80) and suspended in aqueous ethanol (95%; 20 liters) for 24 h. After filtration, the residue was resuspended in aqueous ethanol (95%; 20 liters) for an additional 24 h and refiltered. The combined liquid-phase filtrate was then concentrated to give an initial *B. carterii* ethanol extract (BCE). Based on previous experimentation, 20% by weight of this initial BCE consists of total pentacyclic triterpenic acids. To ensure the presence of a relative proportion of each BA, the following fractionation methods were used for repetitive quality control of each individual preparation.

Isolation scheme to purify α- and β-boswellic acids and related analogs from BCE. The isolation of BAs and derivatives from BCE is summarized in Fig. 1. Forty-five grams of BCE was chromatographed over a 250-g silica gel column using a gradient solvent system consisting of *n*-hexane with increasing amounts of ethyl acetate. A total of 120 fractions, 250 ml/fraction, were collected. The following methods were used to subpurify the BAs.

(i) Method for α- and β-boswellic acid acetates. Fractions 33 through 68 were combined and concentrated. This yielded a 12.2-g residue that was recrystallized in 200 ml of hot methanol and allowed to precipitate at 2°C (± 2) for 12 h; the precipitate was then mesh filtered. The final mixture yielded 6 g of acetylated α- and β-BA. This mixture was further separated by preparative high-performance liquid chromatography (HPLC) (25- by 250-mm Varian Prostar HPLC/ODS column,; mobile phase of 100% MeOH; wavelength of 210 nm; flow rate of 7 ml/min; injected amount of 60 mg), yielding 27 mg of acetyl-α-BA, 22 mg of acetyl-β-BA, and 1.8 mg of acetyl-11-dien-β-BA.

(ii) Method for α- and β-BAs. Fractions 69 through 85 were combined and concentrated, yielding a 13.3-g crystalline residue. This was chromatographed on a 150-g silica gel column chromatograph, eluting with *n*-hexane-ethyl acetate (EtOAc; 5:1), to provide 42 fractions (200 ml/fraction). Of these, fractions 7 through 25 were combined and concentrated, yielding an 8.9-g residue. This was dissolved in 200 ml of hot acetonitrile and precipitated at 2°C (± 2) for 12 h; the precipitate was mesh filtered, yielding a crystalline mixture of 3.0 g of α- and β-BAs. Preparative HPLC (Varian Prostar HPLC/ODS column; mobile phase of 100% MeOH; wavelength of 210 nm; flow rate of 6 ml/min; injected amount of 60 mg) yielded 15 mg of α-BA and 20 mg of β-BA.

(iii) Method for 11-keto-β-BA acetate. Fractions 86 through 98 were combined and concentrated, yielding a 6.8-g residue. This was chromatographed on a 150-g silica gel column chromatograph, eluting with *n*-hexane-EtOAc (4:1). Of the 80 fractions (150 ml/fraction) collected, fractions 30 through 65 were combined and concentrated, yielding a 4.5-g residue. This was dissolved in 100 ml of hot methanol and precipitated at 2°C (± 2) over 12 h. The precipitate was mesh filtered, yielding 1.8 g of acetyl-11-keto-β-BA.

(iv) Method for 11-keto-β-BA. Fractions 99 through 118 were combined and concentrated, yielding a 6.0-g residue. This was chromatographed on a silica gel (150 g) column chromatograph, eluting with *n*-hexane-EtOAc (3:1), and provided 50 fractions (150 ml/fraction). Fractions 15 through 35 from this fourth chromatography were combined and concentrated to yield a 2.1-g residue. This was dissolved in 50 ml of hot methanol and cooled to 2°C (± 2) over 12 h, yielding a final precipitate. The precipitate was mesh filtered, yielding 0.5 g of 11-keto-β-BA.

