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Clove extract and eugenol suppress inflammatory responses elicited by *Propionibacterium acnes* *in vitro* and *in vivo*

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ABSTRACT

Acne vulgaris is a multifactorial inflammatory disease involving an activation of the immune response to *Propionibacterium acnes*. The aim of this study is to determine the effects of clove (*Syzygium aromaticum* L.) extract and eugenol against *P. acnes*-induced inflammatory responses. The results showed that treatments of ethanolic clove extract (ECE) or eugenol at noncytotoxic concentrations significantly suppressed *P. acnes*-stimulated NF- κ B-mediated tumor necrosis factor- α , interleukin (IL)-1 β , and IL-8 productions and matrix metalloproteinase-9 expression in THP-1 cells *in vitro*. In order to investigate their effects *in vivo*, histological assessment was examined in the *P. acnes*-induced mouse ear edema model. The concomitant intradermal injection of ECE and eugenol resulted in reduction of ear swelling in mice along with microabscess. These observations demonstrate that ECE and eugenol inhibit *P. acnes*-induced inflammatory responses and suggest that ECE and eugenol might be a beneficial agent in treatment of skin inflammation induced by *P. acnes*.

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Acne; *Propionibacterium acnes*; clove; eugenol; anti-inflammation

Introduction

Acne vulgaris is an inflammatory skin disease. Inflammation has been demonstrated in all acne lesions (the preclinical microcomedo, comedones, inflammatory lesions, post-inflammatory erythema or hyperpigmentation, and scarring). Inflammation occurs in the very beginning and throughout all stages of acne lesions (Dreno et al., 2015). *Propionibacterium acnes* is a trigger for innate immunity both in very early (microcomedogenic) and in late (inflammatory) acne lesions and promotes both acute and chronic inflammation (Dreno et al., 2015; Tanghetti, 2013). *P. acnes* may contribute to the development of inflammatory acne lesions by releasing chemotactic substances via the activation of toll-like receptor 2, and trigger the release of pro-inflammatory cytokines, including tumor

necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-1 β , and IL-12 (Kim et al., 2002; Kurokawa et al., 2009; Tanghetti, 2013). Pro-inflammatory cytokines are considered to be responsible for the further follicular hyperkeratinization and the characteristic of inflammatory acne lesions (Tanghetti, 2013). Inflammation is critical to all types of acne lesion; therefore, anti-inflammatory agents can be expected to be effective for treating acne.

Extracts from traditional herbs have been applied to the treatment of a variety of inflammatory diseases. Ethanolic extract of saffron, a well-known spice, could be used as an anti-arthritic agent in control of inflammation in rheumatoid arthritis (Zamani Taghizadeh Rabe et al., 2015). The extract from *Sasa veitchii* leaves has been shown to suppress skin inflammation elicited by contact sensitizers (Usuda, Fujii, & Nonogaki, 2016). The natural remedies may serve as an alternative treatment in acne therapy. The anti-acne effects of these medicinal plants may be involved in various distinct mechanisms, including anti-bacterial, anti-inflammatory, anti-oxidant, and anti-androgen activities (Azimi, Fallah-Tafti, Khakshur, & Abdollahi, 2012). We previously reported that plant extracts such as rosemary and wild bitter melon, and their bioactive constituents such as rosmarinic acid, phytol, lutein, and phenolic compounds suppress *P. acnes*-induced pro-inflammatory cytokine releases (Hsu et al., 2012; Huang et al., 2015; Tsai et al., 2013). As part of our continuing efforts to discover promising bioactive natural agents having anti-acne activity, the effect of clove extract on *P. acnes*-induced inflammation was examined in this study. Cloves are the dried flower buds of clove tree, *Syzygium aromaticum* (synonym: *Eugenia caryophyllata*). Clove has been added to foods not only as a flavoring agent, but also as a preservative due to its anti-oxidant and anti-bacterial properties. In Chinese traditional medicine, clove is used to treat dyspepsia, acute/chronic gastritis, and diarrhea. Clove oil has been revealed with various pharmacological and biological properties such as anesthetic and analgesic effects as well as anti-microbial, anti-oxidant, anti-inflammatory, anti-convulsant, anti-carcinogenic, anti-mutagenic, and neuro-protective activities (Bachiega, de Sousa, Bastos, & Sforcin, 2012; Kamatou, Vermaak, & Viljoen, 2012; Kannappan, Gupta, Kim, Reuter, & Aggarwal, 2011). Eugenol (4-allyl-2-methoxyphenol), a component found in clove oil, is commonly used as a flavoring agent in cosmetics and food products and, in particular, in zinc oxide-eugenol chelating cement in dentistry. Previous studies demonstrated that eugenol exhibits anti-inflammatory, anti-bacterial, anti-oxidative, and neuro-protective activities (Bachiega et al., 2012; Devi, Nisha, Sakthivel, & Pandian, 2010; Nagababu, Rifkind, Boindala, & Nakka, 2010; Prasad & Muralidhara, 2013). Although clove has been used worldwide for a long period, however, its effects on *P. acnes*-induced inflammatory skin disease have not yet been fully elucidated. In this study, we aimed to reveal possible anti-inflammatory effects of clove extract and eugenol against *P. acnes*.

Materials and methods

Materials

The strain of *P. acnes* (BCRC10723, isolated from facial acne) was obtained from the Bior-source Collection and Research Center, Hsinchu, Taiwan. *P. acnes* was cultured in an anaerobic condition using BBL GasPak systems (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Briefly, brain heart infusion (BHI) broth (Difco,

Detroit, MI, USA) supplemented with 1% glucose was streaked with *P. acnes* for overnight. Single colonies of bacteria were isolated and used to inoculate BHI broth with 1% glucose. Bacterial log phase was monitored by spectrophotometer OD₆₀₀ value. The human monocytic THP-1 cell line (BCRC 60430) was also obtained from the Bioresource Collection and Research Center. Cells were maintained in RPMI 1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% CO₂.

