PHYTOTHERAPY RESEARCH
Phytother. Res. (2013)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5080

Suppression of Lipopolysaccharide-stimulated Cytokine/Chemokine Production in Skin Cells by Sandalwood Oils and Purified α-santalol and β-santalol

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Medicinally, sandalwood oil (SO) has been attributed with antiinflammatory properties; however, mechanism(s) for this activity have not been elucidated. To examine how SOs affect inflammation, cytokine antibody arrays and enzyme-linked immunosorbent assays were used to assess changes in production of cytokines and chemokines by co-cultured human dermal fibroblasts and neo-epidermal keratinocytes exposed to lipopolysaccharides and SOs from Western Australian and East Indian sandalwood trees or to the primary SO components, α-santalol and β-santalol. Lipopolysaccharides stimulated the release of 26 cytokines and chemokines, 20 of which were substantially suppressed by simultaneous exposure to either of the two sandalwood essential oils and to ibuprofen. The increased activity of East Indian SO correlated with increased santalol concentrations. Purified α-santalol and β-santalol equivalently suppressed production of five indicator cytokines/chemokines at concentrations proportional to the santalol concentrations of the oils. Purified α-santalol and β-santalol also suppressed lipopolysaccharide-induced production of the arachidonic acid metabolites, prostaglandin E2, and thromboxane B2, by the skin cell co-cultures. The ability of SOs to mimic ibuprofen non-steroidal antiinflammatory drugs that act by inhibiting cyclooxygenases suggests a possible mechanism for the observed antiinflammatory properties of topically applied SOs and provides a rationale for use in products requiring antiinflammatory effects. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: sandalwood oils; antiinflammatory; dermal fibroblasts; neo-epidermal keratinocytes; prostaglandin.

INTRODUCTION

Santalum album L. (Santalaceae), commonly known as East Indian sandalwood, is a slow-growing hemiparasitic tree widely distributed in South Asia. Another species, Western Australian sandalwood (*S. spicatum)*, is native to the arid and semi-arid regions of Western Australia. Both species have contributed substantially to the fragrance market (Jones *et al*., 2009). The East Indian sandalwood tree has been used since ancient times for religious purposes as both an incense and for carvings, and used in fragrances, flavorings, and as a traditional medicine. The essential oil of sandalwood is usually obtained by steam distillation of the heartwood and is widely used in aromatherapy, as an antidepressant, antiinflammatory, antifungal, astringent, sedative, insecticide, and antiseptic (Burdock and Carabin, 2008; So *et al*., 2010). The sandalwood oil (SO) emulsion is routinely used in India as an Ayurvedic medicine for inflammatory and eruptive skin diseases. Its antiseptic and antiinflammatory properties make it an effective remedy for various skin problems. The potential antiinflammatory benefit was recently shown in a Phase 2 clinical study in 50 acne patients wherein 90% of patients benefited after 2 months of treatment (Moy *et al*., 2012). The treatment was well tolerated and rapidly reduced lesion redness and inflammation.

The sesquiterpene alcohols (*Z*)-α-santalol and (*Z*)-β-santalol together constitute approximately 70% of the essential oil obtained from mature trees (Christenson *et al*., 1981). The current international standard (ISO 3518:2002(E)) for East Indian sandalwood oil (EISO) specifies 41–55% (*Z*)-α-santalol and 16–24% (*Z*)-β-santalol. Both (*Z*)-α-santalol and (*Z*)-β-santalol are considered to be the primary active components of SOs and have attracted increasing attention for their possible anticancer activity and inhibitory properties against Herpes simplex and other viruses (Dwivedi *et al*., 2006; Paulpandi *et al*., 2012). However, much confusion still exists concerning the therapeutic efficacy of this botanical agent as a consequence of inadequate standardization of most preparations used in clinical trials, and consequently much of the information available in this context is anecdotal.

In this study, we evaluated the ability of well-characterized sandalwood essential oil preparations from Western Australian and East Indian trees to antagonize production of proinflammatory cytokines and chemokines by human dermal fibroblasts and epidermal keratinocytes

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Received 25 March 2013
Revised 15 September 2013
Accepted 14 October 2013

stimulated with lipopolysaccharides (LPS) by screening fluorescent cytokine antibody arrays. Additionally, the ability of purified α-santalol and β-santalol to suppress secretion of key cytokine-related factors and proinflammatory arachidonic acid metabolite production were compared with that of the SOs by enzyme-linked immunosorbent assays (ELISAs).

