



Effects of *Catalpa ovata* stem bark on atopic dermatitis-like skin lesions in NC/Nga mice

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ABSTRACT

Ethnopharmacological relevance: The stem bark of *Catalpa ovata* has been used as a traditional herbal medicine for the treatment of various inflammatory diseases such as itching and scabies.

Aim of the study: In the present study, we investigated the anti-AD effects of *Catalpa ovata* stem bark on *Dermatophagoides farinae*-induced AD in a NC/Nga mouse AD model. We determined dermatitis score, histology, IgE, cytokines, and chemokines related to hypersensitive immune responses in AD. The mechanism of action was also investigated using HaCaT cells.

Materials and methods: We investigated the topical effects of *Catalpa ovata* stem bark on AD-like skin lesions in NC/Nga mice. Five category-experiments were performed, including assessment of dermatitis score; histological analysis of dorsal skin lesions; quantitative measurement of serum total IgE; quantitative measurement of cytokines (IL-1 β , IL-4, IL-5, IL-6, IL-13, TNF- α) from dorsal tissue; and RT-PCR analysis of for TSLP and TARC mRNA expression in HaCaT cells.

Results: The clinical dermatitis score was significantly lower in *Catalpa ovata* extract (COE) groups than in the control group. Histological analysis showed that COE inhibited hypertrophy and hyperkeratosis of the epidermis, intracellular edema, and reduced the infiltration of inflammatory cells. COE significantly inhibited serum total IgE; Th2 cytokines IL-4, IL-5 and IL-13; pro-inflammatory cytokines IL-1 β , IL6 and TNF- α ; the Th2 chemokine TARC and the pro-Th2 cytokine TSLP.

Conclusion: These results demonstrate that *Catalpa ovata* stem bark may be a useful external medicine for treatment of AD. Further investigation is necessary to determine appropriate COE dosage and to evaluate the safety of this medicinal herb.

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1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease that is accompanied by severe itching (Schultz-Larsen, Hanifin, 2002). It is one of the most common skin disorders in developed countries, affecting approximately 20% of children and 1–3% of adults. Clinical symptoms include skin erythematous plaques, eruption and/or lichenification accompanied by intense pruritus and cutaneous hypersensitivity (Spergel and Paller, 2003). A hallmark of AD is dry itchy skin, due at least in part to defects in skin genes that are important for maintaining the barrier function of the skin. The pathogenesis of AD remains incompletely understood (Jin et al., 2009). For humans, house dust mites, such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the most common environmental allergens associated with AD (Matsuoka et al., 2003).

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AD is a biphasic inflammatory skin disease that is provoked by an imbalance between T-helper (Th) 1 and Th2 immune responses (Jin et al., 2009). In particular, AD has been associated with the Th2 phenotype, in which interleukin (IL)-4, IL-5, and IL-13 secretion, and Th2-type cytokine-mediated IgE production are dominant (Leung et al., 2003a). In addition, accumulation of large numbers of eosinophils, mast cells, and dendritic cells (DCs) has been observed in both the epidermis and the dermis of patients with AD (Leung and Bieber, 2003b).

Many trials of therapies intended to modulate AD have been performed with limited success. These include trials of therapies that prevent Th2 responses, enhance Th1 responses, and decrease IgE concentration (Choi et al., 2011). Topical glucocorticoids are important and effective treatments for AD. However, prolonged use of high-dose glucocorticoids causes various adverse effects (Furie et al., 2003). Recently, medicinal herbs have emerged as therapeutic alternatives for the treatment immune disorders because they have potent immuno-modulatory effects and have been proven safe. There are many reports of medicinal herbs as treatment for AD (Kotani et al., 2000; Kang et al., 2008; Yang et al., 2011).

Catalpa ovata (Bignoniaceae) is a deciduous tree that is widely distributed in Korea, China and Japan (Lee, 1996). The stem bark of *Catalpa ovata* has been used as a traditional herbal medicine for the treatment of various inflammatory diseases such as itching and scabies (Administration of Traditional Chinese Med., 1999). *Catalpa ovata* stem bark contains iridoids (Lino von Poser et al., 2000) and naphthoquinones (Fujiwara et al., 1998; Park et al., 2010).