The assay kits for TNF- α , IL-8, and IL-1 β were purchased from Invitrogen (Carlsbad, CA, USA). An NF- κ B/p65 ActivELISA kit was purchased from Imgenex (San Diego, CA, USA). Eugenol and isoeugenol were purchased from Sigma (St. Louis, MO, USA). All chemicals are analytical-grade purity.

Preparation of clove extract

Dried clove buds were purchased from Tomax Enterprise Co. (Taipei, Taiwan). Voucher samples are stored in the author's laboratory. Briefly, 10 g of finely ground clove was extracted with 100 mL of ethanol at room temperature for 4 h. After extraction, the mixture was filtered, and the residue was re-extracted with 100 mL of fresh ethanol overnight. After centrifugation at 12,000 \times g for 10 min, the combined ethanol solution was collected and evaporated in a rotary evaporator. The ethanol extract was reconstituted with dimethyl sulfoxide (DMSO) to a concentration of 400 mg/mL for the subsequent experiments. The yield of the ethanol extract from clove buds was 13.2%.

High-performance liquid chromatography analysis of ethanolic clove extract

The ethanolic clove extract (ECE) (0.1 g) was dissolved in 10 mL of methanol, and passed through a 0.2- μ m membrane filter (Millipore, MA, USA). HPLC analysis was used to separate and determine individual phenolic compounds of ECE. HPLC analysis was performed using HPLC pumps (Ecom LCP 4100, Czech Rep.) equipped with a UV detector (Ecom LCD 2084, Czech Rep.) and chromatographic separations were performed on a C-18 reversed-phase silica Bondclone column (3.9 \times 300 mm i.d., 10 μ m, Phenomenex, Torrance, CA, USA). Chromatographic processing was done using the Peak-ABC Chromatography Data Handling System. The mobile phase was a mixture of solvent A (water), and solvent B (acetonitrile) according to a linear gradient elution from 80% A / 20% B (v/v) to 60% A / 40% B (v/v) during 0–20 min, at a flow-rate of 1 ml/min. The following gradient was used from 60% A / 40% B (v/v) to 60% A / 40% B (v/v) during 20–25 min, from 60% A / 40% B (v/v) to 40% A / 60% B (v/v) during 25–30 min, from 40% A / 60% B (v/v) to 80% A / 20% B (v/v) during 30–35 min. The absorbance of elutes was measured at 280 nm. The injection volume was 20 μ L and all samples were analyzed in triplicate. Identification of the individual compounds was based on the comparison of the retention times of unknown peaks to those of reference authentic standards.

Determination of the viability of THP-1 cells

A suspension of THP-1 cells (1 \times 10⁶ cells/ml) was cultured in 96-well culture plates with treatment of various concentrations of the clove extract or eugenol. After 24 h of

incubation, 20 μ L of Alamar blue reagent (Invitrogen, Carlsbad, CA, USA) was added to each well. After 2 h of incubation, the optical density of medium was measured, and the difference in the absorbance values at 570 and 600 nm was calculated.

Measurement of cytokine production in human monocytic THP-1 cells

To prepare *P. acnes* suspension for subsequent experiments, *P. acnes* culture was harvested at log phase of growth, washed with PBS, and then centrifuged at 10,000 rpm for 5 min. After two additional washes with PBS, the *P. acnes* pellet was re-suspended in RPMI medium. THP-1 cells were seeded at 1×10^6 cells/mL in 24-well plates with serum-free medium, and were treated with tested samples alone or stimulated with live *P. acnes* (wet weight 200 μ g/mL of bacteria; 7.5×10^7 colony-forming units (CFU)/mL) alone or in combination with different concentrations of tested samples for a 24-h incubation. Cell-free supernatants were collected, and concentrations of TNF- α , IL-1 β , and IL-8 were analyzed with respective enzyme immunoassay kits.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Complementary (c)-DNA was generated from 2 μ g of total RNA, with the oligo (dT) primer and 1 μ L of reverse transcriptase (Promega, Madison, WI, USA). Primers and probes were selected for the following cytokines and chemokines: IL-1 β , TNF- α , and IL-8. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene. We used the AAGCTGAGGAAGATGCTG and ATCTACACTCTCCAGCTG primers for IL-1 β , the TCTTCTGCCTGCACTTTGG and ATCTCTCAGCTCCACGCCATTG primers for TNF- α , the TGCCAAGGAGTGC-TAAAG and CTCCACAACCTCTGCAC primers for IL-8, and the GTGAAGGTGCGAGTCAACG and TGAGGTCAATGAAGGGGTC primers for GAPDH. These primer pairs amplified, respectively, a 300 bp fragment of the IL-1 β cDNA, a 224 bp fragment of the TNF- α cDNA, a 157 bp fragment of the IL-8 cDNA, and a 113 bp fragment of the GAPDH cDNA. cDNA was prepared, diluted, and subjected to real-time polymerase chain reaction (PCR) using an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA, USA) and iQTM SYBR Green Supermix (Bio-Rad). The relative amounts of the PCR products were analyzed by iQTM5 optical system software, version 2.1. The messenger (m)RNA level of each sample for each gene was normalized to that of the GAPDH mRNA.

