International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 5 (1): 37-43, 2009
ISSN 1811-7775

Torreya nucifera Essential Oil Inhibits Skin Pathogen Growth and Lipopolysaccharide-Induced Inflammatory Effects

[1]W.J. Yoon, [2]S.S. Kim, [2]T.H. Oh, [2]N.H. Lee and [1]C.G. Hyun
[1]Research Group for Cosmetic Materials, Jeju Biodiversity Research Institute and
Jeju Hi-Tech Industry Development Institute, Jeju 697-943, Korea
[2]Department of Chemistry, Cheju National University, Jeju 690-756, Korea

Abstract: In this study, the chemical composition of *Torreya nucifera* essential oil (TEO) and its biological activities were analyzed. TEO was obtained by steam distillation from leaves collected from Jeju Island and analyzed using gas chromatography (GC)-flame ionization detection (FID) and GC-MS. dl-Limonene (30.1%), δ-3-carene (15.37%) and α-pinene (11.5%) were the major components in TEO. The antibacterial and anti-inflammatory activities of TEO against skin pathogens have not previously been reported. Thus, we assessed the antibacterial activities of TEO using the disk diffusion method. TEO showed excellent antibacterial activities against *Propionibacterium acnes*, *Propionibacterium granulosum*, *Malassezia furfur*, *Staphylococcus epidermidis* and *Candida albicans*. The minimum inhibitory concentration (MIC) of TEO against these skin pathogens ranged from 2.5 to 20.0 μL mL^{-1}. In addition, TEO reduced the LPS-induced secretion of interleukin-1β (IL-1β), IL-6, NO and PGE_2 in RAW 264.7 cells, indicating that it has anti-inflammatory effects. Therefore, we suggest that TEO may be an attractive candidate for promoting skin health.

Key words: Acne, essential oil, inflammation, MIC, skin pathogen, *Torreya nucifera*

INTRODUCTION

A range of microorganisms are found on normal human skin, some of which are present as part of the resident commensal flora. The dominant microbial groups include *Propionibacteria*, *Staphylococci* and *Malassezia* sp. (Bojar and Holland, 2002; Holland *et al*., 2008). On normal undamaged skin these resident species are considered nonpathogenic. However, under certain conditions that are not fully understood, they cause opportunistic infections that may present serious medical problems such as acne.

The pathogenesis of acne involves multiple interrelated factors and events (Tom and Barrio, 2008; Webster, 2005). Upon puberty onset, an increase in androgen production leads to increased sebum production. Abnormal follicular cornification and desquamation cause occlusion of the pilosebaceous duct. As a result, sebum is trapped, with consequent formation of open and closed comedones. *P. acnes*, a normal anaerobic resident of the skin surface, colonizes the occluded pilosebaceous follicles and breaks down sebum to free fatty acids and peptides. An inflammatory response to the bacterium and these metabolic byproducts leads to formation of papules, pustules and nodules (Bergfeld, 2004; Tom and Barrio, 2008; Webster, 2005). It is therefore important to screen remedies that may control the colonization of these microorganisms, either as commensals or pathogens, in the skin environment. Antibiotics continue to be the most widely prescribed therapy for acne vulgaris, a multifactorial disease. Because *Propionibacteria*, especially *Propionibacterium acnes* and *P. granulosum* are implicated in the pathogenesis of acne (Coates *et al*., 2002; Eady and Cove, 2000; Ingham, 1999; Webster, 1995), it is assumed that antibiotics work primarily as anti-propionibacterial agents; however, direct or indirect anti-inflammatory activity is another possible, but disputed, mode of action (Coates *et al*., 2002; Webster, 1995). Topical antibiotics reduce the population of *P. acnes* and other skin pathogens and exert anti-inflammatory activity, but feature the major disadvantage of dramatically increasing bacterial resistance. A new approach for preventing antibiotic resistance is the use of essential oil therapy. Although trials have shown that treatments with essential oils prevent the emergence of resistance and sometimes enhance the synergic effects, some limitations remain in essential oil treatment (Loughlin *et al*., 2008;

Corresponding Author: Hyun Chang-Gu, Research Group for Cosmetic Materials, Jeju Biodiversity Research Institute and Jeju Hi-Tech Industry Development Institute, Jeju 697-943, Korea
Tel: +82-64-720-2811 Fax: +82-64-720-2801

Shahverdi *et al*., 2007; Si *et al*., 2008). Thus, further studies of essential oils are needed. Earlier studies have suggested that several essential oils possess important antimicrobial activity against acne-causing pathogens (Baik *et al*., 2008; Kim *et al*., 2008a) and have therapeutic potential, mainly in diseases involving skin infections. The major constituents of many of these oils are phenolic compounds (terpenoids and phenylpropanoids) like thymol, carvacrol or eugenol, the antimicrobial and antioxidant activities of which are well documented (Lawrence, 2005; Lopes-Lutz *et al*., 2008).