LDI/MS and LDI/MS/MS analysis. Laser desorption/ionization mass spectrometry (LDI/MS) was performed with sufficient resolution of MH^+, MNa^+, and MK^+ ions to reveal isotopic distributions. BCE and its BA subextracts were

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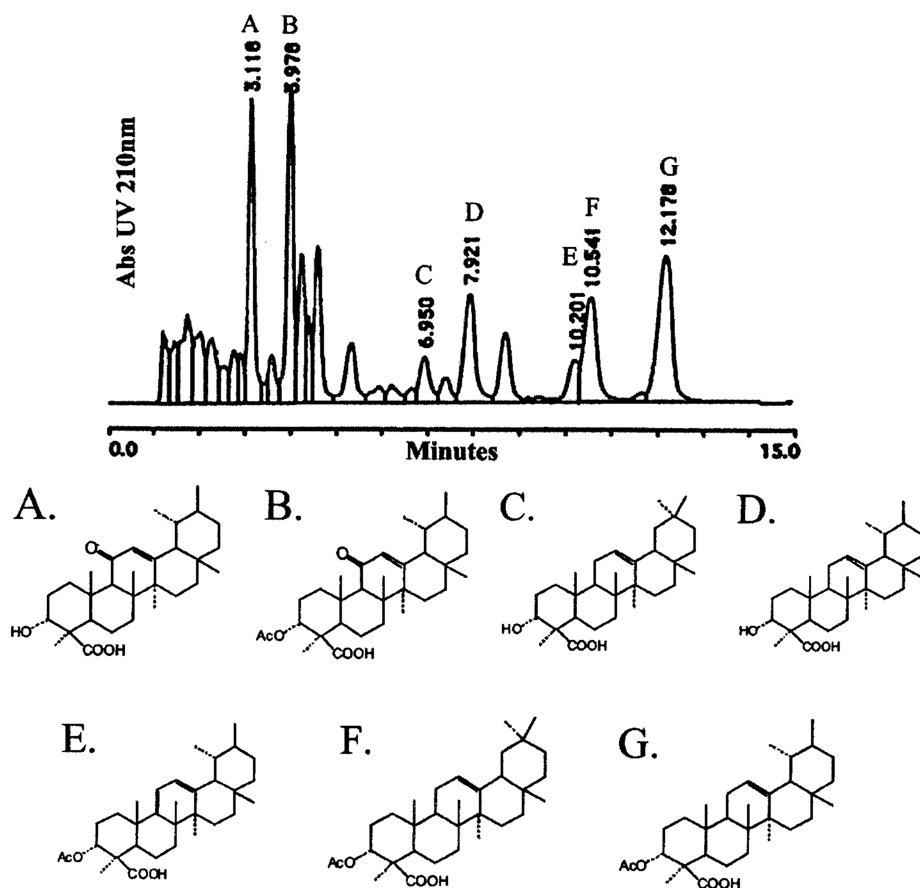

FIG. 2. Identification of BAs present in the initial *B. carterii* ethanol extract by HPLC. Shown in the upper panel is a representative analytical HPLC tracing. The lower panel contains the molecular structure of the eluted compounds. Compounds (retention time [range] in min) are as follows: A, 11-keto-β-BA (2.6-3.4); B, acetyl-11-keto-β-BA (3.5-4.2); C, α-BA (6.6-7.9); D, β-BA (7.5-8.6); E, acetyl-11-dien-β-BA (9.5-10.4); F, acetyl-α-BA (9.9-10.6); G, acetyl-β-BA (11.5-12.2).

dissolved in 70% USP ethyl alcohol–30% distilled H_2O at 20°C and pipetted onto metal sample platens of UV laser desorption/ionization-time of flight mass spectrometers (Kratos MALDI IV or AXIMA; Shimadzu Scientific, Manchester, England). During analysis, peaks corresponding to the predicted molecular mass/charge ratio (*m/z*) of BAs or their NA^+/K^+ adducts were gated and subjected to LDI tandem mass spectrometry (LDI/MS/MS) analysis, revealing characteristic degradation product ions to confirm their composition.