Detection of matrix metalloproteinase-9 level by western blot analysis

THP-1 cells were seeded at 2×10^6 cells/mL in 6-cm dishes and were stimulated with viable *P. acnes* (wet weight 200 μ g/mL) alone or co-incubated with various concentrations of tested samples. After 24 h of treatment, cells were harvested and washed with PBS. Whole-cell lysates were prepared in a lysis buffer (Cell Signaling, Beverly, MA, USA) containing 10 mM phenylmethylsulfonyl fluoride. The cell lysates were sonicated and centrifuged at 4°C, 14,000 rpm for 10 min. The protein concentration was determined by DC protein assay (Bio-Rad). Aliquots of the lysates (each containing 50 μ g of protein) were boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. The resolved

proteins were then blotted to polyvinylidene difluoride (PVDF) membranes (Millipore) and incubated with gelatin-NET (0.25% gelatin, 0.15 M NaCl, 5 mM EDTA-Na, 0.05% Tween 20, 50 mM Tris) buffer at room temperature to block nonspecific binding. Then primary antibodies anti-human matrix metalloproteinase (MMP)-9 (Epitomics, Burlingame, CA, USA) and anti-human β -actin (Sigma) diluted in gelatin-NET buffer were added, and incubated for another hour. This anti-human MMP-9 monoclonal antibody recognizes both the latent (92 kDa) and active (83 kDa) forms of human MMPs-9. The bound primary antibody was detected using a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA) and the enhanced chemiluminescence kit (Bio-Rad) according to the manufacturer's instructions. Immuno-reactive proteins were detected with the enhanced ECL chemiluminescence Western blotting detection system (Chemidoc XRS, Bio-Rad). Signal strengths were quantified using densitometric program (Image Lab™ Software, Bio-Rad).

NF- κ B activation assay

NF- κ B activation was analyzed using quantification of nuclear-translocated p65 subunit with the NF- κ B/p65 ActivELISA kit (Imgenex; San Diego, CA, USA). To determine the effect of the clove extract and eugenol on *P. acnes*-induced activation of NF- κ B, THP-1 cells (3×10^6 cell/mL) cultured in serum-free medium were stimulated with *P. acnes* (200 μ g/mL) alone or in combination with the indicated concentrations of tested samples. After 16 h of incubation, cytoplasmic and nuclear extracts were prepared according to the manufacturer's instructions. Briefly, the cytoplasmic fraction was collected in the supernatant of whole-cell lysates after centrifugation at $12,000\times g$ for 30 s at 4°C. The nuclear pellet was re-suspended in 100 μ L nuclear lysis buffer at 4°C for 30 min, and the suspension was centrifuged at $12,000\times g$ for 10 min at 4°C. The supernatant containing the nuclear fraction was subjected to an enzyme-linked immunosorbent assay (ELISA) to detect the active form of the p65 subunit, according to the manufacturer's instructions. The absorbance was read at 405 nm using a Synergy HT multidetection microplate reader (BioTeck).

***P. acnes*-induced inflammation in vivo**

The animal use protocol was reviewed and approved by the Animal Care Committee of the National Taiwan Normal University. Eight-week-old male ICR mice were purchased from the BioLASCO Taiwan Co., Ltd., Yilan, Taiwan and kept on a 12 h light/12 h dark cycle at $21 \pm 2^\circ\text{C}$ with food and water ad libitum. In the preliminary test, intradermal injection of ECE (0.5 mg in 10 μ L PBS) and eugenol (2 μ g in 10 μ L PBS) to mice ears did not cause noticeable skin irritation (data not shown). Then, we chose the nonirritated dosages for the following experiments. ICR mice were randomly grouped ($n = 8$ per group). Live *P. acnes* (6×10^7 CFU/10 μ L in PBS) was intradermally injected into the right ear of ICR mice to induce edema, while PBS (10 μ L) was injected into the left ear as a PBS vehicle control. In the treatment groups, clove extract (0.5 mg) or eugenol (2 μ g) in 10 μ L PBS was injected into the same location of right ear after *P. acnes* inoculation, while the left ear received intradermal PBS injection. In the negative control group, the right ear remained untreated and the left ear received intradermal PBS. After 24-h

injection, ear thickness was measured using a micro-caliper (Mitutoyo, Kanagawa, Japan). Mice were then sacrificed with carbon dioxide asphyxiation. The punch biopsies of 5.0 mm diameter were taken from the ears and weighed. The ear thickness and biopsy weight were calculated and expressed as the percentage of the PBS-injected ear. For histological observation, the paraffin embedded ears were vertically cut into cross-sections. The cross-sections were stained with hematoxylin and eosin (H&E) and then viewed on a microscope for the evaluation of inflammatory responses.

Statistical analysis

All data are presented as the mean \pm standard deviation (SD). Statistical analyses were performed using the SPSS 19.0 statistical package (Chicago, IL, USA). The data were evaluated for statistical significance with the one-way ANOVA followed by Duncan's multiple range tests. A *p* value of $<.05$ was considered statistically significant.

Results

Characterization of phenolic compounds in clove extract

Identification of eugenol in ECE was made by comparing the chromatographic feature of the peak and retention time 20.4 min for the standard reference as presented in [Figure 1](#). The amount of eugenol in ECE was 26.86 mg/g dry weight. HPLC analysis revealed that eugenol was the major component found in ECE.