The extract of *Catalpa ovata* stem bark inhibits the expression of inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs) stimulated with tumor necrosis factor- α (TNF- α) (Cho et al., 2007). This extract also inhibits the production of TNF- α and nitric oxide (NO) in lipopolysaccharide-stimulated RAW 264.7 macrophages (Pae et al., 2003). Catalposide, an iridoid isolated from the stem bark of *Catalpa ovata*, suppresses inducible nitric oxide synthase (iNOS) expression, nuclear factor-kappa B (NF- κ B) activation, and production of pro-inflammatory mediators such as TNF- α , IL-1 β , and IL-6 (An et al., 2002; Oh et al., 2002). Catalposide also suppresses TNF- α /interferon- γ (IFN- γ)-induced expression of thymus and activation-regulated chemokine (TARC/CCL17), and macrophage-derived chemokine (MDC/CCL22) in human keratinocyte (HaCaT) cells (Yang, 2009). A naphthoquinone derivative that is isolated from the stem bark of *C. ovata* exerts anti-inflammatory effects through the inhibition of NO production (Park et al., 2010). Therefore, *Catalpa ovata* stem bark may be able to inhibit AD. However, there have not yet been any further investigations of the inhibitory potential of *Catalpa ovata* stem bark in a specific AD animal model.

Several animal models of AD have been developed. The NC/Nga mouse AD model is clinically and histologically very similar to human AD, when mice are raised under conventional conditions (Suto et al., 1999; Matsuoka et al., 2003). In the present study, we investigated the anti-AD effects of *Catalpa ovata* stem bark on *Dermatophagoides farinae*-induced AD in a NC/Nga mouse AD model. We determined dermatitis score, histology, IgE, cytokines, and chemokines related to hypersensitive immune responses in AD. The mechanism of action was also investigated using HaCaT cells.

2. Materials and methods

Plant material: *Catalpa ovata* stem bark (1.5 kg) was collected on April 7, 2012 from trees in Hamyang County, Gyeongsangnamdo, Republic of Korea. Professor Ho-young Choi of Kyung Hee University identified the plants. A voucher specimen of stem bark from *Catalpa ovata* was deposited at the College of Oriental Medical Science, Kyung Hee University, Seoul, Republic of Korea. The stem bark of *Catalpa ovata* was extracted twice with 10 L MeOH for 2 h in a reflux apparatus. After reflux and filtration, the extract was evaporated *in vacuo*, and lyophilized at -50°C using a freeze dryer (FD-series, IlshinBiobase, Korea) to yield 42.5 g of crude extract. The dried extract yield from starting crude materials was approximately 2.8% (w/w). We prepared samples for topical application using an ointment base (pure vaseline jelly, Sung Kwang Pharm Co., Korea). We prepared *Catalpa ovata* stem bark extract (COE) 1% and COE 2% ointments by adding 1 g and 2 g of COE, respectively, to 100 g of ointment base.

Animals: NC/Nga male mice (14–22 g body weight, 4 weeks of age) were purchased from Central Lab. Inc. (Seoul, Korea), a branch of Charles River Japan (Kanagawa, Japan). NC/Nga male mice were housed in specific pathogen-free (SPF) conditions in a controlled environment ($22 \pm 2^{\circ}\text{C}$; lighting 0700–1900; humidity 30–70%). Food and tap water were provided *ad libitum*. After acclimating for 2 weeks, the mice were randomly divided into

5 groups. The animal experiments in this study were approved by the animal care committee of Kyung Hee University (Protocol Number KHUASP (SE)-11-009).

Mite antigen: We used an ointment made from an extract of house dust mite, *Dermatophagoides farinae* extract ointment (DfE; Biostir[®] AD, Biostir, Japan) (Koda et al., 1987).

Induction of AD and drug treatment: AD-like skin lesions were induced using a modification of the procedure described by Gao et al. (2004). The experimental schedule is provided in Fig. 1. The hair on the back of 6-week old NC/Nga mice was removed with no further treatment for 24 h. Then, mice were assigned to one of 5 groups (NOR, CON, Protopic[®], COE1%, COE2%) at the beginning of the experiment. We applied 100 mg Biostir[®] AD (DfE) to the dorsal skin and ears of each mouse (except those in the NOR group) two times each week (Monday and Thursday) for 3 weeks. Prior to each DfE application, we shaved any hair regrowth on the back of the mice, and applied 4% sodium dodecyl sulfate (SDS) to disrupt the skin barrier. Between the 21st day after the first application of DfE and the 35th day after the first application of DfE, the 5 groups were treated everyday as follows:

Normal group (NOR)—no treatment (7 mice).

Control group (CON)—application of 100 mg ointment base only (7 mice).

Protopic[®] group—application of 100 mg Protopic[®] (tacrolimus ointment 0.1%, Astellas Pharm Inc., IL, USA) (7 mice).

COE1% group—application of 100 mg COE 1% ointment (7 mice).

COE2% group—application of 100 mg COE 2% ointment (7 mice).

