Anti-inflammatory effects of ethanol extract from the leaves and shoots of *Cedrela odorata* L. in cytokine-stimulated keratinocytes

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Abstract. *Cedrela odorata* L. is a native plant of the Amazon region. The bark is used in folk remedies for the treatment of diarrhea, vomiting, fever and inflammation. Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease accompanied by itching. It is a complex disease involving environmental factors and genetic factors. In the present study, the anti-inflammatory and anti-allergic effects of *C. odorata* L. methanol extract (COEE) on tumor necrosis factor (TNF)-α and interferon (IFN)-γ-stimulated HaCaT keratinocyte cells were investigated. ELISA and RT-PCR analysis revealed that the extract had anti-inflammatory effects, and reduced the interleukin (IL)-6 and IL-8 levels of the HaCaT cells. In addition, COEE exhibited anti-allergic effects, comprising a reduction in the thymus and activation-regulated chemokine and macrophage-derived chemokine levels. In addition, pathway analysis and comparison with Bay11-7082 indicated that these effects are due to the inhibition of nuclear factor (NF)-κB in TNF-α/IFN-γ-induced HaCaT cells. Therefore, the results of the present study suggest that COEE has anti-inflammatory and anti-allergic properties in TNF-α and IFN-γ-stimulated HaCaT cells, which are associated with the inhibition of pro-inflammatory cytokines and chemokines via the NF-κB pathway.

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Introduction

Atopic dermatitis (AD) is caused by a disturbance of the immune system. Therefore, to treat AD, it is necessary to normalize the immune system in addition to treating the external skin manifestations (1-3). Characteristic symptoms of AD are pruritus, pus, erythema and chronic skin bacterial infection. Skin barrier defects are recognized as one of the most important features of AD (4-6). The abnormal differentiation of skin epithelial cells causes skin barrier defects. These defects enable the infiltration of allergens, which induce an inflammatory reaction and systemic immunological reaction. These skin barrier defects are usually caused by genetic and acquired factors (7,8).

Keratinocytes are keratin-producing epidermal cells, which account for ~90% of epidermal cells (9,10). The main function of the epidermis is to provide a barrier that protects the human body from environmental factors, including pathogens, heat, ultraviolet rays and moisture loss. Thymic stromal lymphopoietin (TSLP) present in keratinocytes stimulates dendritic cells to increase the production of thymus and activation-regulated chemokine [TARC; also known as chemokine (C-C motif) ligand 17, CCL17] and macrophage-derived chemokine (MDC; also known as CCL22) (11,12). TARC and MDC are typical type 2 helper T cell (Th2 cell)-secreted chemokines that induce Th2 cell recruitment at inflammatory sites (13). High concentrations of TARC, MDC and TSLP have been detected in patients with AD (14). These biomarkers are known to be very closely associated with atopic disease (15,16).

Cedrela odorata L. is a plant of the genus *Cedrela* and is distributed across tropical climate regions, such as the Amazon (17,18). Its wood is mainly used as raw material for household furniture or musical instruments (19). Traditionally, *C. odorata* L. has been utilized in folk remedies for diarrhea, fever, inflammation, vomiting, hemorrhage, and indigestion (20,21). However, since there is no literature regarding this plant in relation to inflammation or AD, it was investigated in the present study. The aim of the study was to examine the biochemical activity of *C. odorata* L. ethanol extract (COEE)

using HaCaT cells induced with a mixture of tumor necrosis factor (TNF)-α and interferon (IFN)-γ.

Materials and methods

Preparation of COEE. The leaves and shoots of *C. odorata* L. were collected in Palo Verde National Park, Costa Rica, in 2014. A voucher specimen (KRIB 0056657) has been deposited at the International Biological Material Research Center (IBMRC) in the Korea Research Institute of Bioscience and Biotechnology (Daejeon, South Korea). The dried and refined leaves and shoots of *C. odorata* (100 g) were extracted with 700 ml 95% ethanol for 2 h, three times. The extract was percolated through filter paper (3 mm; Whatman PLC, Kent, UK), condensed using a rotary evaporator (Büchi AG, Flawil, Switzerland) and lyophilized using a freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany).

Cell culture. The human keratinocyte HaCaT cell line was purchased from the American Tissue Culture Center (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin, and maintained using an incubator at temperature 37˚C with a 5% CO_2 atmosphere while maintaining a confluency of 60-80%.