Effects of ECE and eugenol on *P. acnes*-induced cytokine production and MMP-9 level

The ECE (up to 200 $\mu\text{g}/\text{mL}$) and eugenol (up to 200 μM) had no significant cytotoxicity toward THP-1 cells and did not affect the basal (constitutive) levels of IL-8, IL-1 β , or TNF- α by THP-1 cells (data not shown). Upon treatment of *P. acnes*, the secretions of IL-8, IL-1 β , and TNF- α significantly increased in THP-1 cells ([Figure 2](#)). While, treatment with ECE ([Figure 2\(a\)](#)) and eugenol ([Figure 2\(b\)](#)) significantly suppressed the *P. acnes*-induced IL-8, IL-1 β , and TNF- α production. To further investigate the effect of clove extract and eugenol on *P. acnes*-activated induction of cytokines, mRNA expressions of these cytokines were evaluated by quantitative real-time PCR. As shown in [Figure 3](#), whenever cells were treated with clove extract ([Figure 3\(a\)](#)) and eugenol ([Figure 3\(b\)](#)), the cytokine mRNA levels induced by *P. acnes* were significantly decreased. The effects of clove extract and eugenol on *P. acnes*-induced MMP-9 level were performed. Results of the Western blot analysis are shown in [Figure 4](#). Induced MMP-9 levels by *P. acnes* were reduced significantly by either treatment of clove extract and eugenol.

ECE and eugenol inhibited *P. acnes*-induced NF- κ B activation

Activation of the transcription factor NF- κ B is an essential step for activating the transcription of various cytokines and chemokines. We evaluated the NF- κ B p65 translocation to the nucleus in *P. acnes*-stimulated THP-1 cells after treatment with clove extract and

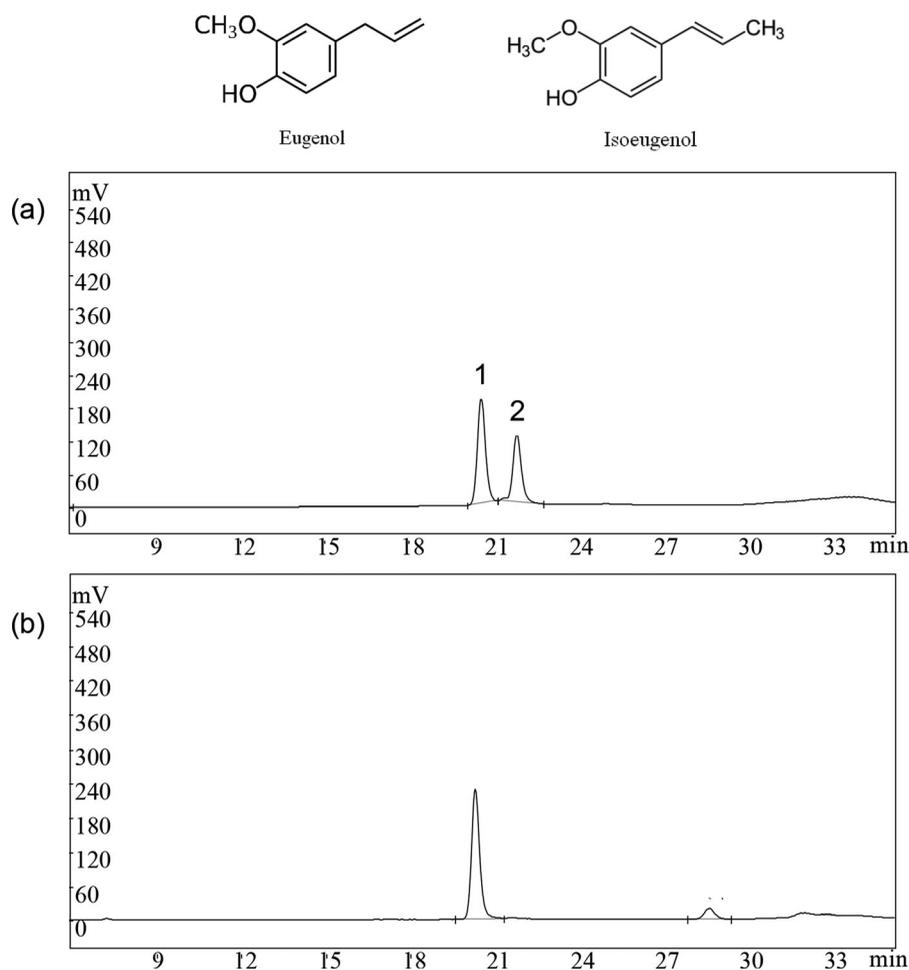


Figure 1. HPLC chromatograms of standard compounds (a) and compounds in ECE (b). Detection was at 284 nm. Peak 1: eugenol; peak 2: isoeugenol.

eugenol. Stimulation of THP-1 cells with *P. acnes* resulted in a significant increase of NF- κ B translocation after 16 h of incubation (Figure 5). However, treatment with clove extract (Figure 5(a)) and eugenol (Figure 5(b)) significantly attenuated the increased NF- κ B translocation in *P. acnes*-stimulated THP-1 cells after 16 h of incubation.

Effect of ECE and eugenol on *P. acnes*-induced inflammation in vivo

Prior to the determination of the anti-inflammatory effect of clove extract and eugenol *in vivo*, an intradermal injection test was performed to evaluate the skin irritation effect of clove extract and eugenol. No visible irritation, such as ear swelling, redness, and cutaneous erythema, and no histological increasing infiltrated neutrophil response by intradermal administration of either clove extract (0.5 mg per site) and eugenol (2 μ g per site) alone (data not shown). To examine the *in vivo* anti-inflammatory effect of clove extract and eugenol, mouse ears were intradermally injected with live *P. acnes* for one day. Infiltrated neutrophils were observed at an H&E-stained frozen cross-section