Preparation of extract for in vitro use. Pulverized BCE stock was prepared by mortar and pestle and irradiated to promote sterility. On the day of use, a stock solution prepared using the fine powder was dissolved in autoclaved solvent of either 100% USP ethanol or sesame oil (SO). Using equivalent amounts of solvent, extract was used at final concentrations of 10, 50, or 200 μg/ml. Controls consisted of unstimulated cells plus solvent (negative control) and stimulant plus solvent without BCE (positive control).

In vitro generation and measurement of cytokine production. Normal male C57BL/6 (B6), DBA/2, or B6D2F1 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in an animal facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care. Splenocytes from 6- to 12-week-old mice were tested for their ability to produce in vitro IL-2, IL-4, IL-10, and IFN-γ following stimulation with soluble anti-CD3 or concanavalin A (ConA) as previously described (19). Briefly, spleen cells (4×10^6 cells/well) were cultured in 24-well plates (Costar, McLean, VA) in a total volume of 2 ml of culture medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, antibiotics, 2-mercaptoethanol, nonessential amino acids, and L-glutamine. Cells were cultured in a 7% CO_2-humidified air mixture at 37°C. Measurements of the TH2 cytokines IL-4 and IL-10 were performed in both unstimulated cultures and cultures stimulated with 10 μg/ml (final concentration) of soluble anti-CD3 monoclonal antibody (2C11) (14). Measurements of TH1 cytokines IFN-γ and IL-2 were performed in cultures stimulated with 5 μg/ml (final concentration) of ConA. Prior experiments determined optimal time points for supernatant harvesting to be 24 h for TH1 cytokines or 48 h for TH2 cytokines. Supernatants were frozen at −20°C until testing. Cytokine content was determined by enzyme-linked immunosorbent assay (ELISA) using commercial anticytokine sandwich monoclonal antibody (BD-Pharmingen, San Diego, CA). Beginning at a dilution of 1:2, supernatants were tested, and optical density values were converted to U/ml by comparison with results using recombinant cytokine standards (BD-Pharmingen, San Diego, CA). Supernatant cytokine assays were performed in duplicate, with results being confirmed in two additional, independent experiments.

Viability analysis. Cell viability was assessed by trypan blue exclusion. Results are expressed as the percent of trypan blue excluding cells (live cells) per total cells counted.

Statistical analysis. Groups shown in Fig. 5 and 6 were compared by repeated measures analysis of variance.

RESULTS

Analysis of BAs in the extract. The BCE was prepared as described in Materials and Methods and then subjected to further fractionation. The fractionation scheme and yields are shown in Fig. 1. Figure 2 depicts a representative analytical HPLC tracing showing the elution of the BAs in BCE. The identities of the BAs in the initial extract were further confirmed by initial LDI/MS and gating of appropriate peaks to

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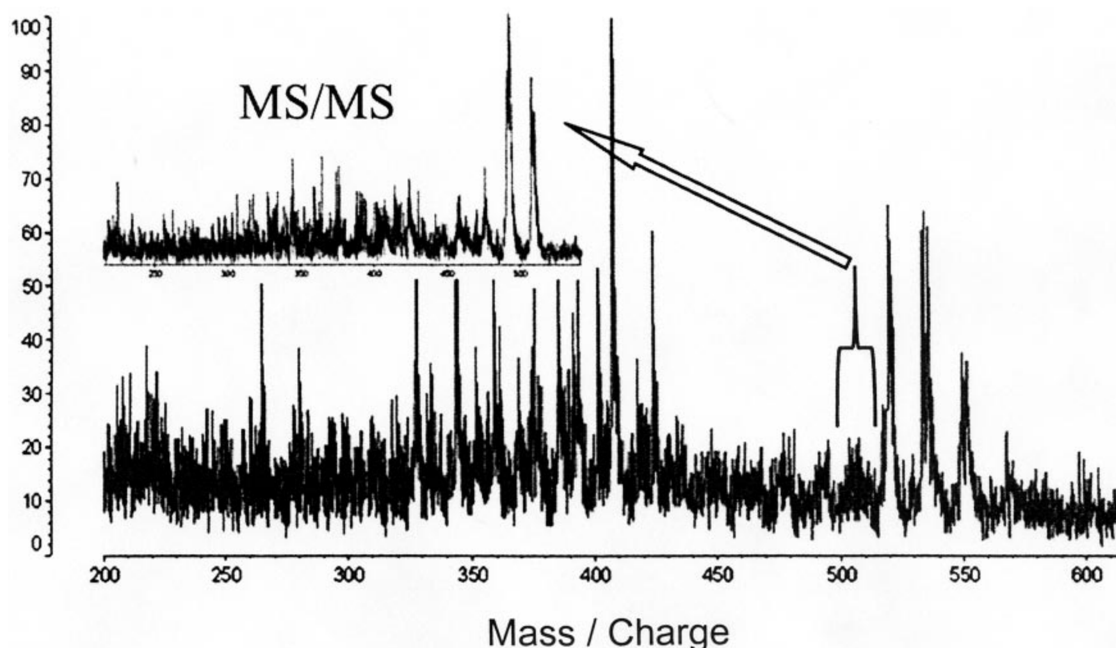