MTT assay. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HaCaT cells were seeded in 96-well plates (SPL Life Sciences, Pocheon, Korea) at a density of $1x10^4$ cells/well. After 6 h of incubation, COEE (1.25, 2.5, 5, 10 and 20 μg/ml) was administered and the cells were incubated for 24 h at 37˚C. Untreated cells were defined as the control group. Next, 5 μl MTT solution (5 mg/ml; Amresco, LLC, Solon, OH, USA) was added to the cell supernatant, and the mixture was incubated for 4 h at 37˚C. After removing the medium, DMSO was added to dissolve the formazan. A microplate reader was used to measure the absorbance at 570 nm, and the untreated formazan value was set at 100%.

Cytokine assay. HaCaT cells were cultured in 96-well plates at a density of $5x10^4$ cells/well. After an incubation for 6 h at 37˚C, COEE (2.5, 5, 10 and 20 μg/ml) and Bay11-7082 (5 μM) were administered. TNF-α/IFN-γ (10 ng/ml of each; TNF-α cat. no. 300-01A; IFN-γ cat. no. 300-02; PeproTech, Inc., Rocky Hill, NJ, USA) was applied 1 h later. The next day, the supernatant was harvested. The inhibitory effect of COEE on the secretion of interleukin (IL)-6, IL-8, TARC and MDC into the supernatants was evaluated using the following ELISA kits: IL-6 (cat. no. 555220; BD Biosciences, Santa Clara, CA, USA), IL-8 (cat. no. DY208), TARC (cat. no. DY364) and MDC (cat. no. DY336; all R&D Systems, Inc., Minneapolis, MN, USA). Samples were analyzed according to the manufacturer's protocol.

RT-PCR analysis. Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Inc.). Following isolation of the RNA, cDNA synthesis was performed using a QuantiTect Reverse Transcription kit (cat. no. 205310; Qiagen GmbH, Hilden, Germany). RNA, gDNA Wipeout Buffer and RNase-free water were mixed and incubated at 42˚C for 2 min. Then, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT River Mix were mix with the aforementioned reagents and incubated at 42˚C for 15 min. Finally, the mixture was incubated at 95˚C for 3 min to inactivate Quantiscript Reverse Transcriptase. The synthesized cDNA was amplified by PCR using a GoTaq® Green Master mix (Promega Corporation, Madison, WI, USA) with 11 pmol of each primer. The sequences of the RT-PCR primers used in the present study are listed in Table I. β-actin was used as the reference gene. The thermocycling conditions were as follows: Pre-denaturation at 94˚C for 5 min, then 25 cycles of denaturation at 94˚C for 20 sec, annealing at 56˚C for 20 sec and extension at 72˚C for 45 sec. The reaction products were separated by electrophoresis on a 1.5% agarose gel and stained with RedSafe™ kits (Intron Biotechnology, Inc., Seongnam, Korea). Images were captured using an Olympus C4000 zoom camera system (Olympus Corporation). The densitometry of the bands were measured using ImageJ 1.50i software (National Institutes of Health, Bethesda, MD, USA).

Immunoblotting. Immunoblotting of the cells was performed as previously described (22). The HaCaT cells were pre-treated with the indicated concentrations of COEE (2.5, 5. 10 and 20 μg/ml) for 1 h and stimulated with TNF-α/IFN-γ (10 ng/ml each) for 20 min at 37˚C. Immunoblots were created using anti-nuclear factor (NF)-κB p65 (cat. no. sc-8242; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phospho-NF-κB inhibitor α (anti-p-IκBα; cat. no. 2859), anti-IκB-α (cat. no. 9242), anti-NF-κB p-p65 (cat. no. 3033) and anti-β-actin (cat. no. 4967; all 1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA). The secondary antibodies were horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. sc-2030; 1:5,000 in 5% skimmed milk; Santa Cruz Biotechnology, Inc.). The densitometry of the bands were measured using ImageJ 1.50i software.

Luciferase assay. HaCaT cells were transfected with 0.1 μg pGL4.32 (luc2P/NF-κB-RE/Hygro) plasmids (Promega Corporation), using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 24 h after transfection, the cells were pretreated with COEE (2.5, 5, 10 and 20 μg/ml) and Bay11-7082 (5 μM) for 1 h at 37˚C, stimulated with TNF-α/IFN-γ for 20 h at 37˚C, harvested and then assessed for *luc2P* luciferase activity using the ONE-Glo™ luciferase reporter assay system (Promega Corporation) according to the manufacturer's instructions. Normalization was performed by comparison with *Renilla* luciferase activity.