Mice were sacrificed on the 35th day and blood was collected from the vena cava. The dorsal skin tissues of mice were excised and subjected to histological examination.

Evaluation of dermatitis severity: Clinical dermatitis severity was tested using the method described by Yamamoto et al. (2007). The severity of dermatitis was evaluated once each week (7th, 14th, 21st, 28th, and 35th day). The development of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion was scored as 0 (none), 1 (mild, < 20%), 2 (moderate, 20–60%) and 3 (severe, > 60%). The sum of the individual scores was used as the dermatitis score.

Histological analysis of skin lesions: On the 35th day after DfE application, the mice were sacrificed and skin samples were collected from the dorsal area. The samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned into 4 μm slices

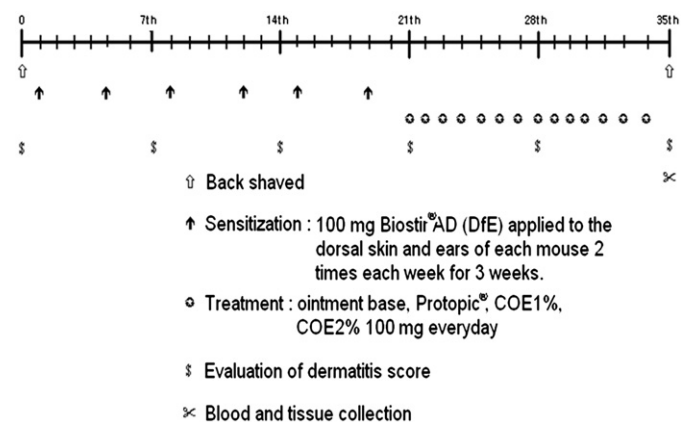


Fig. 1. Schedule for sensitization and treatment of NC/Nga mice. Biostir[®] AD (DfE) was used for sensitization and treatments included ointment base alone, Protopic[®], COE1% and COE2%.

and stained with hematoxylin and eosin (H&E). Pathologic changes (epidermal and dermal hyperplasia, parakeratosis, hyperkeratosis, dermal edema, vesicular formation, and inflammation) were evaluated and compared between groups. Selected sections were stained with toluidine blue for evaluation of mast cell infiltration.

Measurement of IgE concentration in serum: Serum samples were obtained from blood collected on the 35th day after the first DfE application by centrifugation ($1700 \times g$, 10 min) and stored at -70°C until analysis. The level of serum total IgE was measured using an IgE ELISA Kit For Mouse (Shibayagi Co., Ltd., Gunma, Japan) according to the manufacturer's instructions.

Measurement of cytokines production: Cytokine levels were determined using ELISA. Dorsal skin samples ($n=6$, 100 mg) of the inflamed region were homogenized in 1 ml of T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail. Homogenates were then centrifuged at $12,000 \times g$ for 20 min at 4°C . Cytokine levels of IL-1 β , IL-4, IL-5, IL-6, IL-13, TNF- α and Regulated on Activation, Normal T-cell Expressed, and Secreted in the supernatant were analyzed using Quansys reagents and instruments (Quansys Biosciences, Logan, UT, USA). The Quansys Imager, driven by an 8.4 megapixel Canon 20D digital SLR camera, supports 96-well plate-based chemiluminescent imaging. The Q-Plex™ (16-plex) Mouse Cytokine – Screen (Quansys Biosciences, Logan, UT, USA) is a quantitative ELISA – based test in which sixteen distinct capture antibodies have been absorbed to each well of a 96-well plate in a defined array. Cytokine levels were normalized with a protein assay kit and calculated as pg per mg total protein.

HaCaT cell culture: Human keratinocyte cell line HaCaT was kindly provided by Dr. W.M. Yang (Kyung Hee University, Seoul, Korea). HaCaT cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (Gibco BRL, Grand Island, NY, USA), 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco BRL, Grand Island, NY, USA), and 100 $\mu\text{g}/\text{ml}$ penicillin (Gibco BRL, Grand Island, NY, USA). HaCaT cells were cultured in 96-well plates and stimulated with 10 ng/ml human recombinant IFN- γ (R&D Systems Inc., Minneapolis, MN, USA) and 10 ng/ml human recombinant TNF- α (R&D Systems Inc., Minneapolis, MN, USA) in the presence or absence of COE (50, 100, or 200 $\mu\text{g}/\text{ml}$) for 24 h, as previously described (Vestergaard et al., 2000). Culture supernatants were collected and stored at -70°C for ELISA.