FIG. 3. Identification of BAs present in the initial *B. carterii* ethanol extract by laser desorption MS/MS analysis. Spectra were performed on the BCE and fragmentation ions were determined in MS/MS (reflectron mode) to confirm their identification by observing characteristic fragment ions. The inset shows an example of the MS/MS confirmation of the 11-keto-β-BA. The ion current corresponding to the MNa^+ distribution is gated and subjected to a second mass analysis, thereby demonstrating the relative selectivity of the technique. MNa^+ predicted and observed masses are monoisotopic since these peaks were resolved to an isotopic distribution. The observed versus expected masses are shown in Table 1.

yield LDI/MS/MS. Figure 3 shows an overall mass spectrum of BCE and summarizes several BA parent ions identified by mass spectrometry. The peaks corresponding to potential BA molecular ions were gated using a ~30 atomic mass unit (AMU) *m/z* window to capture the entire MH^+/MNa^+ isotopic mass distribution. The entire mass window was analyzed for spontaneous degradation products in a tandem (LDI/MS/MS) mode to reveal characteristic progeny ions. Structures of the parent ions were confirmed by progeny ion spectrums. The spectral inset in Fig. 3 is an example of an LDI/MS/MS spectrum of 11-keto-β-BA and demonstrates the characteristic fragmentation observed in this mode. The observed versus