Statistical analysis. Data are presented as the mean ± SEM. Statistical differences among groups were determined by one-way ANOVA with repeated measures followed by Newman-Keuls testing using SPSS version 14.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Sequences of the reverse transcription PCR primers used in the current study.

Gene	Direction	Primer sequences (human; 5'-3')	Fragment size (bp)
TARC	Forward	CAC GCA GCT CGA GGG ACC AAT GTG	222
	Reverse	TCA AGA CCT CTC AAG GCT TTG CAG G	
MDC	Forward	AGG ACA GAG CAT GGC TCG CCT ACA GA	362
	Reverse	TAA TGG CAG GGA GGT AGG GCT CCT GA	
IL-6	Forward	GAC AGC CAC TCA CCT CTT CA	124
	Reverse	AGT GCC TCT TTGCTG CTT TC	
IL-8	Forward	ATG ACT TCC AAG CTG GCC GTG GCT	299
	Reverse	TTA TGA ATT CTC AGC CCT CTT CAA AAA	
β-actin	Forward	CAT GTA CGT TGC TAT CCA GGC	250
	Reverse	CTC CTT AAT GTC ACG CAC GAT	

IL, interleukin; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine.

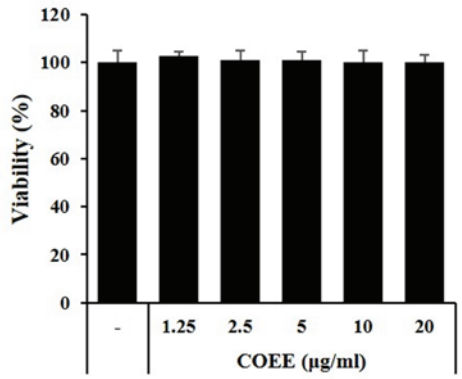

Figure 1. Cytotoxicity of COEE to HaCaT cells. Cells were seeded in 96-well plates and treated with COEE (1.25, 2.5, 5, 10 and 20 μg/ml) for 24 h. Cell viability was assessed using the MTT assay. COEE, *Cedrela odorata* L. ethanol extract.

Results

Cytotoxic effects of COEE in HaCaT cells. Whether COEE affects the viability of HaCaT cells was analyzed using the MTT assay. As shown in Fig. 1, COEE did not exhibit cytotoxicity and did not affect cell viability even when used at a high concentration of 20 μg/ml for 24 h. This confirmed experimentally that COEE does not exhibit toxicity in HaCaT cells at concentrations ≤20 μg/ml.

COEE inhibits TNF-α/IFN-γ-induced IL-6 and IL-8 expression in HaCaT cells. Next, ELISA and RT-PCR assays were used to study the inhibitory effect of COEE on the production of IL-6 and IL-8 in HaCaT cells stimulated with TNF-α/IFN-γ. The RT-PCR results confirmed that the levels of IL-6 and IL-8 were significantly increased in the group treated with TNF-α/IFN-γ compared with those in the untreated group. Similarly, when TNF-α/IFN-γ was added after the introduction of COEE to HaCaT cells, the mRNA expression levels of IL-6 and IL-8 decreased in an apparently concentration-dependent manner compared with those in the group treated with TNF-α/IFN-γ without COEE (Fig. 2A). Furthermore, the ELISA results demonstrated that COEE inhibited the expression of the IL-6 and IL-8 proteins in HaCaT cells stimulated with TNF-α/IFN-γ compared with those in the cells treated with TNF-α/IFN-γ without COEE (Fig. 2B and C).

COEE inhibits TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 expression in HaCaT cells. Chemokines are significant mediators of the inflammatory reaction and immune response. Exposure of keratinocytes to TNF-α/IFN-γ induces the increased expression of chemokines, leading to the infiltration of leukocytes into inflammatory lesions in the skin (23,24). In the present study, ELISA and RT-PCR were used to investigate the suppressive effect of COEE on TARC and MDC production in TNF-α/IFN-γ-stimulated HaCaT cells. The RT-PCR results confirmed that TARC and MDC mRNA levels were significantly increased in the cells treated with TNF-α/IFN-γ compared with those in the untreated group. Similarly, when TNF-α/IFN-γ was added after the application of COEE to the HaCaT cells, the mRNA expression levels of TARC and MDC decreased compared with those in the group treated with TNF-α/IFN-γ without COEE, and the reduction appeared to be concentration-dependent (Fig. 3A). Furthermore, the ELISA results indicated that COEE inhibited the expression of the TARC and MDC proteins in HaCaT cells induced with TNF-α/IFN-γ (Fig. 3B and C).