MATERIALS AND METHODS

Sandalwood oil preparations. East Indian SO and Western Australian SO (WASO) were obtained by steam distillation of the heartwood from Santalis Pharmaceuticals (San Antonio, TX, USA). WASO (lot: 110308SDD) is the essential oil from wild grown *Santalum spicatum syn Fusanus spicatus*. EISO (lot: PISO-110904SD/SA) is the essential oil from plantation-grown *S. album*. The chemical composition of oils is shown in Table 1 and are in agreement with generally accepted standards for this kind of preparation. The α-santalol and β-santalol were purified from EISO by a combination of flash chromatography on silver nitrate impregnated silica followed by a second purification using supercritical fluid chromatography on a chiral support by Santalis Pharmaceuticals. The purified santalols were demonstrated to be 96.8% and 91.5% pure by a variety of analytical methods, including gas chromatography/flame ionization detector, liquid chromatography–mass spectrometry, Fourier transform infrared spectroscopy, carbon nuclear magnetic resonance (NMR), two-dimensional NMR, hydrogen NMR, and elemental analysis. The oils or santalols were diluted in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the experiments (1:100,000) had no adverse effects on the cells.

Cell lines and reagents. Primary cultures of human dermal fibroblasts and neo-epidermal keratinocytes (Lonza, Walkersville, MD, USA) were cultured in KBM-SFM (Invitrogen, Grand Island, NY, USA) supplemented with human recombinant EGF and bovine pituitary extract. Cells were maintained in a 37 °C, 5% CO_2 atmosphere. LPS from *Escherichia coli* 0111:B4 and the non-steroidal antiinflammatory drug (NSAID), ibuprofen (IB), were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Chemical composition of Western Australian sandalwood oil and East Indian sandalwood oil

Sesquiterpenes	WASO (%)[a]	EISO (%)
α-santalol	16	49
epi-α-bisabolol	6	ND[b]
z-α-(trans)bergamotol	4	6
cis-β-santalol	6	21
epi-β-santalol	2	4
trans, trans farnesol	13	ND
z-β-curcumen-12-ol	7	ND
cis nuciferol	6	ND
z-γ-curcumen-12-ol	5	ND
z-lanceol	4	ND
E-nerolidol	3	ND
B-bisabolol	2	ND
dendrolasin	1	ND
Total sesquiterpenoid alcohols	99	

[a]% concentration of sesquiterpenes mass per volume

[b]ND: component composition not determined

Cell viability assay. Cytotoxic profiles of LPS, SOs, and IB were assessed using the MTS™ [(3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent (phenazine methosulphate; PMS) cell viability assay] cell viability assay (Promega, Madison, WI, USA). Briefly, 5000 cells seeded in wells of 96 well plates (Corning, NY, USA) were allowed to attach overnight, exposed to 0.5–5.0 μg/ml LPS and 11.25–360 μM SOs or IB for 24 h. A proportional volume of DMSO was added to 'untreated' experiments as vehicle control. The tetrazolium dye was added to each well of the plate and incubated for a further 4h. Optical density was then measured at 490 nm using a EL_X 808 Automated Microplate Reader (Bio-TEK® Instruments, Winooski, VT, USA). All treatments were performed in triplicate. The percentage change in viable cell mass was presented as the OD_{490} ratio between the untreated and treated cells at the indicated concentrations.

Lipopolysaccharide-stimulated cytokine-related factor profiling. Dermal fibroblasts and keratinocytes were grown in 12 well plates (Corning) to produce confluent monolayers. For co-culture experiments, dermal fibroblasts grown on 3.0 μm pore cell culture inserts (BD Falcon, Franklin Lakes, NJ, USA) were added to keratinocytes grown in 12 well plates in KBM-SFM. LPS was added to the co-culture media at 1 μg/ml for 24 h ± 45 or 360 μM WASO, EISO, or IB. Conditioned media was harvested for ELISA tests and Quantibody cytokine array analysis. In data not shown, we observed that medium alone, with or without an equivalent volume of DMSO, and cell-free supernatant derived from control, uninfected cells exhibited indistinguishable levels of basal cytokine/chemokine production.

Enzyme-linked immunosorbent assays. Sandwich ELISAs for IL-6, IL-8, MCP-1, CXCL5 (ENA-78), and GM-CSF (RayBiotech, Norcross, GA, USA) and for prostaglandin E2 and thromboxane B2 (Cayman Chemical, Ann Arbor, MI, USA) were performed on culture supernatants from the indicated treatments according to manufacturer's instructions. Standard curve was constructed with supplied standards to allow conversion of OD_{450} absorbance readings of experimental samples to pg/ml. All samples were assayed in triplicate.

Cytokine antibody arrays. Fluorescent antibody array systems (RayBiotech, Inc. Norcross, GA, USA) were used to screen for secretion of cytokine-related factors in response to LPS treatment ± WASO, EISO, or IB. The array format QAH-INF-3 contained quadruplicate antibody spots for 40 cytokines and inflammation-related mediators and array format QAH-CHE-1 contained quadruplicate antibody spots for 40 chemokines.

The array slides were processed according to the manufacturer's instructions. Data acquisition was performed using a GenePix 4000B microarray scanner (Axon GenePix 4000B, Molecular Devices, LLC1311 Orleans Drive, Sunnyvale, CA 94089-1136 United States) and subsequent quantification performed using Bio Discovery ImaGene® 9.0 microarray analysis software (Bio Discovery, Inc. 5155 Rosecrans Avenue, Suite 310 Hawthorne, CA 90250). Signal intensity medians were background corrected, and the mean of the replicates was calculated. Signal intensities among the quadruplicates varied by less than ±10%. Some of the slide wells were treated with provided antigen standards in order to construct a standard curve. Prism 4 software (GraphPad Software, Inc. 7825 Fay Avenue, Suite 230 La Jolla, CA 92037 USA) was used to convert the calculated mean intensities of test samples to concentration (pg/ml).