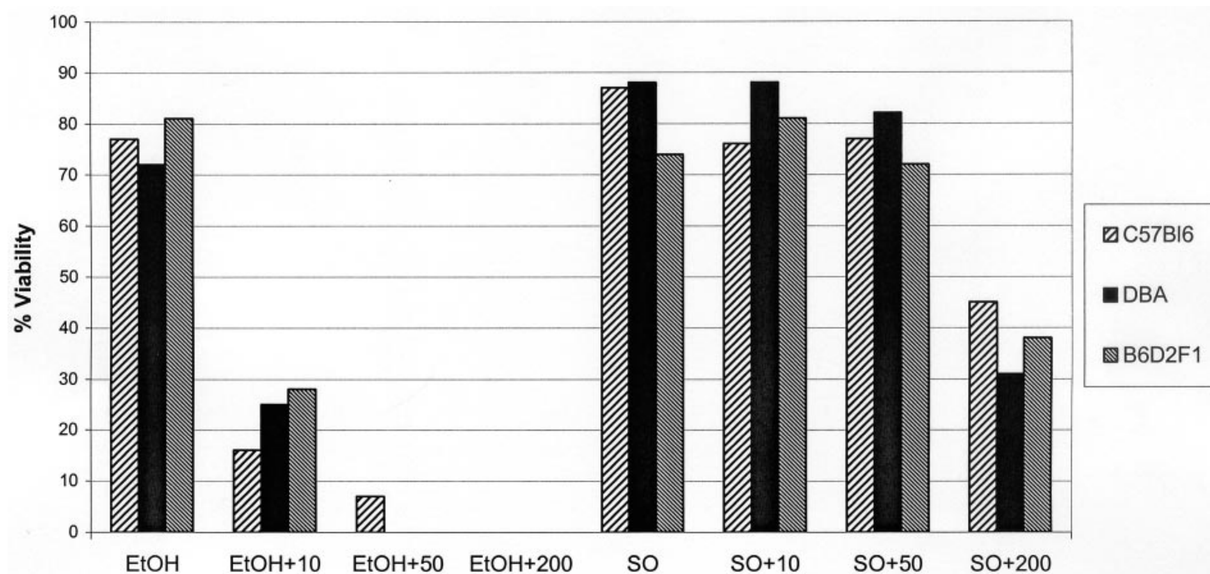

FIG. 4. BCE in ethanol exhibits cellular toxicity not seen with SO as a carrier or with ethanol alone. Murine splenocytes from B6, DBA/2, or B6D2F1 mice were cultured in vitro with either ethanol (EtOH) alone (final concentration, 0.1%), SO alone (final concentration, 0.5%), or three concentrations of BCE in their respective solvents (final concentrations of 10, 50, and 200 μg/ml). Viability was determined by trypan blue exclusion at 24 h of culture (see Materials and Methods).

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expected masses of the parent BA molecular ions in the initial BCE are summarized in Table 1. Although there are techniques available to enhance analysis of molecules of <1,500 AMU using exogenous UV-adsorbent matrix (3), no matrix was necessary because the analyte had sufficient UV-absorbent properties to promote ionization and transfer into the gas phase. This reduced concerns regarding chemical reactions with the BA under analysis or additional background signal due to the addition of the UV-absorbent matrices that are typically utilized in matrix-assisted LDI/MS (13).

In vitro effects of BCE: cellular toxicity with ethanol but not SO as a solvent. The BCE was dissolved in either sterile ethanol or sesame oil, and its effect on cell viability in vitro was assessed using splenocytes from B6, DBA/2, and B6D2F1 mice. Splenocytes were cultured with three concentrations of BCE (final concentration of 10, 50, or 200 μg/ml), and viability was determined by trypan blue exclusion at 24 h. As shown in Fig. 4, when ethanol was used as a solvent, BCE exhibited 100% toxicity (0% viable cells) at concentrations of 50 μg/ml and 200 μg/ml for both the DBA/2 and B6D2F1 splenocytes. This profound degree of cellular toxicity was not seen when SO was used as a solvent or with the addition of ethanol alone (no BCE). Based on these results, subsequent studies of the immunomodulatory properties of BCE were carried out using SO as a solvent.

In vitro effects of BCE: BCE in SO exhibits immunomodulatory activity. In a dose-response experiment, BCE dissolved in sesame oil (SO-BCE) was tested for its ability to alter cytokine production of ConA-stimulated splenocytes. Three responder strains were tested: B6 mice, which make a strong TH1 response (10); DBA/2 mice, which make a strong TH2 response (6); and the B6D2F1 hybrid mice. As shown in Fig. 5, SO-BCE exhibits a dose-dependent inhibition of the TH1 cytokines IFN-γ (panel A) and IL-2 (panel B) from all three strains tested. In vitro detection of IL-4 and IL-10 was possible for TH2 response-prone DBA/2 mice following anti-CD-3 stimulation (Fig. 6) but not for B6 or B6D2F1 mice (data not shown). Moreover, the addition of SO-BCE in vitro potentiated TH2 cytokine production by DBA/2 splenocytes in a dose-dependent fashion; however, cytokine potentiation was lost at the 200 μg/ml concentration, a dose at which significant cellular toxicity is evident (Fig. 4). Taken together, these results indicate that BCE dissolved in sesame oil exhibits immunomodulatory properties in vitro and promotes a shift in the cytokine production profile away from a TH1 response and toward a TH2 response.