COEE inhibits the phosphorylation of NF-κB p65 in HaCaT cells. The nuclear factor NF-κB signaling pathway is considered a prototypical pro-inflammatory pathway, mainly due to the role of NF-κB in the expression of pro-inflammatory genes, for example, adhesion molecules, chemokines and cytokines (25). Therefore, NF-κB p65 phosphorylation in TNF-α/IFN-γ-treated HaCaT cells was analyzed in the present study. The western blotting results indicated that the phosphorylation of IκBα and NF-κB p65 was significantly increased by TNF-α/IFN-γ-treatment, whereas pretreatment with COEE attenuated the TNF-α/IFN-γ-induced increase in p-IκBα and p-p65 levels (Fig. 4).

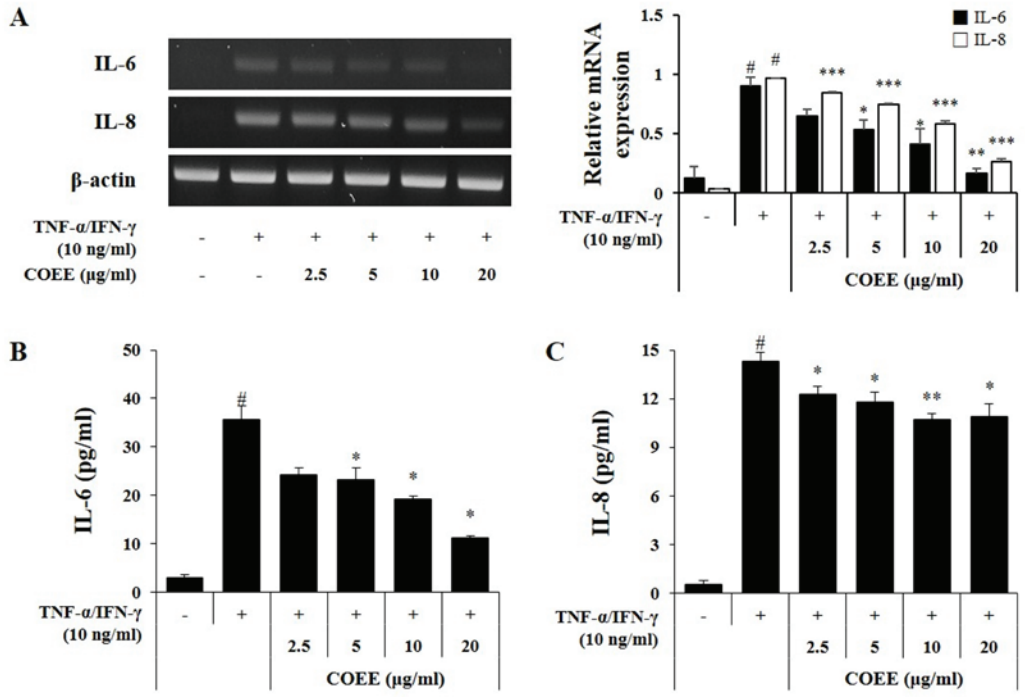

Figure 2. Effect of COEE on the expression of cytokines by HaCaT cells. HaCaT cells were pretreated with COEE (2.5, 5, 10 and 20 μg/ml) and then induced with TNF-α (10 ng/ml)/IFN-γ (10 ng/ml) for 24 h. (A) IL-6 and IL-8 mRNA expression by HaCaT cells, as detected by RT-PCR. (B) IL-6 and (C) IL-8 expression levels in the culture supernatants of cells treated with COEE and TNF-α/IFN-γ for 24 h, as detected by ELISA. Each bar represents the mean of three independent experiments. Data are presented as the mean values ± SEM of three samples. $^{\#}$P<0.01 vs. the untreated control; *P<0.05, **P<0.01 and ***P<0.001 vs. the TNF-α/IFN-γ group. COEE, *Cedrela odorata* L. ethanol extract; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