Statistical analysis. MTS assay: Differences in cell viability, expressed as the OD ratio between treated and untreated cells at the indicated concentrations, were calculated using a Student's two-tailed *t*-test assuming equal variances.

Enzyme-linked immunosorbent assays: Results were compared using a one-way analysis of variance followed by a Bonferroni post-test comparing only the pairs of interest. All analysis of variance *p*-values were significant, and the post-test results are shown in the respective tables.

Cytokine antibody arrays: Prism 4 software was used to calculate differences in concentration of a given chemokine/cytokine across treatments using a two-sided *t*-test not assuming the variance to be equal followed by correction for multiple testing using the method of Benjamini Hochberg. Data acquisition for the RayBiotech fluorescent cytokine/chemokine antibody arrays and statistical analysis was performed by the Laboratory for Advanced Genome Analysis at the Vancouver Prostate Centre.

RESULTS

Cytotoxic profile of lipopolysaccharides and sandalwood oils

The WASO and EISO preparations were first assessed for cytotoxicity on keratinocyte and dermal fibroblast monocultures (Fig. 1A and B). At concentrations below 90 μM, the oils showed no apparent cytotoxic effects by MTS or microscopic examination (data not shown). At a concentration of ≥180 μM, dermal fibroblast viability was decreased 90%, while keratinocyte viability was decreased 55–60% at 180 μM and 80–90% at ≥ 360 μM of EISO or WASO; thus both populations exhibited acute cytotoxic effect at ≥180 μM. IB, used as an antiinflammatory positive control, exhibited no cytotoxic effects on either cell type at doses up to 360 μM (data not shown). All subsequent experiments for antiinflammatory properties of SOs were performed at ≤90 μM. In response to treatment with LPS for 24 h, dermal fibroblasts exhibited no adverse effects at doses up to 5 μg/ml, while viability of keratinocytes was decreased 20% at 3 μg/ml and >50% at ≥ 4 μg/ml. In all subsequent experiments, LPS was used at a final concentration of 1 μg/ml.

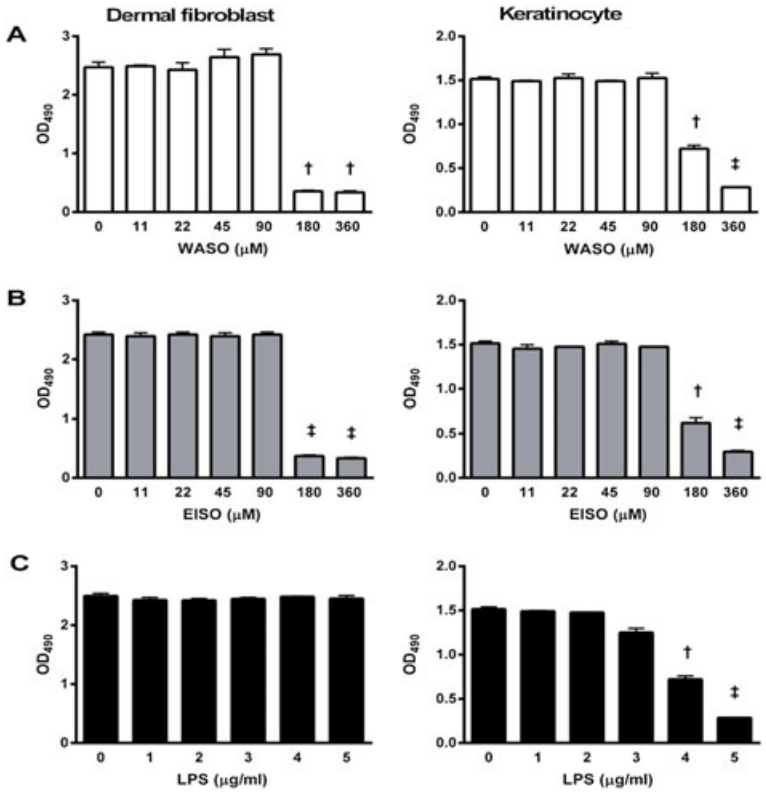

Figure 1. Cytotoxicity analysis of Western Australian sandalwood oil (WASO), East Indian sandalwood oil (EISO), and lipopolysaccharide (LPS) on dermal fibroblast and keratinocyte monocultures. Confluent dermal fibroblast (left column) and keratinocyte (right column) cultures were treated with the indicated concentrations of WASO (A), EISO (B), and LPS (C) for 24 h. Cell viability, expressed as OD_{490}, was measured by modified trichrome stain analysis relative to vehicle control (0) samples. †: $p < 0.01$ compared with control sample; ‡: $p < 0.001$ compared with control sample.