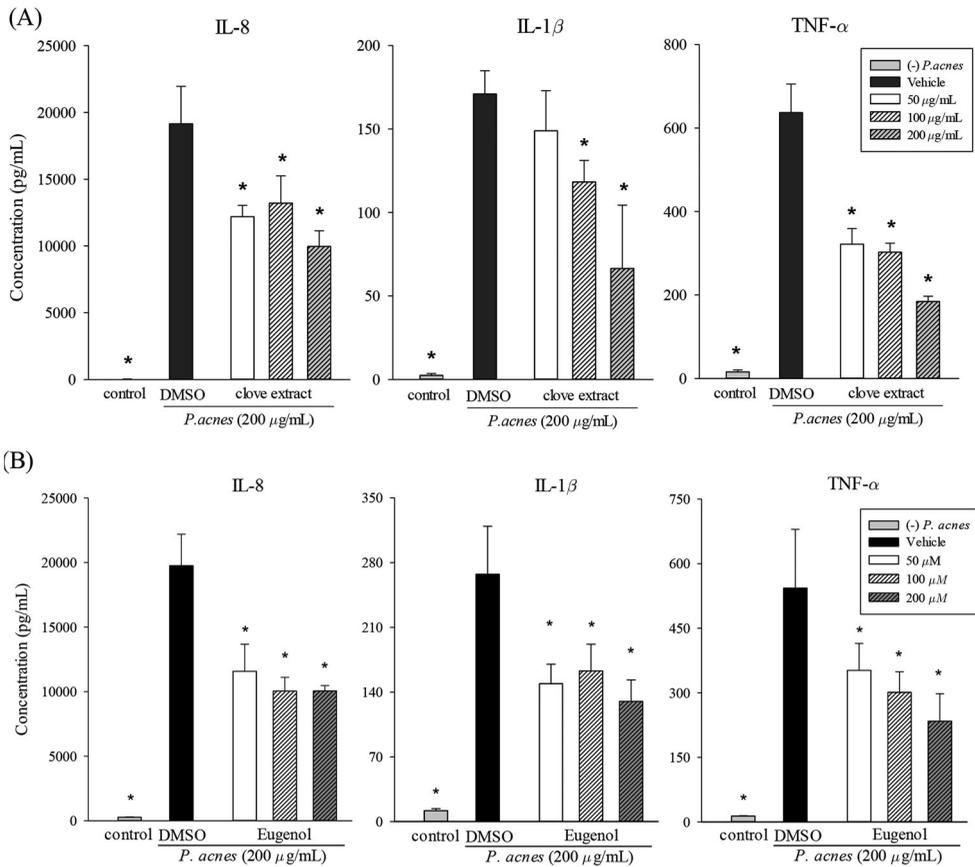


Figure 2. Effects of ECE (a) and eugenol (b) on the productions of pro-inflammatory cytokines in *P. acnes*-stimulated monocytic THP-1 cells. THP-1 cells were incubated for 24 h without *P. acnes* (control) and with *P. acnes* alone (DMSO vehicle) or with *P. acnes* in the presence of clove extract or eugenol. Data are expressed as the mean \pm SD. * $p < .05$ compared to the DMSO vehicle.

of the *P. acnes*-injected ear (Figure 6(a), magnification panel). When being co-injection with bacteria suspension, both ECE and eugenol attenuated the size of *P. acnes*-induced microabscess (Figure 6(a)). In addition, the *P. acnes*-induced ear swellings were reduced significantly by simultaneous injections of clove extract and eugenol, and shown as ear thickness (Figure 6(b)) and ear biopsy weight (Figure 6(c)).

Discussion

Inflammation plays a key role in the development of acne vulgaris. The expression of TNF- α , IL-1 β , and IL-8 are significantly up-regulated in acne-involved skin (Kang et al., 2005). TNF- α is one of the key pro-inflammatory cytokines that plays a central role in mediating inflammatory responses and innate immunity (Sabio & Davis, 2014). IL-1 β has been shown as a potent inducer of pro-inflammatory cytokines IL-6 and IL-8 in sebocytes, suggesting a potential role in diseases of the pilosebaceous unit such as acne. Recently, active form of IL-1 β has been proven abundant in inflammatory acne lesions (Kistowska et al., 2013). IL-8 is a CXC chemokine and a chemoattractant involved in the recruitment of neutrophils that is