The genus *Torreya nucifera* is an ornamental, usually aromatic herb, belonging to the Taxaceae family. It is a slow-growing coniferous tree native to southern Japan and to South Korea's Jeju Island. It grows to 15-25 m tall with a trunk up to 1.5 m diameter. The leaves are evergreen, needle-like, 2-3 cm long and 3 mm broad, with a sharply spined tip and two whitish stomatal bands on the underside; they are spirally arranged but twisted at the base to lie horizontally either side of the stem. In Korea, the fruits of *T. nucifera* have been used since ancient times as an ingredient in folk remedies for hookworm. Recent reports identified several medicinal functions for *T. nucifera*, such as anti-oxidant and anti-inflammatory effects (Cho *et al*., 2004). In addition, *Torreya nucifera* have shown hepatoprotective and neuroprotective effects (Jang *et al*., 2001; Kim *et al*., 2003).

In contrast, anti-acne and anti-inflammatory effects for TEO have not been described. In this study, we demonstrate that TEO has high antibacterial and anti-inflammatory effects against skin pathogens. Therefore, we suggest that TEO may be employed as an effective therapeutic agent to ameliorate skin disease. To the best of our knowledge, this is the first report demonstrating the *in vitro* anti-inflammatory activity of TEO and providing a scientific basis for its cosmetic use.

MATERIALS AND METHODS

Plant material and extraction: An ethnobotanical survey was carried out on Jeju Island of South Korea in October 2006. Voucher specimens were identified by Dr. G. Song and deposited in the JeJu Biodiversity Research Institute (JBRI) (Jeju, South Korea). The essential oil of *T. nucifera* leaves (mature) was extracted by hydrodistillation as described by Kim *et al*. (2008). Briefly, approximately 1 kg of fresh *T. nucifera* leaves was immersed in 3.5 L of distilled water in a 5 L three-neck flask. Steam distillation was carried out for 12 h at atmospheric pressure.

The essential oil was analyzed by GC/MS on a Hewlett-Packard mass spectrometer 5975 at 70 eV coupled to an HP 6890 GC equipped with a DB1-HT column (30×0.32 mm × 0.1 μm). The oven temperature was programmed from 40 to 100°C at a rate of 2°C min^{-1}, 100 to 230°C at a rate of 5°C min^{-1} and held at 230°C for 5 min (71 min analysis time). The injector and detector temperatures were 240 and 280°C, respectively. The flow rate of the carrier gas (He) was 1.5 mL min^{-1} and the split ratio was 1:10. For the injection (splitless), 10 μL of essential oil was diluted in 500 μL of CH_2Cl_2 and 1 μL of this diluted solution was injected. The volatile constituents were identified on the basis of their mass spectra, which were compared to those in the literature. The retention indices were calculated using a homologous series of n-alkanes C_6-C_{25}. The peak areas of individual compounds were related to total peak areas of compounds detected by GC.

Pathogens: Three Gram-positive bacterial species and one yeast strain that are each involved in acne, *P. acnes* ATCC6919, *Propionibacterium granulosum* ATCC25564, *Staphylococcus epidermidis* KCTC 3958 and *Malassesia furfur* KCCM 12679, were selected as test microorganisms according to their pathological capacity. We also used *Candida albicans* KCCM11282 as a test pathogen. *Propionibacterium* strains were cultured at 37°C for 48 h in GAM broth (Nissui, Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions before the assay. *S. epidermidis* KCTC3958 was cultured at 37°C for 24 h with *Corynebacterium* media (casein peptone 10.0 g, yeast extract 5.0 g, glucose 5.0 g, NaCl 5.0 g L^{-1}). *M. furfur* was grown on YM agar containing 1% olive oil at 37°C for 24 h. *C. albicans* was also cultured at 37°C for 24 h in YM broth.