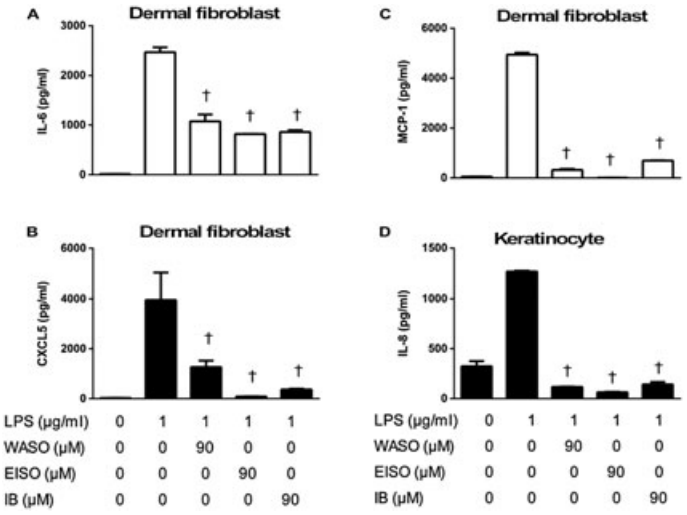

Figure 2. Lipopolysaccharide (LPS) stimulates, and Western Australian sandalwood oil (WASO), East Indian sandalwood oil (EISO), and ibuprofen (IB) suppress sentinel cytokines/chemokines production by dermal fibroblast/keratinocyte co-cultures. Confluent cultures of dermal fibroblasts (A–C) and keratinocytes (D) were treated ± LPS, WASO, EISO, and IB at the indicated concentrations for 24 h. Accumulation of IL-6 (A), CXCL5 (B), MCP-1 (C), and IL-8 (D) was determined from conditioned media by enzyme-linked immunosorbent assay. Cytokine/chemokine accumulation was compared with levels of LPS-stimulated samples, expressed as pg/ml, †: $p < 0.01$.

Table 2. Cytokine and chemokine production by lipopolysaccharide-stimulated dermal fibroblast/keratinocyte co-cultures treated with Western Australian sandalwood oil, East Indian sandalwood oil, or ibuprofen[a]

LPS (1 µg/ml):	−	+	+	+	+	+	+	+
Treatment:	DMSO[b]	DMSO	WASO	WASO	EISO	EISO	ibuprofen	ibuprofen
Concentration:	0.001%[c]	0.00%	45 µM	90 µM	45 µM	90 µM	45 µM	90 µM
GM-CSF	BD4	108	123	BD‡	9 ‡	BD ‡	152*	BD ‡
I-309	2	53	17 ‡	ND‡	0.55 ‡	7 ‡	3 †	19*
ICAM-1	BD	609	10 ‡	608 ‡	950 ‡	2505 ‡	330	BD†
IL-1a	12	114	71 ‡	155 ‡	122 †	285 ‡	54 †	879*
IL-6	28	3397	3397 †	118 ‡	1928 ‡	102 ‡	3094*	170 ‡
IL-8	37	778	823	484 ‡	634	483 ‡	997*	498 †
MCP-1	157	1786	2119	1939*	1953	399 †	1671 †	1110 ‡
MIP-1a	BD	178	36 ‡	BD	0.60 ‡	1 ‡	46 ‡	BD‡
RANTES	7	957	326†	23 ‡	37 ‡	27 ‡	146 ‡	38 ‡
TNF RI	2	152	464 ‡	204 †	130*	105	48*	BD ‡
TNF RII	BD	28	13 ‡	BD‡	BD ‡	BD‡	10 ‡	BD †
CXCL16	BD	334	12‡	BD‡	BD‡	BD*	BD‡	BD‡
CXCL5	341	10392	11341	140*	299*	BD*	9982	BD*
GCP-2	17	11009	574‡	488‡	384‡	300‡	3819	157‡
LIF	12	161	88	BD†	BD‡	BD‡	BD*	BD†
MCP-2	4	558	48†	10‡	12‡	9‡	30†	4‡
MCP-3	9	1225	63‡	19‡	14‡	20‡	353	52‡
MCP-4	BD	19	BD‡	BD‡	BD‡	BD	BD‡	BD‡
MIP-3a	4	125	4‡	4‡	4‡	4‡	12‡	3‡
NAP-2	BD	13	BD†	BD†	BD‡	BD‡	BD‡	BD‡

DMSO, dimethyl sulfoxide; WASO, Western Australian sandalwood oil; EISO, East Indian sandalwood oil; IB, ibuprofen.

[a]Cytokine (above dashed line) and chemokine (below dashed line) levels in conditioned media 24 h after treatment expressed as pg/ml from antibody array analysis as described in Materials and methods.

[b]DMSO added to cultures as vehicle control.

[c]DMSO final concentration in culture media expressed as percent (v/v).

[d]Below limit of detection (BD) when agents added.

For statistical analysis, a value of 1 pg/ml was used when cytokine concentration was below limit of detection. All LPS-treated values are significantly greater than DMSO alone samples ($p < 0.001$). Differences from LPS-treated samples: * = $p < 0.05$, † = $p < 0.01$, ‡ = $p < 0.001$.