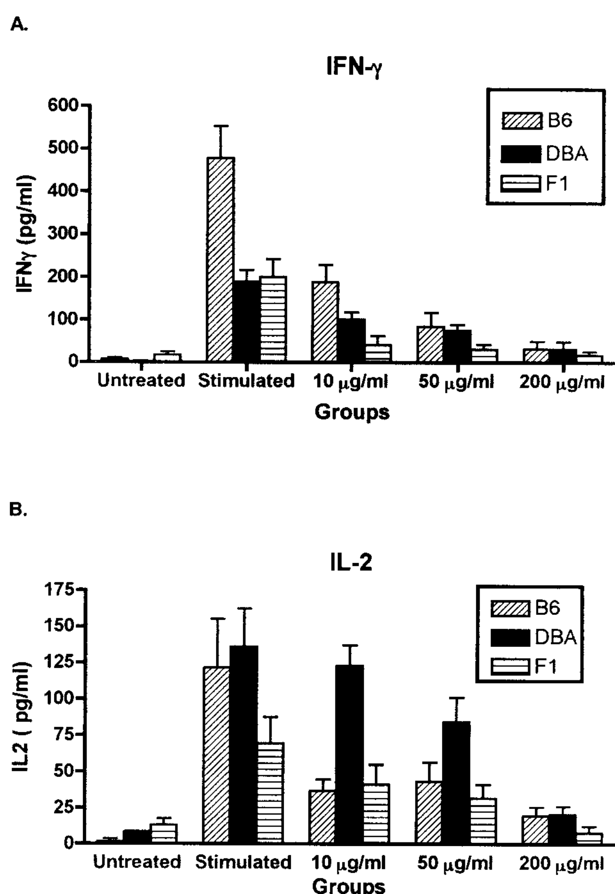

FIG. 5. BAs in SO inhibit TH1 cytokine production in vitro. IFN-γ (A) and IL-2 (B) levels in culture supernatants were determined by ELISA at 24 h following stimulation with ConA as described in Materials and Methods. Similar results were seen in two additional independent experiments, and standard errors were calculated on replicate analyses. Untreated, SO without BCE (negative control); Stimulated, SO plus ConA without BCE (positive control). The final concentrations of BCE plus SO plus ConA medium are indicated as 10 μg/ml, 50 μg/ml, and 200 μg/ml. F1, B6D2F1 mice; DBA, DBA/2 mice.

DISCUSSION

Alternative medicine treatments frequently incorporate herbal therapies and plant resins from species of the *Boswellia* family e.g., *B. serrata* and B. *carterii*. Both have anti-inflammatory properties and contain α- and β-BAs (5, 8, 12, 15); however they differ in their proportion of acetylated acids. In this study, we focused on the less studied *B. carterii* resin. Herbal therapeutics are typically formulated with less quality control than commercially prepared pharmacologic agents and may contain multiple bioactive components (9). Moreover, *B. carterii* is widely used in unpurified or minimally purified extracts. For these reasons, we used both chromatography and mass spectrometry for specific identification of the BAs present in the initial *B. carterii* extract. This analysis was aided by the use of an intrinsic 337-nm UV absorbance, promoting soft desorption and permitting the production of molecular ions. These relate to the initial species that is present in analyte rather than fragments. With this ionization strategy, ample parent ions were generated. Progeny fragment ions could be generated to confirm the parent ion composition. Quality control was optimized by tracking multiple components within the complex mixture through the combination of direct laser desorption/ionization coupled with a high-resolution tandem time of flight mass analysis. This analysis not only identified the BAs present in the initial extract but also ensured the similarity of separate batches tested in vitro.