Disc diffusion assay: The inhibitory effect of TEO on test bacteria was determined by the agar diffusion method. The culture suspensions were adjusted to 4 McFarland. The wells (Φ 7.0 mm) were prepared and the essential oil, diluted in ethanol to the test concentration, was added to the wells (20 μL); an equal volume (20 μL) of ethanol was used as a control. The inoculated plates were incubated at 37°C for 48 h under anaerobic conditions for *Propionibacterium* sp. (BBL GasPak System). Other pathogens were incubated at 37 °C for 24 h under aerobic conditions. After incubation, the diameter of the inhibition zone was measured with calipers.

Minimum inhibitory concentration (MIC) determination: The microdilution broth susceptibility assay was used in the determination of MIC. A stock solution of essential oil was prepared in 10% dimethylsulfoxide (DMSO) and serially diluted to concentrations ranging

from 2.5 to 40 μL mL^{-1}. The 96-well plates were prepared by dispensing 95 μL of culture broth, 100 μL of essential oil and 5 μL of the inoculants into each well. A positive control (containing inoculum but no essential oil) and negative control (containing essential oil but no inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at proper temperature and incubation times. The MIC was defined as the lowest concentration of the compounds that inhibited the growth of microorganisms. The experiment was performed in triplicate.

Determination of nitric oxide (NO) and prostaglandin ($PGE)_2$ production: Nitrite production, an indicator of NO synthesis, was measured in the supernatant of cultured macrophages by the Griess reaction (Hevel and Marletta, 1994). After pre-incubation of RAW 264.7 cells (1.5×10^5 cells mL^{-1}) with LPS (1 μg mL^{-1}) for 24 h, the quantity of nitrite in the culture medium was measured as an indicator of NO production. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid], the mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined from a sodium nitrite standard curve. All experiments were performed in triplicate. PGE_2 levels in macrophage culture medium were measured with a commercially available ELISA kit according to the manufacturer's instructions (RandD Systems, Minneapolis, MN, USA).

Measurement of pro-inflammatory cytokine (TNF-α, IL-1β and IL-6) production: The inhibitory effect of TEO on pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α) production in LPS-treated RAW 264.7 cells was determined as described by Cho *et al.* (2000). Supernatants were used for pro-inflammatory cytokine assays with mouse ELISA kits (R and D Systems Inc., MN, USA). All experiments were performed in triplicate.

Lactate dehydrogenase (LDH) cytotoxicity assay: Lactate dehydrogenase (LDH) leakage provides a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. LDH activity was determined following the production of NADH during the conversion of lactate to pyruvate (Fernandez *et al.*, 2006). The release of LDH from RAW 264.7 cell was used to detect cytotoxicity and was measured at the end of each proliferation experiment. The LDH activity was determined using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, culture medium was centrifuged at 12,000 rpm for 3 min at room temperature to ensure accumulation of cells. The cell-free culture medium (50 μL) was collected and then incubated with 50 μL of the reaction mixture from the cytotoxicity detection kit for 30 min at room temperature in the dark. 1N HCl (50 μL) was added to each well to stop the enzymatic reaction. The optical density of the solution was then measured using an ELISA plate reader at 490 nm. Percent cytotoxicity was determined relative to the control group. All experiments were performed in triplicate.

Statistical analysis: The Student's t-test and one-way ANOVA were used to determine the statistical significance of differences between the values for the various experimental and control groups. Data were expressed as Means±Standard errors (SEM) and the results were taken from at least three independent experiments performed in triplicate. p-values of 0.05 or less were considered statistically significant.

RESULTS AND DISCUSSION

The pale-yellow essential oil was obtained by hydrodistillation in a Clevenger-type apparatus from leaves of *T. nucifera* at a yield of 0.1% (v/w) on dry weight basis. The TEO was analyzed by GC-MS with DB1-HT column. The general chemical profile of the essential oil, the percentage content and the retention indices of the constituents are shown in Table 1. A total of 32 components were characterized on the basis of a typical library search and literature data; only components showing matches that exceeded 80% were selected and these represented about 95% of the TEO. Three compounds were identified that represent more than 56% of the TEO: dl-limonene (30.1%), δ-3-carene (15.37%) and α-pinene (11.5%). Other chemical components included δ-cardinene (6.87%), germacrene D (3.89%), α-bisabolol (3.62%), β-farnesene (3.22%) and α-copaene (3.09%). The chemical class distribution of the essential oil components of the plants are reported in Table 2. The compounds were separated into four classes: monoterpenes, sesquiterpenes, diterpenes and others. As shown in Table 2, monoterpene components were the main constituents of TEO, accounting for 63.7%. The major compounds for the chemical class distributions in the TEO are reported in Table 3. dl-Limonene was the most abundant constituent at 30.1%.