Antiinflammatory properties of Western Australian sandalwood oil and East Indian sandalwood oil on monoculture of dermal fibroblast and keratinocytes

Prior to assessing antiinflammatory properties of the respective SO preparations, the ability of LPS to induce secretion of key cytokine-related factors (IL-6, IL-8, MCP-1, and CXCL5) was assessed in keratinocyte and dermal fibroblast monocultures. After 24 h of LPS stimulation, dermal fibroblast conditioned media contained substantially elevated levels of IL-6, MCP-1, and CXCL5 (Fig. 2A-C), but not IL-8 (data not shown). Conversely, keratinocyte-conditioned media contained substantially increased IL-8 levels after LPS stimulation (Fig. 2D), while IL-6, MCP-1, and CXCL5 levels were unchanged (data not shown). As a preliminary assessment of potential antiinflammatory properties of the SO preparations, we observed that accumulation of the indicated cytokines in conditioned media of the respective cells was significantly suppressed by WASO, EISO, or IB treatment. MTS assays performed on dermal fibroblast and keratinocyte at the end of the experiment indicated that none of the LPS, WASO, EISO, or IB treatment combinations affected cell viability relative to vehicle controls (data not shown). Additional control tests showed that the sandalwood oils did not interfere significantly in any of the ELISA steps. On the basis of these results, we decided to use the 24 h time point to evaluate the effects of SOs and IB on LPS-stimulated cytokine/chemokine profile in dermal fibroblasts and keratinocytes co-culture.

Cytokine and chemokine antibody arrays

To investigate the possibility that sandalwood essential oils might affect production of additional cytokines/chemokines, we analyzed similar cell-free conditioned media prepared from LPS induction in dermal fibroblast/keratinocyte co-cultures by fluorescent antibody arrays. Images of the arrays are provided in the Supporting Information (Figs S1 and S2). LPS treatment stimulated increased production of 16 of the 40 cytokines spotted on the arrays. Of these, G-CSF, GM-CSF, ICAM-1, IL-6, MIP-1α, and RANTES were detected at the highest concentrations (≥100-fold increase). I-309, IL-8, IL-11, TNF RI, and TNF RII were detected at moderate concentrations (≥20-fold increase). IL-1α, IL-6sR, MCP-1, MCSF, and MIP-1β were up regulated by greater than or equal to ninefold, while production of the other 24 cytokines present on the array was not affected greater than twofold by LPS stimulation. LPS treatment also stimulated production of ten of the 40 chemokines spotted on the arrays. Of these, CTACK, CXCL16, GCP-2, MCP-2, and MCP-3 were increased ≥100-fold, while CXCL5 was increased 30-fold and LIF, MCP-4, MIP-3a, and NAP-2 were increased greater than or equal to tenfold. The other 30 chemokines showed no significant response to LPS induction.

Table 2 summarizes the data for the 11 cytokines and nine chemokines elevated by LPS stimulation that were significantly suppressed by at least by of the EISO, WASO, or IB treatments. On average, treatment with WASO or EISO suppressed LPS-stimulated production of cytokines and chemokines by >75% at 45 μM and >90% at 90 μM, some less than or equal to the untreated levels (Table 2). The SOs alone showed no effect on cytokine production (data not shown). Because SOs have been previously ascribed to have antiinflammatory properties, we compared the SO effects with the ability of the NSAID, IB, to suppress LPS-stimulated cytokines and chemokines. Similar to the effects of the SOs, IB also significantly suppressed production of all the LPS-stimulated and SO-suppressed cytokine and chemokines (Table 2). IB suppressed LPS-stimulated CXCL5 by 4% and 98% at 45 and 90 μM, respectively. IL-6 was suppressed 3% at 45 μM and 32% at 90 μM. MCP-1 was suppressed 2% at 45 μM and 7% at 90 μM. IB suppressed LPS-stimulated production of the other 16 cytokines/chemokines by ≥90%.