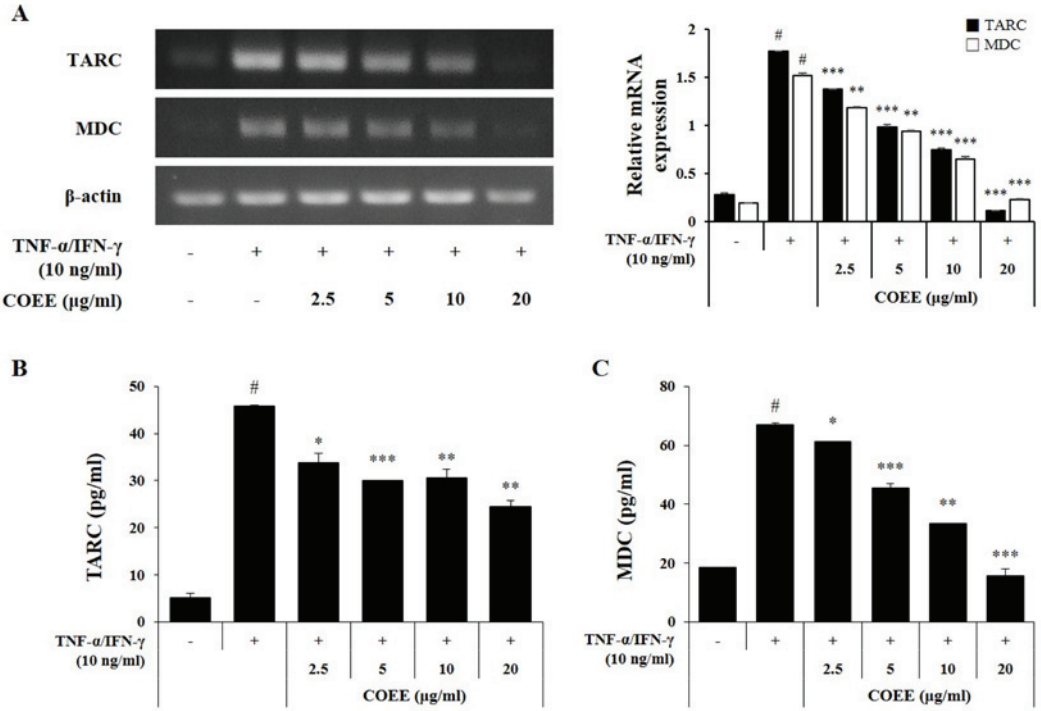

Figure 3. COEE affects the expression of chemokines by HaCaT cells. The inhibitory effects of COEE on TNF-α/IFN-γ-induced pro-inflammatory chemokine production by HaCaT cells were investigated. HaCaT keratinocytes were pretreated with COEE (2.5, 5, 10 and 20 μg/ml) and stimulated with TNF-α (10 ng/ml)/IFN-γ (10 ng/ml) for 24 h. (A) TARC and MDC mRNA expression in HaCaT cells, as detected using RT-PCR. (B) TARC and (C) MDC levels were measured by ELISA in the culture supernatants of cells treated with COEE and TNF-α/IFN-γ for 24 h. Each bar represents the mean of three independent experiments. Data are presented as the mean values ± SEM of three samples. $^{\#}$P<0.01 vs. the untreated control. *P<0.05, **P<0.01 and ***P<0.001 vs. the TNF-α/IFN-γ group. COEE, *Cedrela odorata* L. ethanol extract; TNF, tumor necrosis factor; IFN, interferon; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine.