The antimicrobial activities of essential oils extracted from many plants have been recognized, albeit empirically, for centuries; only recently have such properties been confirmed (Lee *et al.*, 2007). The

Table 1: Chemical composition (%) of *Torreya nucifera* essential oil

Retention time (min)	Constituent	Peak area (%)	Retention time (min)	Constituent	Peak area (%)
3.371	α-Pinene ($C_{10}H_{16}$)	11.50	12.754	trans-Caryophyllene ($C_{15}H_{24}$)	2.22
3.498	Camphene ($C_{10}H_{16}$)	1.24	12.875	Caryophyllene ($C_{15}H_{24}$)	0.75
4.143	cis-Ocimene ($C_{10}H_{16}$)	0.70	12.974	γ-Elemene ($C_{15}H_{24}$)	0.27
5.014	β-Myrcene ($C_{10}H_{16}$)	1.61	13.160	α-Humulene ($C_{15}H_{24}$)	1.79
5.663	δ-3-Carene ($C_{10}H_{16}$)	15.37	13.456	β-Farnesene ($C_{15}H_{24}$)	3.22
6.394	dl-Limonene ($C_{10}H_{16}$)	30.10	13.555	Germacrene D ($C_{15}H_{24}$)	3.89
6.823	β-Phellandrene ($C_{10}H_{16}$)	0.12	13.643	β-Cubebene ($C_{15}H_{24}$)	1.19
7.543	α-Terpinolene ($C_{10}H_{16}$)	2.42	13.719	Germacrene B ($C_{15}H_{24}$)	1.11
8.125	1-Octyne ($C_{10}H_{16}$)	0.12	13.796	α-Muurolene ($C_{15}H_{24}$)	1.10
9.268	α-Terpineol ($C_{10}H_{18}O$)	0.14	13.906	α-Amorphene ($C_{15}H_{24}$)	0.47
10.132	4-Chlorocctylacetate ($C_{19}H_{17}O_2Cl$)	0.10	14.158	δ-Cardinene ($C_{15}H_{24}$)	6.87
10.823	4-Methyl-bicyclo[3.2.1]oct-3-en-2-one ($C_9H_{12}O$)	0.15	14.367	β-Bisabolene ($C_{15}H_{24}$)	0.36
11.449	Undecane ($C_{11}H_{24}$)	0.01	15.365	α-Copaene ($C_{15}H_{24}$)	3.09
11.778	α-Terpinene ($C_{10}H_{16}$)	0.48	15.859	α-Bisabolol ($C_{15}H_{26}O$)	3.62
11.920	α-Cubebene ($C_{15}H_{24}$)	0.28	16.210	(E)-Farnesol ($C_{15}H_{26}O$)	0.32
12.403	Valencene ($C_{15}H_{24}$)	0.15	21.398	Ferruginol ($C_{20}H_{30}O$)	1.02

Table 2: The chemical class distribution of the components of *Torreya nucifera* essential oil

Compound class	Area (%)	No. of compounds
Monoterpenes	63.70	10
Sesquiterpenes	30.70	17
Diterpenes	1.02	1
Others	0.38	4

Table 3: The major components in the chemical classes of the constituents of *Torreya nucifera* essential oil

Compound class	Major component	Area (%)
Monoterpenes	dl-Limonene	30.10
Sesquiterpenes	α-Cubebene	16.21
Diterpenes	Ferruginol	1.02
Others	4-Methyl-bicyclo[3.2.1]oct-3-en-2-one	0.15

Table 4: Antimicrobial activity of *Torreya nucifera* essential oil

Skin pathogen	Inhibition zone (mm)	MIC value (μL mL^{-1})
Propionibacterium acnes	9	20.0
Propionibacterium granulosum	9	20.0
Malassezia furfur	12	2.5
Staphylococcus epidermidis	12	5.0
Candida albicans	11	10.0