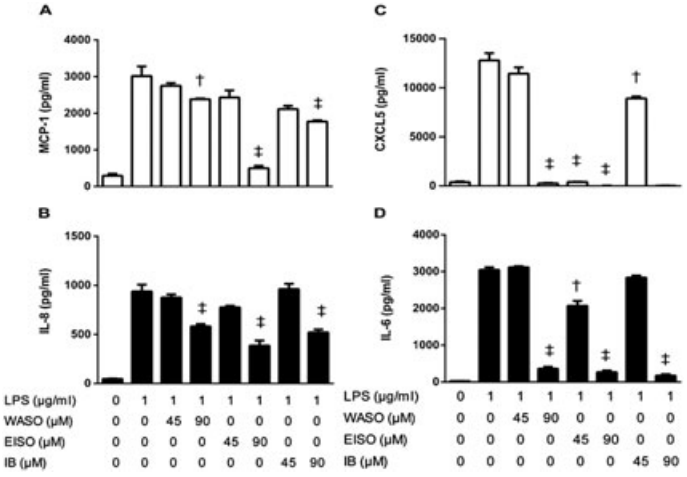

Figure 3. Enzyme-linked immunosorbent assay validation of sentinel lipopolysaccharide (LPS)-stimulated and Western Australian sandalwood oil (WASO), East Indian sandalwood oil (EISO), and ibuprofen (IB)-suppressed cytokine/chemokine production in dermal fibroblast/keratinocyte co-cultures. In transwell culture systems, confluent cultures of dermal fibroblast cultured on inserts and keratinocyte on lower chamber were treated ± LPS, WASO, EISO, and IB at the indicated concentrations for 24 h. Accumulation of MCP-1 (A), IL-8 (B), CXCL5 (C), and IL-6 (D) were determined from conditioned media by enzyme-linked immunosorbent assay. Cytokine/chemokine accumulation was compared with levels of LPS-stimulated samples, expressed as pg/ml, †: $p < 0.01$; ‡: $p < 0.001$.

Enzyme-linked immunosorbent assay validation of suppressed IL-6, IL-8, MCP-1, and ENA-78 production by WASO, EISO, and α-santalol and β- santalol

A subset of the cytokines and chemokines that were detected at high concentrations in response to LPS stimulation and suppressed by SOs and IB by fluorescent antibody array system was selected for validation by ELISA (Fig. 3). Consistent with the antibody array results, LPS-stimulated production of IL-6, lL-8, MCP-1, and CXCL5 by dermal fibroblast/keratinocyte co-cultures, and treatment with WASO or EISO significantly suppressed LPS-stimulated levels at 45and 90 μM.

In order to evaluate the contribution of the respective santalol isomers to the effect of the SOs on LPS-induced chemokine and cytokine production, co-cultures were similarly treated with LPS and 45 or 90 μM α-santalol or β-santalol (Fig.4). Both santalols exhibited equivalent dose dependent suppression of the five tested LPS-stimulated chemokines and cytokines. At 45 μM, α-santalol suppressed IL-6 (40%), IL-8 (91%), MCP-1 (84%), CXCL5 (99%), and GM-CSF (75%), while β-santalol suppressed IL-6 (51%), IL-8 (74%), MCP-1 (78%), CXCL5 (99%), and GM-CSF (71%). At 90 μM, both α-santalol and β-santalol reduced LPS-stimulated production of these cytokines/chemokines to levels indistinguishable from control values. These results demonstrate that the santalols are the major antiinflammatory agents in SOs and show for the first time that β-santalol was as effective as α-santalol as an antiinflammatory agent in equimolar concentrations.