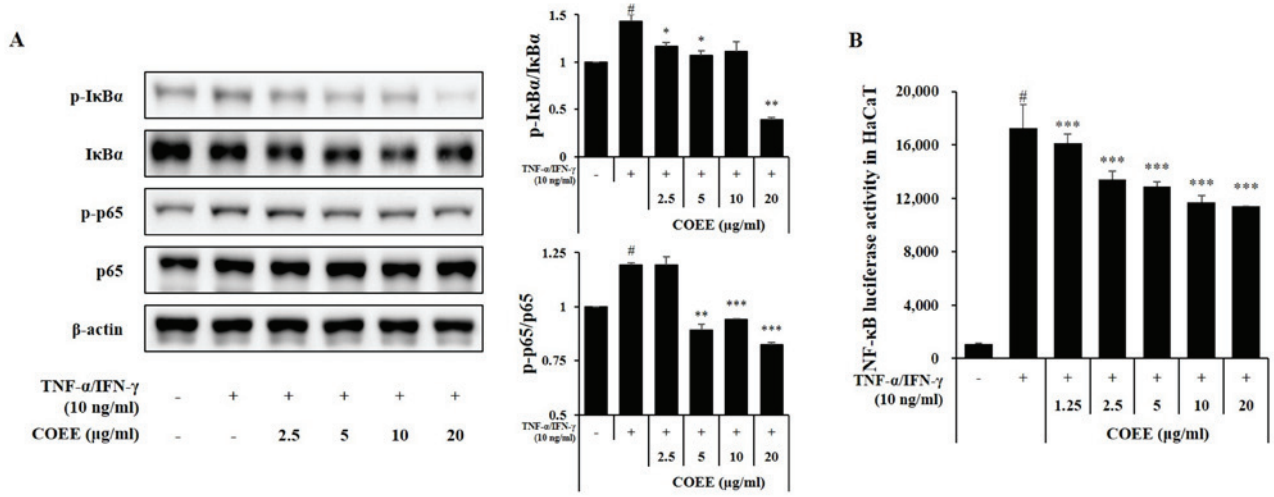

Figure 4. Effect of COEE on TNF-α/IFN-γ-induced NF-κB activation in HaCaT cells. (A) Phosphorylation of p65 and IκBα was analyzed by western blotting. (B) HaCaT cells were transfected with the expression vector luciferase reporter plasmid (0.1 μg). At 24 h after transfection, HaCaT cells were treated with COEE for 1 h, and the *luc2P/Renilla* luciferase activity was then measured. All data represent three independent experiments. Data are presented as the mean values ± SEM of three samples. $^{\#}$P<0.01 vs. the untreated control. *P<0.05, **P<0.01 and ***P<0.001 vs. the TNF-α/IFN-γ group. COEE, *Cedrela odorata* L. ethanol extract; TNF, tumor necrosis factor; IFN, interferon; NF-κB, nuclear factor-κB; p65, NF-κB subunit p65; p, phospho; IFN, interferon; IκBα, NF-κB inhibitor α.

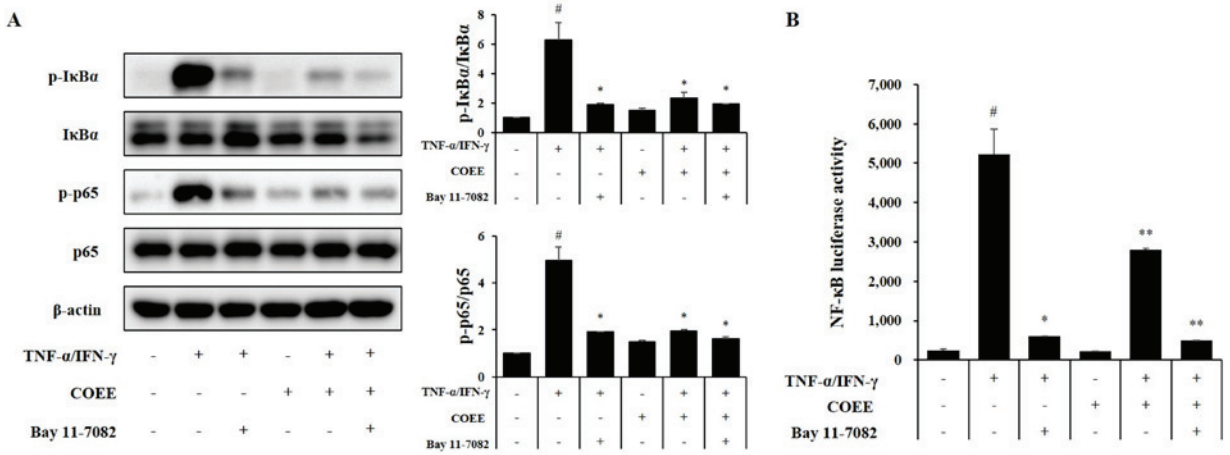

Figure 5. Effect of COEE and Bay11-7082 on TNF-α/IFN-γ-induced NF-κB activation in HaCaT cells. (A) Phosphorylation of p65 and IκBα was analyzed by western blotting. (B) HaCaT cells were transfected wih the luciferase reporter plasmid (0.1 μg). At 24 h after transfection, HaCaT cells were treated with COEE and Bay11-7082 for 1 h, and the *luc2P/Renilla* luciferase activity was then measured. All data represent three independent experiments. Data are presented as the mean values ± SEM of three samples. $^{\#}$P<0.01 vs. the negative control. *P<0.05 and **P<0.01 vs. the TNF-α/IFN-γ group. COEE, *Cedrela odorata* L. ethanol extract; TNF, tumor necrosis factor; IFN, interferon; NF-κB, nuclear factor-κB; p65, NF-κB subunit p65; p, phospho; IκBα, NF-κB inhibitor α.