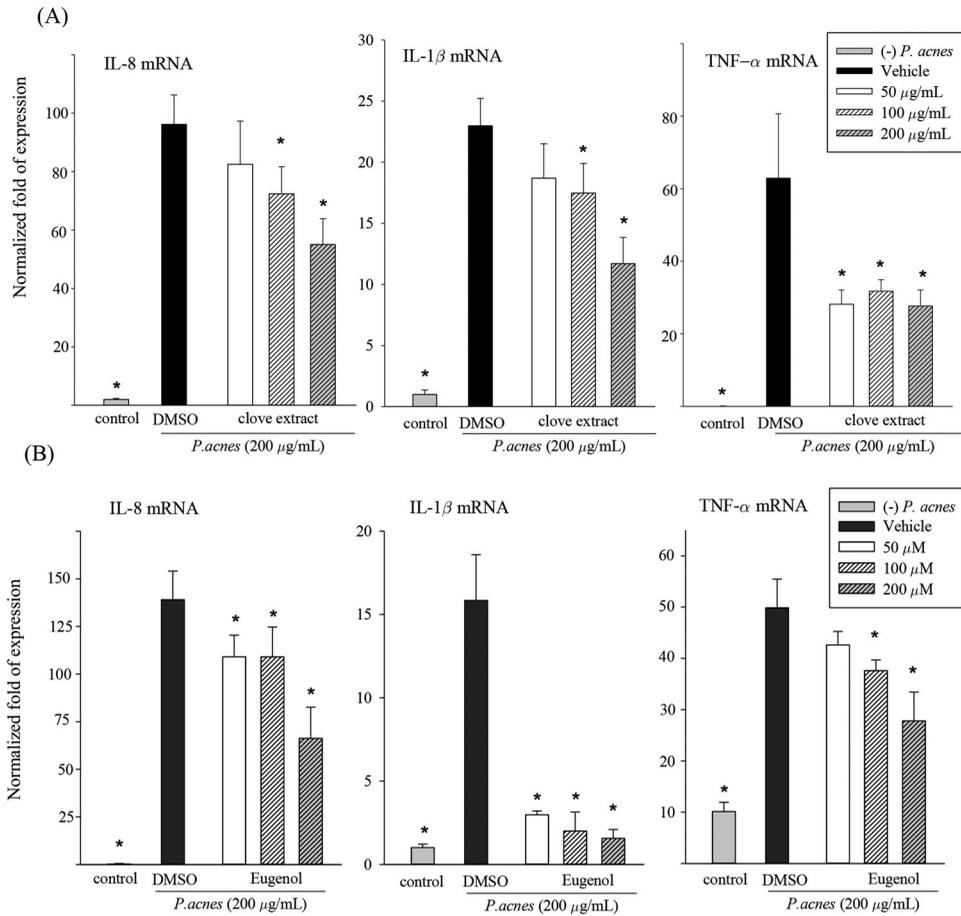


Figure 3. Effects of ECE (a) and eugenol (b) on mRNA expression of pro-inflammatory cytokines in *P. acnes*-stimulated monocytic THP-1 cells. THP-1 cells were incubated for 16 h without *P. acnes* (control) and with *P. acnes* alone (DMSO vehicle) or with *P. acnes* in the presence of clove extract or eugenol. The expression level of mRNA was determined using quantitative real-time PCR. The expression of cytokine mRNA was normalized with GAPDH mRNA and expressed as fold-change with untreated THP-1 cell as control. Data are presented as the means \pm SD. * $p < .05$ compared to the DMSO vehicle.

found to occur in acne-related lesions (Kim, 2005; Trivedi, Gilliland, Zhao, Liu, & Thiboutot, 2006). Taken altogether, targeting of production and secretion of pro-inflammatory cytokines may be a therapeutic option for acne vulgaris.

The preventive effects of clove extract and eugenol have been shown on endotoxin lipopolysaccharide (LPS)-induced the productions of PGE₂, nitric oxide, and pro-inflammatory cytokines by mouse macrophages cells *in vitro* (Hong et al., 2002; Kim et al., 2003; Rodrigues, Fernandes, Sousa, Bastos, & Sforcin, 2009). In addition, eugenol has been demonstrated to inhibit the formation of leukotriene-C4 in human polymorphonuclear leukocytes with stimulation of arachidonic acid and calcium ionophore A23187 (Raghavenra, Diwakr, Lokesh, & Naidu, 2006). Although the anti-inflammatory and cancer chemopreventive activities of clove and its components have been reported, little is known

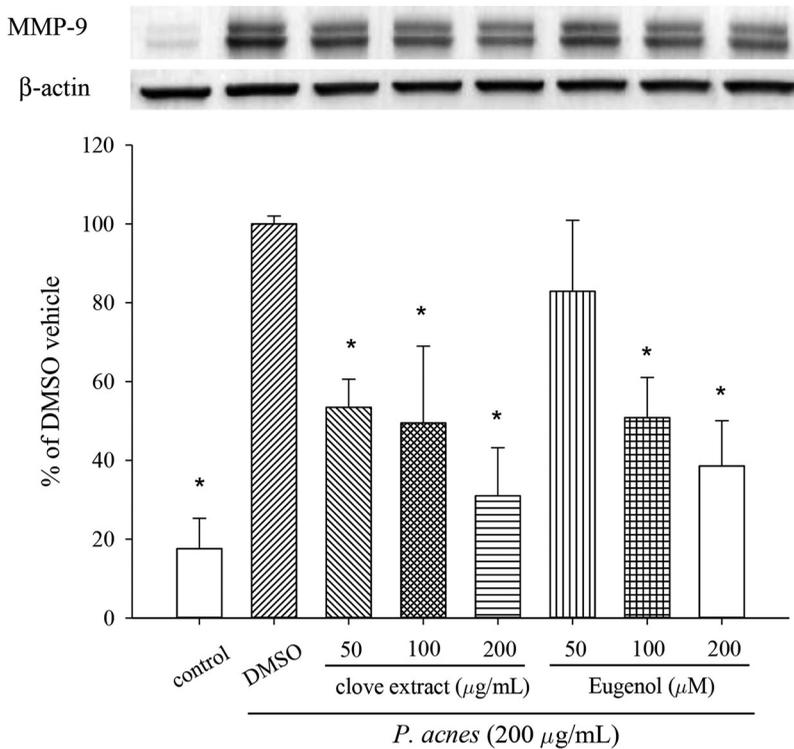


Figure 4. Inhibitory effect of ECE (a) and eugenol (b) on *P. acnes*-induced expression of matrix metalloproteinase (MMP)-9 by THP-1 cells. Cells were co-incubated with DMSO (as the vehicle) or the indicated concentration of samples and live *P. acnes* for 24 h. A control experiment without *P. acnes* treatment was conducted in parallel. Each column shows the mean \pm SD. The relative density of each band after normalization for β -actin was shown under each immunoblot as a fold-change compared with DMSO treatment. * $p < .05$ compared to the DMSO vehicle.

about their effects on *P. acnes*-induced inflammatory responses. Our current results demonstrate that the simultaneous treatments of clove extract or eugenol with *P. acnes* significantly inhibited pro-inflammatory cytokine protein level and mRNA expression (Figures 2 and 3). These results suggested that ECE and eugenol suppress cytokine expression, at least partially, at the transcriptional level.