To elucidate the antibacterial activity of TEO against skin pathogens, we introduced a disk diffusion method. TEO was found to have significant antibacterial activities. Erythromycin was employed as a positive control. The inhibitory diameters against *P. acnes* and *P. granulosum* were 22 and 24 mm at concentration of 25 μg per disk

botanical source, provenance of the plant, harvest time or development stage, extraction technique, use of fresh or dried plant material, test microorganisms and antimicrobial methodology are all factors that influence the antimicrobial activity (Cosentino *et al*., 1999; Janssen *et al*., 1987; Kim *et al*., 2008b) and must therefore be taken into account whenever antimicrobial assays are performed with these oils. Here, the antimicrobial activities of TEO were determined by paper disc diffusion and MIC tests against a panel of skin pathogens. As shown in Table 4, TEO showed excellent activity against the test pathogens. The results from the disc diffusion method and measurements of MIC indicate that *M. furfur* was the most sensitive pathogen with the largest inhibition zone (12 mm) and lowest MIC value (2.5 μL mL^{-1}). Other sensitive pathogens were *S. epidermidis* and *C. albicans*, with MIC values of 5.0 and 10.0 μL mL^{-1}, respectively. TEO also had moderate antibacterial activities against *P. acnes* and *P. granulosum* (MIC, 20 μL mL^{-1}).

Since, acne vulgaris is the result of the combined action of bacterial infection and the inflammatory response to that infection, we next examined whether TEO possessed anti-inflammatory activity. To investigate the effect of TEO on NO production, we measured the accumulation of nitrite, a stable oxidized product of NO, in culture media. NO production was examined in RAW264.7 cells stimulated with LPS for 24 h in the presence or absence of TEO. Nitrite levels in LPS-stimulated cells increased significantly compared to that in control cells. As shown in Fig. 1a, TEO (0.025, 0.05 and 0.1 μL mL^{-1}) markedly inhibited LPS-induced NO production by RAW264.7 cells in a dose-dependent manner. 2-Amino-4-methylpyridine (2-AMP), a standard NOS inhibitor, was used as a positive control and caused significant inhibition. The numbers of viable activated macrophages were not altered by TEO as determined by LDH assays, indicating that the inhibition of NO synthesis by TEO was not simply due to cytotoxic effects.

COX-2 is induced by cytokines and other activators, such as LPS, in a variety of inflammatory cells, including macrophages, resulting in the release of large amounts of PGE_2 at inflammatory sites. Therefore, we examined the effects of TEO on PGE_2 production in LPS-stimulated RAW264.7 macrophages. When macrophages were stimulated with LPS (1 μg mL^{-1}) for 24 h, the levels of PGE_2 increased in the culture medium. As shown in Fig. 1b, TEO (0.025, 0.05 and 0.1 μL mL^{-1}) suppressed LPS-induced PGE_2 production in a dose-dependent manner. NS-398 is a COX-2 selective inhibitor and had significant inhibitory effects on PGE_2 production. Interestingly, 20 μM 2-AMP also showed inhibitory activity. IL-1β, IL-6 and TNF-α are produced primarily by

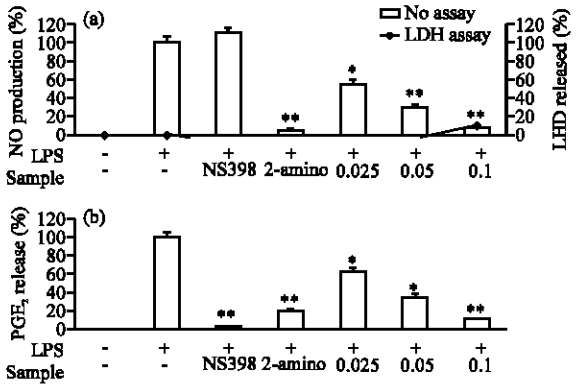

Fig. 1: Inhibitory effects of *T. nucifera* essential oil on (a) nitric oxide and (b) PGE_2 production in RAW264.7 cells. Cells (1.5×10^5 cells mL^{-1}) were stimulated by LPS (1 μg mL^{-1}) for 24 h in the presence of the oil extract from *T. nucifera* (0.025, 0.05 and 0.1 μL mL^{-1}), NS-398 (20 μM) and 2-amino-4-methylpyridine (20 μM). Supernatants were collected and the PGE_2 concentration was determined by ELISA. Cytotoxicity was determined using the LDH method. Values are the Mean±SEM of triplicate experiments. *p<0.05; **p<0.01