Cell viability assay: Cell viability studies were performed in 96-well plates. HaCaT cells were mechanically scraped and plated at 1×10^5 cells/ml in 96-well plates containing 100 μl of DMEM medium with 10% FBS and incubated overnight. COE was dissolved in DMSO, and the DMSO was added to all plates to compensate the same volume of DMSO. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl of FBS-free medium containing MTT 5 mg/ml. After 4 h of incubation at 37°C , the medium was discarded and the formazan blue that formed in the cells was dissolved in DMSO 100 μl . The optical density was measured at 540 nm.

RT-PCR for TSLP, TARC production in HaCaT cells: The level of mRNA expression for thymic stromal lymphopoietin (TSLP) and TARC in HaCaT cells was determined using RT-PCR. Total RNA was isolated using the easy-BLUE total RNA extraction kit (Intron,

Seoul, Korea) according to the manufacturer's instructions. For cDNA synthesis, 1 μg total RNA was mixed with Maxime RT premix (Intron, Seoul, Korea) containing oligo-dT primers and diethyl pyrocarbonate (DEPC)-treated water to a final volume of 20 μl and incubated at 45°C for 60 min. The reaction was terminated by heat inactivation at 95°C for 5 min. cDNA was then amplified with gene-specific primers using a Taq PCR master mix kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The 20 μl amplification mixture was composed of 1 μl cDNA and 10 μl of a $2 \times$ Taq PCR master mix containing 1.5 mM MgCl_2 , 0.1 μM each primer, and water. After a 15 min preincubation at 94°C , PCR amplification occurred in 35 cycles of: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. Primers were designed using primer 3 software, and the sequences are currently available in the GenBank database. Table 1 shows the sequences of the gene-specific PCR primers. The relative expression levels of target genes were normalized using GAPDH as an internal control.

Statistical analysis: Data are presented as mean $\pm$ standard deviation (SD). Data for treatment groups were compared using one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. All statistical analyses were performed using SPSS v.13.0 statistical analysis software (SPSS Inc., USA). The statistical significance of differences was accepted at the level of $p < 0.05$.

3. Results

Dermatitis score: The AD-like skin lesions induced by cutaneous application of DfE resulted immediate itching, erythema, and hemorrhage on the ear and back that was followed by edema, superficial erosion, deep excoriation, scaling and dryness of the skin. In all groups of NC/Nga mice, except the NOR group, clinical signs and symptoms of dermatitis rapidly developed during the 3 weeks of DfE application. However, after the topical application of COE and Protopic® on the 21st day, these skin symptoms were inhibited. Photographs of the dorsal skin of representative mice from each group that were taken at the end of the experiment are presented in Fig. 2. The dermatitis score of the CON group increased rapidly and became significantly different from that of the NOR group by the 7th day after the first application of DfE. However, the dermatitis scores of the COE1%, COE2%, and Protopic® groups, began to decrease on the 28th day, as compared to that of the CON group, and decreased markedly on the 35th day (Fig. 3). The dermatitis score reduction ratios on the 35th day $((\text{CON} - X)/(\text{CON} - \text{NOR}) \times 100$, where $X = \text{COE1\%}$, $\text{COE2\%}$, $\text{Protopic}^\circledast$): COE1% 42.9%, COE2% 44.4%, Protopic®: 81.0%.

Histological analysis: Following H&E staining, hypertrophy and hyperkeratosis of the epidermis, intracellular edema, and infiltration of inflammatory cells were observed in the CON group, while they were not observed in the NOR group. However, treatment with COE and Protopic® inhibited these inflammatory changes (Fig. 4). To investigate whether inflammatory cells had infiltrated into the skin after DfE application, tissue sections of dorsal skin were stained with toluidine blue in order to detect mast cells. The number of mast cells in the dermis was markedly greater in the CON group than in the NOR group, whereas COE and Protopic®

Table 1

Sequences of primers used for RT-PCR analysis of TARC, TSLP expression in human keratinocytes.

Genes	Forward	Reverse	Length (bp)
TARC	5'-CAGGAAGTTGGTGAGCTGGTATA-3'	5'-TTGTGTTTCGCCTGTAGTGCATA-3'	300
TSLP	5'-TATGAGTGGGACCAAAAGTACCG-3'	5'-GGGATTGAAGGTTAGGCTCTGG-3'	97
GAPDH	5'-AAGCTGTGGCGTGATGGCCG-3'	5'-TGGGCCCTCAGATGCTGCT-3'	228

treatment significantly reduced the number of dermal mast cells (COE1%, $p < 0.001$; COE2%, $p < 0.001$; Protopic[®], $p < 0.01$ vs. control; Figs. 4 and 5).