Besides pro-inflammatory cytokines, MMPs may play an important pathological role in inflammatory acne lesions. MMPs not only are crucial effector molecules of inflammatory cells but can modify the biological functions of cytokines and chemokines (Hu, Van den Steen, Sang, & Opdenakker, 2007; Parks, Wilson, & López-Boado, 2004). *P. acnes* could play a role in scar formation due to enhanced extracellular matrix degradation by MMP-stimulated peptidoglycan from the bacterial cell wall (Sato et al., 2011). Increased levels of MMP-1, MMP-3, and MMP-9 have been detected in acne lesions (Kang et al., 2005; Ray Jalian et al., 2008; Trivedi et al., 2006). Monocytes are the infiltrating leukocytes found early in an inflammatory site, and they release MMP-9 during extravasation and in an activated state (Henderson, Hobbs, Mathies, & Hogg, 2003; Lu & Wahl, 2005). It has been demonstrated previously that MMP-9 expression was induced by *P. acnes* in human monocytic THP-1 cells (Hsu et al., 2012) and primary human monocytes (Ray Jalian et al.,

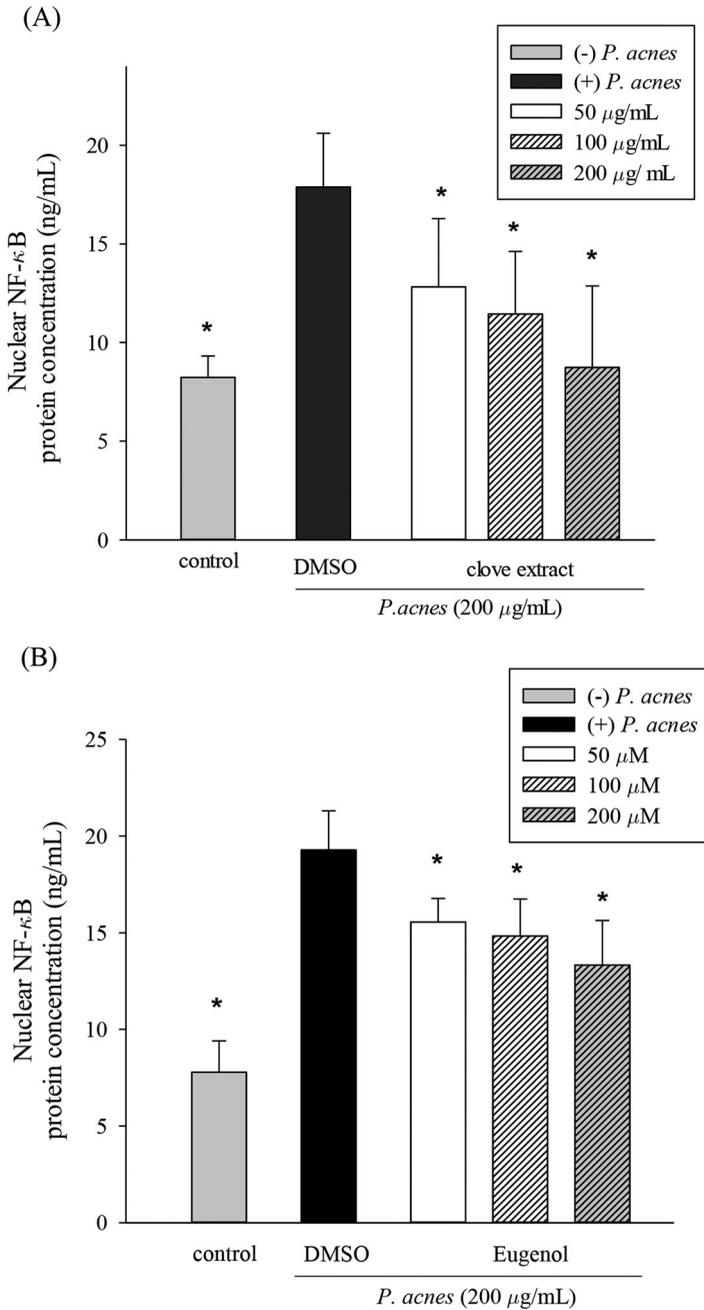


Figure 5. Effect of ECE (a) and eugenol (b) on *P. acnes*-induced NF κ B activation in THP-1 cells. THP-1 cells were incubated for 16 h without *P. acnes* (control) and with *P. acnes* alone (DMSO vehicle) or with *P. acnes* in the presence of clove extract or eugenol. Data are presented as the means \pm SD.* p < .05 compared to the DMSO vehicle.

2008). MMP inhibitors have been extensively studied as a potential new therapeutic agent for inflammatory diseases. For example, chemically modified tetracyclines with MMPs-inhibitory activity are effective against acne (Hu et al., 2007). A previous study reported