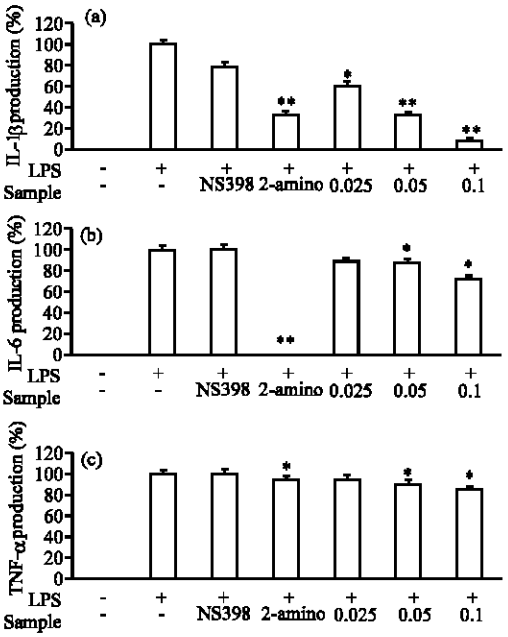

Fig. 2: Inhibitory effect of *T. nucifera* essential oil on (a) IL-1β, (b) IL-6 and (c) TNF-α production in RAW264.7 cells. Cells (1.5×10^5 cells mL^{-1}) were stimulated by LPS (1 μg mL^{-1}) for 24 h in the presence of the oil extract from *T. nucifera* (0.025, 0.05 and 0.1 μL mL^{-1}), NS-398 (20 μM) and 2-amino-4-methylpyridine (20 μM). Supernatants were collected and the IL-1β, IL-6 and TNF-α concentrations in the supernatants were determined by ELISA. Values are the Mean±SEM of triplicate experiments *p<0.05; ** p<0.01

activated monocytes or macrophages. Because TEO potently inhibited pro-inflammatory mediators, we investigated its effects on LPS-induced IL-1β, IL-6 and TNF-α release using enzyme immunoassays (EIA). After 24 h incubation with both LPS (1 μg mL^{-1}) and TEO, there was remarkable inhibition of IL-1β and IL-6 production in RAW264.7 cells (Fig. 2).

As mentioned previously, TEO exhibited antibacterial and anti-inflammatory activities. These activities may be attributed to the presence of limonene, δ-3-carene, α-pinene and bisabolol in TEO. α-Pinene and limonene have moderate antibacterial activities (Pichette *et al*., 2006; Kim *et al*., 2008a). These chemical components exert their toxic effects against these microorganisms by disrupting bacterial membrane integrity. α-Pinene is able to destroy cellular integrity, thereby inhibiting respiration and ion transport processes. It also increases membrane permeability in yeast cells and isolated mitochondria (Andrews *et al*., 1980; Uribe *et al*., 1985). This finding is strongly supported by a study on the effects of different essential oil components on membrane permeability in acne-inducing bacteria (Helander *et al*., 1998). 3-Carene has proved to be an emergent alternative as an antifungal agent against dermatophyte strains (Cavaleiro *et al*., 2006). Low concentrations of 3-carene also stimulate the differentiation of mouse osteoblastic MC3T3-E1 subclone 4 cells (Jeong *et al*., 2008). Bisabolol is the active anti-inflammatory agent in chamomile extract and is widely used in cosmetic formulations (Yarosh *et al*., 2006).

In conclusion, we have demonstrated that TEO has good anti-bacterial and anti-inflammatory effects. Therefore, we suggest that TEO may be an effective therapeutic agent to promote skin health. However, although the antimicrobial and anti-inflammatory effects of TEO against acne-inducing bacteria were identified, the mechanism of action was not determined. Thus, the possible mechanisms of inhibition of pro-inflammatory cytokines remain to be evaluated in further studies.

ACKNOWLEDGMENT

This research was supported by the Regional Technology Innovation Program (RTI04-02-07), which is managed by the Ministry of Knowledge and Economy, Korea.

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