COEE and Bay11-7082 inhibit the phosphorylation of NF-κB in HaCaT cells. Bay11-7082 inhibits IκBα phosphorylation in cells and has been used to indicate the involvement of the canonical IκB kinases and NF-κB in mechanistic analysis (26). A comparative experiment was conducted in the present study, in which the efficacy of COEE (20 μg/ml) was compared with that of Bay11-7082 (5 μM) in the inhibition of NF-κB p65. Phosphorylation of IκBα and NF-κB p65 was significantly increased by TNF-α/IFN-γ-treatment, while pretreatment with COEE and Bay11-7082 decreased the levels of p-p65 and p-IκBα in TNF-α/IFN-γ-treated HaCaT cells, as indicated by the results of immunoblotting and the luciferase assay (Fig. 5).

COEE and Bay11-7082 inhibit the expression of chemokines and cytokines in HaCaT cells. Using ELISA and RT-PCR, the suppressive effects of COEE and Bay11-7082 on TARC, MDC, IL-6 and IL-8 production in HaCaT cells stimulated with TNF-α/IFN-γ were investigated. The results confirmed that the levels of TARC, MDC, IL-6 and IL-8 were significantly increased in the group treated with TNF-α/IFN-γ compared with those in the untreated group. However, when TNF-α/IFN-γ was added after the introduction of COEE and Bay11-7082 to the HaCaT cells, the mRNA and protein expression levels of TARC, MDC, IL-6 and IL-8 decreased in an apparently concentration-dependent manner compared with those in the group treated with TNF-α/IFN-γ without COEE (Fig. 6).