The initial BCE dissolved in SO exerted a dose-dependent inhibitory effect on in vitro IFN-γ and IL-2 production by splenocytes from B6, DBA/2, and B6D2F1 hybrid mice. The TH2 cytokines IL-4 and IL-10 were detected in vitro only from stimulated TH2 response-prone DBA/2 splenocytes (6). In

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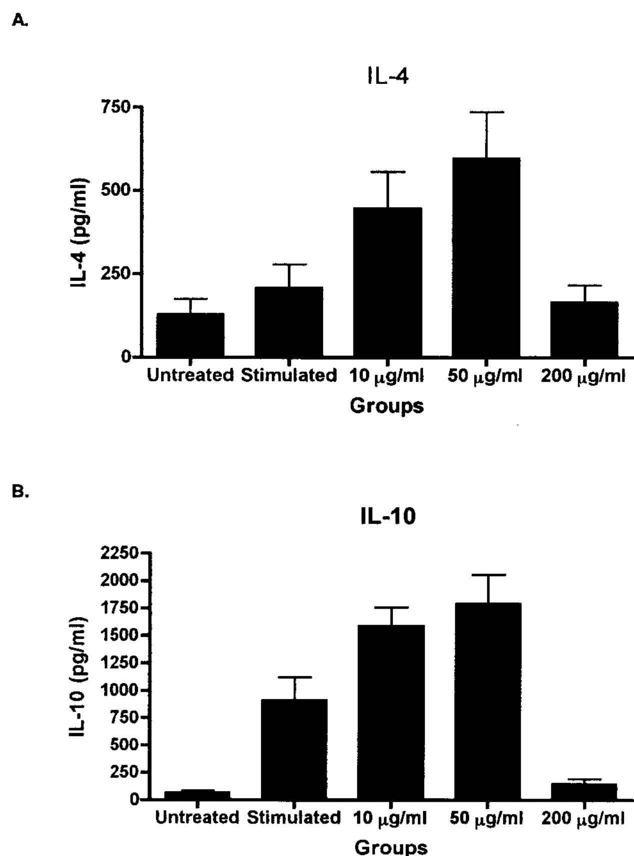

FIG. 6. BAs in SO promote TH2 cytokine production in vitro. IL-4 (A) and IL-10 (B) levels in culture supernatants from DBA/2 splenocytes were determined by ELISA at 48 h after stimulation with anti-CD3 as described in Materials and Methods. Similar results were seen in two additional independent experiments, and standard errors were calculated on replicate analyses. Untreated, SO without BCE (negative control); Stimulated, SO (without BCE) plus anti-CD3 (positive control). The final concentrations of BCE plus SO plus anti-CD3 medium are indicated as 10 μg/ml, 50 μg/ml, and 200 μg/ml.

contrast to the effects on TH1 cytokine production, BCE in SO did not inhibit IL-4 and IL-10 production and, instead, promoted production of TH2 cytokines at the two lower BCE concentrations tested. Very little stimulated TH1 or TH2 cytokine was detected at the highest concentration of BCE-SO (200 μg/ml), consistent with the loss of viability seen at this level (Fig. 4). These data support the conclusion that BCE-SO at concentrations of 10 μg/ml to 50 μg/ml is immunomodulatory rather than immunosuppressive or simply toxic, as these concentrations simultaneously inhibit TH1 cytokine production and promote TH2 cytokine production.

Lastly, we observed that BCE combined with ethanol exhibits significant cellular toxicity that was not seen when it was combined with SO or when ethanol alone was used. We can only speculate as to the mechanism for this differential toxicity, and it very likely may reflect the different kinetics of drug release from the two carriers, resulting in toxic accumulations of BCE with the ethanol solvent and slower release from the SO solvent. Alternatively, BCE may exhibit a toxic synergy with ethanol that is not seen with SO. It is not possible to directly extrapolate the concentrations used in these in vitro studies to those achieved in humans following oral ingestion of *Boswellia* resins; nevertheless, these data raise the question as to whether coingestion of alcohol might promote toxic properties of orally ingested boswellin-containing herbal preparations. Our results not only support the need for standardization of dosage and delivery of phytopharmaceuticals but also underscore the possibility of unexpected, adverse effects.

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