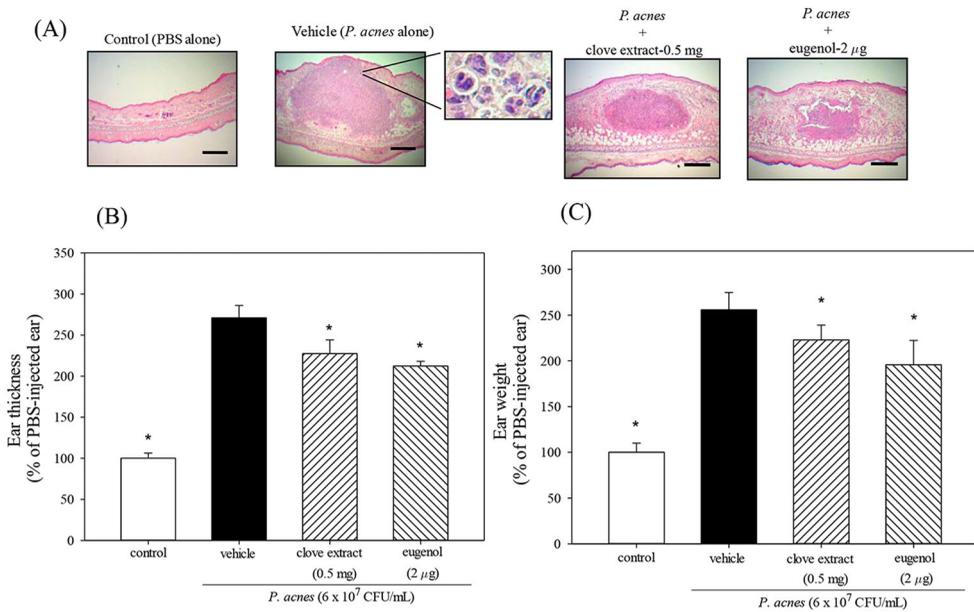


Figure 6. Inhibitory effect of ECE and eugenol on *P. acnes*-induced inflammation *in vivo*. Histology of ear biopsy of ICR mice which were intradermally injected with control (PBS), vehicle (*P. acnes* alone), or 0.5 mg of ECE and 2 μ g of eugenol in the presence of *P. acnes* were observed after hematoxylin and eosin (H&E) staining ($\times 100$ magnifications) (a). Scale bars represent 200 μ m. Infiltrated neutrophils were observed at an H&E-stained frozen cross-section of the *P. acnes*-injected ear (a) ($\times 1000$ magnification panel). The inhibitory effects of ECE and eugenol on *P. acnes*-induced ear edema in mice were evaluated by measuring the ear thickness (b) and ear biopsy weight (c). Uninfected/untreated ears served as a negative control. Data are presented as the means \pm SD. * $p < .05$ compared to the vehicle.

that eugenol inhibits protein expression of MMP-9 in phorbol-myristate-acetate-stimulated human fibrosarcoma HT1080 cells suggesting its application for prevention of metastasis (Nam & Kim, 2013). This study demonstrated ECE and eugenol capable of inhibiting *P. acnes*-induced MMP-9 expression (Figure 4). Taken together, these observations suggested that clove extract and eugenol may be potential candidates for preventing MMP-9-related pathological processes.

NF- κ B pathway plays a critical role in the inflammatory responses and also regularly and robustly activated by *P. acnes* (Kim, 2005; Kim et al., 2002). NF- κ B activation leads to the expressions of pro-inflammatory cytokines and MMPs. Cloves have been reported to suppress NF- κ B activation by suppressing inhibitory kappa B-alpha (I- κ B α) degradation (Kadoma, Murakami, Atsumi, Ito, & Fujisawa, 2009). Eugenol administration has been shown to abrogate NF- κ B activation in alveolar macrophages from BALB/c mice with LPS-induced lung injury and decrease NF- κ B expression in a rat model of gastric carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Magalhães et al., 2010; Manikandan, Vinothini, Vidya Priyadarsini, Prathiba, & Nagini, 2011). The results of treating THP-1 cells with ECE and eugenol inhibiting cytokine (Figure 2) and MMP-9 expressions (Figure 4) suggested the transcription factor NF- κ B as a possible target for ECE and eugenol-mediated effects. Our data supported that ECE and eugenol inhibit the translocation of NF- κ B to the nucleus in THP-1 cells stimulated with

P. acnes (Figure 5). These findings suggested that one mechanism by which ECE and clove reduce the productions of pro-inflammatory cytokines and MMP-9 is partially associated with the inhibition of *P. acnes*-mediated activation of NF- κ B.

Several animal models including rabbit ear model, rhino mouse model, Mexican hairless dog model, hamster model, swine, and guinea pig have been used in the study of acne (Avci et al., 2013). Compared to larger animals, mice have advantages such as small size and low cost. The acne-like inflammatory model was developed in the ears of Sprague-Dawley rats (De Young, Young, Ballaron, Spires, & Madli Puhvel, 1984) and ICR mice (Huang et al., 2015; Nakatsuji et al., 2008; Nakatsuji et al., 2009; Ryu et al., 2015). Intradermal injection with *P. acnes* causes local bacterial colonization and inflammation in the ears of ICR mice, which is similar to that in human acne lesions (Ryu et al., 2015). *P. acnes* injection significantly induces ear swelling, redness, and erythema in ICR mice and considerably increases in the number of infiltrated inflammatory cells such as macrophages, CD45 + leukocytes, and Ly6G + neutrophils (Huang et al., 2015; Nakatsuji et al., 2008). To evaluate the anti-inflammatory effects of clove extract and eugenol *in vivo*, we used a *P. acnes*-induced mouse ear edema model. Previously, oral administration of eugenol exerts *in vivo* anti-inflammatory activity on animal models of inflammation, the carrageenan-induced paw edema in rats (Daniel et al., 2009). Magalhães et al. (2010) reported that intraperitoneal injection of eugenol treatment significantly reduces neutrophil infiltration and TNF- α release in the bronchoalveolar lavage fluid, suggesting that eugenol exhibits *in vivo* anti-inflammatory action in LPS-induced lung injury. In this study, we confirmed that the effect of ECE and eugenol against *P. acnes*-induced inflammatory responses *in vivo*. Concomitant intradermal injection of ECE and eugenol in mice significantly reduced *P. acnes*-induced ear swelling and neutrophil infiltration (Figure 6) compared with the *P. acnes*-challenged group without treatment. Further investigations are required to reveal the mechanism of anti-inflammatory action of ECE and eugenol *in vivo*.

In conclusion, our results demonstrated that ECE and eugenol suppress *P. acnes*-induced inflammatory responses both *in vitro* and *in vivo*. The suppressive effects of the ECE and eugenol on inflammatory responses in monocytic THP-1 cells may be mediated through attenuating NF- κ B activation. Therefore, our current results confirmed that ECE and eugenol have potential in treating acne effectively.

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