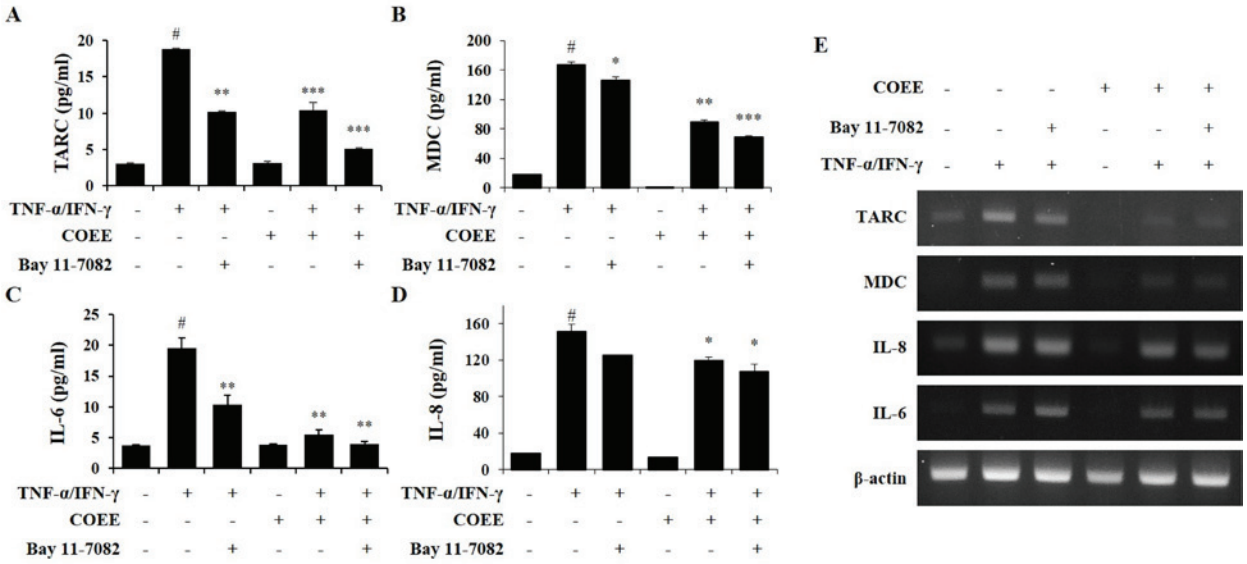

Figure 6. Effect of COEE and Bay11-7082 on TNF-α/IFN-γ induced chemokines and cytokine activation in HaCaT cells. HaCaT cells were pretreated with COEE (20 μg/ml), Bay11-7082 (5 μM) and then induced with TNF-α (10 ng/ml)/IFN-γ (10 ng/ml) for 24 h. At Protein concentrations of (A) TARC, (B) MDC, (C) IL-6 and (D) IL-8. (E) mRNA expression of TARC, MDC, IL-6 and IL-8. Data are presented as the mean values ± SEM of three samples. $^{\#}$P<0.01 vs. the untreated control; *P<0.05, **P<0.01 and ***P<0.001 vs. the TNF-α/IFN-γ group. COEE, *Cedrela odorata* L. ethanol extract; TNF, tumor necrosis factor; IFN, interferon; TARC, thymus and activation-regulated chemokine; MDC, macrophage-derived chemokine; IL, interleukin.

Discussion

AD, also known as eczema, is a common chronic inflammatory skin disease and is characterized by the infiltration of inflammatory cells into the skin (27). Although AD is generally treated with immunosuppressive drugs and anti-inflammatory drugs, these treatments are often ineffective (28). This may cause patients to use alternative treatment strategies, including traditional plant-based remedies.

In the present study, *in vitro* experiments were conducted to determine the effects of COEE on pro-inflammatory chemokine secretion in keratinocytes. Keratinocytes serve a crucial role in inflammatory skin disorders via the production of pro-inflammatory cytokines and chemokines (29). These cells participate in the pathogenesis of AD by secreting various chemokines, among which TARC and MDC selectively attract Th2 cells that are predominant in atopic inflammation (30). IL-8 amplifies the inflammatory response in AD by recruiting neutrophils into the skin lesions (31). Numerous researchers have reported that TNF-α/IFN-γ treatment increases chemokine production in keratinocytes (32,33). The TNF-α/IFN-γ combination activates several intracellular pathways, including NF-κB pathways (34,35). NF-κB pathways have been shown to be involved in the regulation of chemokine and cytokine production in keratinocyte cells; they serve a significant role in the immune response and regulate inflammatory signaling (36,37). Therefore, experiments to investigate the effect of COEE on the TNF-α/IFN-γ-stimulated expression of MDC and TARC in HaCaT cells were conducted in the present study.

The NF-κB family includes critical transcription factors that are activated by various stimuli, including TNF-α, IFN-γ, IL-1 and lipopolysaccharide. Upon stimulation, NF-κB complexes in the cytoplasm translocate into the nucleus, where they participate in the expression of numerous pro-inflammatory genes (22). NF-κB signaling pathways have been shown to be involved in the regulation of TARC and MDC production in HaCaT cells (38). Furthermore, the promoters of TARC and MDC both contain NF-κB-binding sites (39), indicating that these transcription factors may be involved in the modulation of TARC and MDC (38). In the present study, the results indicated that COEE suppressed signaling pathways leading to the activation of TARC and MDC by NF-κB.

Treatment with COEE or the IκBα inhibitor Bay11-7082 reduced the TNF-α/IFN-γ-activated expression of pro-inflammatory cytokines (IL-6 and IL-8) and chemokines (TARC and MDC) to baseline values. These results indicate that COEE reduces the production of the pro-inflammatory cytokines IL-6 and IL-8, and the expression of the Th2 chemokines TARC and MDC in HaCaT cells via inhibition of NF-κB pathways in HaCaT cells. These effects are hypothesized to be closely associated with the suppression of NF-κB activation. Therefore, it is suggested that COEE has the potential to be used as a therapeutic drug for the treatment of AD.

In conclusion, the results of the present study indicate that COEE inhibits the TNF-α/IFN-γ-stimulated expression of TARC and MDC in HaCaT cells via the inhibition of NF-κB pathways. The ability of COEE to suppress the formation of these Th2 chemokines suggests that it may be able to inhibit the infiltration of Th2 cells into skin lesions and thereby reduce skin inflammation. Further investigation of the mechanism by which COEE inhibits the release of these Th2 chemokines may provide insights helpful in the design of targeted treatments for AD. However, additional studies using *in vivo* skin inflammation models are required to support the potential of COEE in the clinical treatment of AD.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

HSL and JWP analyzed the data and wrote the manuscript. OKK, YL, JHK, SYK, NZ and KR prepared the *Cedrela odorata* L., and analyzed and edited the manuscript. SC, SRO, and KSA designed the study and edited the manuscript. All authors critically revised the article and have approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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