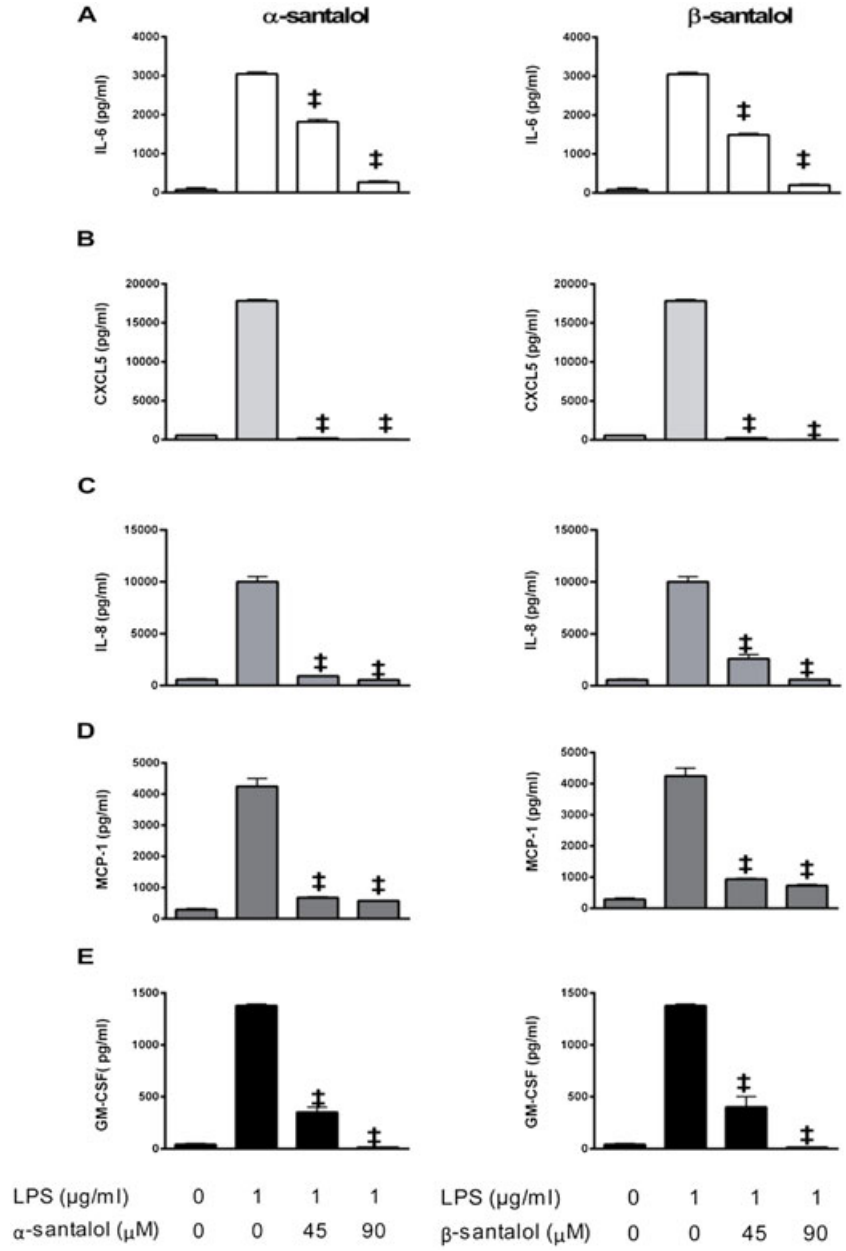

Figure 4. Suppression of lipopolysaccharide (LPS)-stimulated accumulation of sentinel cytokines/chemokines by α-santalol and β-santalol in dermal fibroblast/keratinocyte co-cultures. Transwell co-cultures of confluent dermal fibroblasts and keratinocytes were prepared as in Fig. 3 and treated ± LPS, α-santalol (left column) and β-santalol (right column). Accumulation of IL-6 (A), CXCL5 (B), IL-8 (C), MCP-1 (D), and GM-CSF (E) was determined from conditioned media by enzyme-linked immunosorbent assay. Cytokine/chemokine accumulation was compared with levels of LPS-stimulated samples, expressed as pg/ml, ‡: $p < 0.001$.

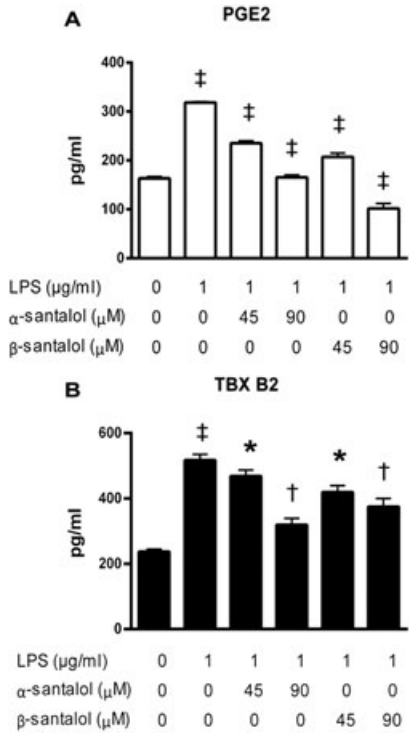

Figure 5. Lipopolysaccharide (LPS) stimulates and α-santalol and β-santalol suppress PGE2 and TBX B2 production dermal fibroblast/keratinocyte co-cultures. Transwell co-cultures of confluent dermal fibroblasts and keratinocytes were prepared as in Figure 3 and treated ± LPS, α-santalol and β-santalol at the indicated concentrations. Accumulation of PGE2 (A) and TBX B2 (B) were determined from conditioned media by enzyme-linked immunosorbent assay. Accumulation of these cyclooxygenase-mediated arachidonic acid metabolites was compared with levels of LPS-stimulated samples, expressed as pg/ml, *: $p < 0.05$; †: $p < 0.01$; ‡: $p < 0.001$.

Cyclooxygenase inhibition by α-santalol and β-santalol in dermal fibroblast/keratinocyte co-culture

The mechanism of action, as well the side effects, of NSAIDs such as IB is explained by inhibition of cyclooxygenase (COX)-mediated arachidonic acid metabolism (Rao and Knaus, 2008). Because IB mimicked the antiinflammatory effects of SOs on LPS-stimulated cytokine and chemokine production, we next investigated whether purified α-santalol and β-santalol could mimic NSAID activity and disrupt arachidonic acid metabolism, by evaluating the ability of the santalol isomers to suppress LPS-induced prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) production in dermal fibroblast/keratinocyte co-cultures (Fig. 5). LPS induced an approximate twofold increase in steady state PGE2 and TXB2 levels, which was then dose-dependently suppressed by α-santalol and β-santalol at 45 and 90 μM, respectively. While statistically indistinguishable, surprisingly, the β-santalol appeared to be marginally more effective at suppressing production of these arachidonic acid metabolites. These results suggest that α-santalol and β-santalol may exert antiinflammatory effects by down-regulating COX activity like NSAID.

DISCUSSION

While the essential oil emulsion or paste of sandalwood is routinely used in India as an Ayurvedic medicine to treat inflammatory skin diseases, such as acne, much of the information available about the antiinflammatory properties of SOs is anecdotal, as a consequence of inadequate standardization and poor characterization of most preparation. The SO preparations used here, α-santalol represents ~50% of the EISO by weight as compared with ~20% of WASO, while β-santalol represents ~25% of EISO by weight as compared with ~10% of WASO. In this study, we addressed the antiinflammatory effects of SO preparations on skin, using human epidermal keratinocyte/dermal fibroblast co-culture models. We determined that the SO preparations were well tolerated by the respective monoculture with essentially no cytotoxic effects observed at concentrations of up to 90 μM. LPS, used as an inflammatory stimulus, and IB, used as an antiinflammatory positive control, had no cytotoxic effects at the doses used. The antiinflammatory activity we observed for WASO, EISO, and IB in a pilot test of LPS-stimulated dermal fibroblasts and keratinocytes indicated that both the SOs and IB substantially suppressed expression of indicator cytokine/chemokines. These findings are consistent with the previously reported antiinflammatory properties of SO preparations seen in a recently completed clinical trial of SO-containing acne products (Moy *et al.*, 2012).

On the basis of evaluation of LPS stimulation and SO suppression kinetics (data not shown), we decided to use 24 h following LPS induction to evaluate the effects of Sos and IB on a panel of 40 cytokines and chemokines in dermal fibroblast/keratinocyte co-cultures. The similar ability of the SO preparations and IB to suppress expression of the 19 of the 80 LPS-stimulated chemokine and cytokines included in the antibody arrays suggests that the SOs may have a similar mechanism of action. The ability of purified α-santalol and β-santalol used at the same concentrations as WASO and EISO to significantly suppress expression of five sentinel chemokines/cytokines indicates that the santalols are the primary factors contributing to the antiinflammatory properties of SOs and, for the first time, it was shown that β-santalol is as effective as α-santalol at suppressing LPS-stimulated proinflammatory events.

The mechanism of antiinflammatory action of IB is inhibition of COX and production of the resulting arachidonic acid metabolites (Rao and Knaus, 2008). In this study, suppression of LPS-stimulated PGE2 and TXB2 production was evaluated as an indication of COX inhibition in skin cell co-cultures. The ability of α-santalol and β-santalol at 45 and 90 μM to equivalently suppress LPS-stimulated PGE2 and TXB2 production suggests that santalols may function by inhibiting COX activity in cells. Combined with the lack of cytotoxicity of SO preparations, these observations support the supposition that SOs may be beneficial alternatives for treating the UV light-induced acute sunburn reactions without photosensitization or other side effects of NSAIDs.

The distinct immunomodulating activities of WASO, EISO, and α-santalol and β-santalol are demonstrated here by suppressed expression of the majority of LPS-stimulated cytokines and chemokines. Keratinocytes play an important role in the regulation of skin inflammation. For example, strong expression of IL-8 and related chemokines by psoriatic keratinocytes is responsible for neutrophil recruitment to lesions (Nickoloff and Turka, 1994). MCP-1, RANTES, MCP-4, MIP-1β, and MIP-3α are also strongly expressed by the psoriatic

epidermis (Gillitzer *et al*., 1993; Dieu-Nosjan *et al*., 2000). Furthermore, CXCL5/ENA-78 in humans can be expressed by cells of the epidermis such as keratinocytes (Boniface *et al*., 2005), as well as infiltrating monocytes (Walz *et al*., 1997), and it is plausible that both of these cell types contribute to the increased expression of CXCL5/ENA-78 after ultraviolet B irradiation (Dawes *et al*., 2011) The ability of the WASO and EISO preparations to suppress LPS-induced secretion of numerous proinflammatory cytokines and chemokines supports the concept that such extracts could be beneficial in treating various inflammatory cutaneous diseases, such as psoriasis, acne, and skin warts. EISO was more effective than WASO, presumably because of the higher concentrations of the α-santalol and β-santalol, because purified α-santalol and β-santalol showed the same antiinflammatory effect as the sandalwood essential oils.

These data demonstrate that sandalwood essential oils, as well as purified α-santalol and β-santalol, are capable of significantly suppressing the expression of specific proinflammatory signaling molecules in co-cultures of stimulated skin cells. Inflammation, and associated discomfort, is a hallmark of many skin conditions and diseases, and there is a need for better methods of safely and effectively reducing or preventing inflammation. The study also explains usefulness of LPS-stimulated *in vitro* systems for the evaluation of the antiinflammatory properties of plant extracts using this methodological approach (Magni *et al*., 2012; Yan *et al*., 2013). The novel mechanism of biological action described herein for santalols provides an impetus for conducting additional preclinical studies with sandalwood oil and its constituents with the goal of further evaluating these materials in the clinical setting. The data presented in this paper, as well as the recently completed clinical trial of SO-containing acne products that yielded promising results (Moy *et al*., 2012), provide further support for the concept that traditional medicines, such as SO, are valuable additions to the modern therapeutic armamentarium.

Acknowledgements

This work was supported by an Industrial Research Award from Santalis Pharmaceuticals to M.E.C. and by support from the Laboratory for Advanced Genome Analysis at the Vancouver Prostate Centre.

Conflict of Interest

C.L. is a paid employee of Santalis Pharmaceutical Co.

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