Serum total IgE: The serum total IgE of each group is listed in illustration Fig. 6. Application of DfE for 21 days considerably increased the serum total IgE levels in the CON group, as compared to the NOR group. COE treatment significantly reduced serum total IgE levels as compared to controls (COE1%, $p < 0.05$ vs. control; COE2%, $p < 0.05$ vs. control). The serum total IgE level in the Protopic[®] group was not significantly lower than that of controls.

Pro-inflammatory cytokine (IL-1 β , IL-6, TNF- α) production: The IL-1 β level was significantly higher in the CON group than in the NOR group after DfE treatment. Protopic[®] treatment significantly reduced IL-1 β level ($p < 0.001$), as compared to the levels in the CON group, as did COE1% treatment ($p < 0.05$) and COE2% treatment ($p < 0.05$; Table 2). The IL-6 level was significantly higher in the CON group than in the NOR group after DfE treatment. Protopic[®] treatment significantly reduced IL-6 levels ($p < 0.001$), as compared to the levels in the CON group. Similarly, COE2% treatment significantly reduced IL-6 levels ($p < 0.05$), as compared to the CON group. However, COE1% treatment did not significantly reduce IL-6 levels (Table 2). The TNF- α level was significantly higher in the CON group than in the NOR group after DfE treatment. Treatment with Protopic[®] ($p < 0.001$), COE1% ($p < 0.05$), and COE2% ($p < 0.001$) significantly reduced TNF- α levels, as compared to CON TNF- α levels (Table 2).

Th2-type cytokine (IL-4, IL-5, IL-13) production: The IL-4 level was significantly higher in the CON group than in the NOR group after DfE treatment. Protopic[®] treatment ($p < 0.001$), COE1% treatment ($p < 0.05$), and COE2% treatment ($p < 0.01$) significantly reduced IL-4 levels, as compared to CON levels (Table 3). The IL-5 level was significantly higher in the CON group than in the NOR group after DfE treatment. Protopic[®] treatment ($p < 0.001$) and COE2% treatment ($p < 0.05$) significantly reduced IL-5 levels, as compared to CON levels. However, COE1% treatment did not significantly reduce IL-5 level (Table 3). The IL-13 level was significantly higher in the CON group than in the NOR group

after DfE treatment. Protopic[®] treatment ($p < 0.001$), COE1% treatment ($p < 0.001$), and COE2% treatment ($p < 0.001$) significantly reduced IL-13 levels, as compared to CON levels (Table 3).

TSLP and TARC mRNA expression in HaCaT cells treated with IFN- γ /TNF- α : To investigate the inhibitory effects of COE on TNF- α /IFN- γ -induced expression of TSLP and TARC, HaCaT cells were pretreated with COE for 1 h and then stimulated with TNF- α and IFN- γ for 20 h. COE significantly suppressed TNF- α and IFN- γ -induced mRNA expression of TSLP and TARC in a dose-dependent manner ($p < 0.001$; Fig. 7). The cytotoxic effects of COE were also evaluated using MTT assays, and this extract had no cytotoxicity at the concentrations of 1000 μ g/ml (Fig. 7A). Accordingly, we used 50, 100, and 200 μ g/ml COE to investigate TSLP and TARC mRNA expression regardless of concentration. Thus, the inhibitory effects of COE on TSLP and TARC mRNA expression were not attributable to any cytotoxic effect.

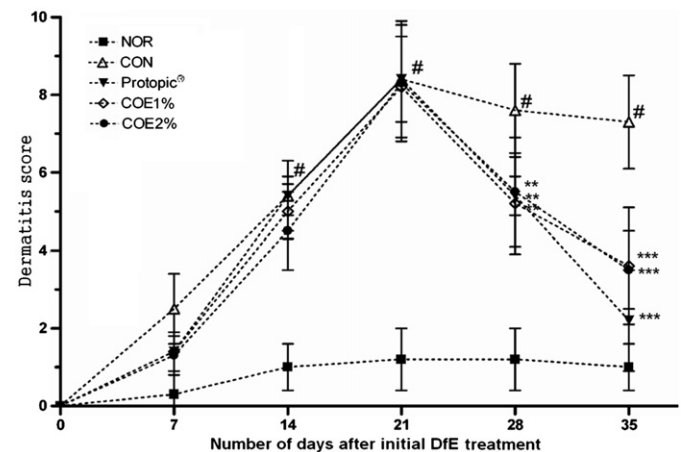


Fig. 3. Effect of COE on time-dependent dermatitis score of NC/Nga mice with AD-like skin lesions. Data were collected at 1-week intervals after the first cutaneous application of DfE. Data are presented as means $\pm$ SD ($n=6$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control; # $p < 0.05$ control vs. normal.

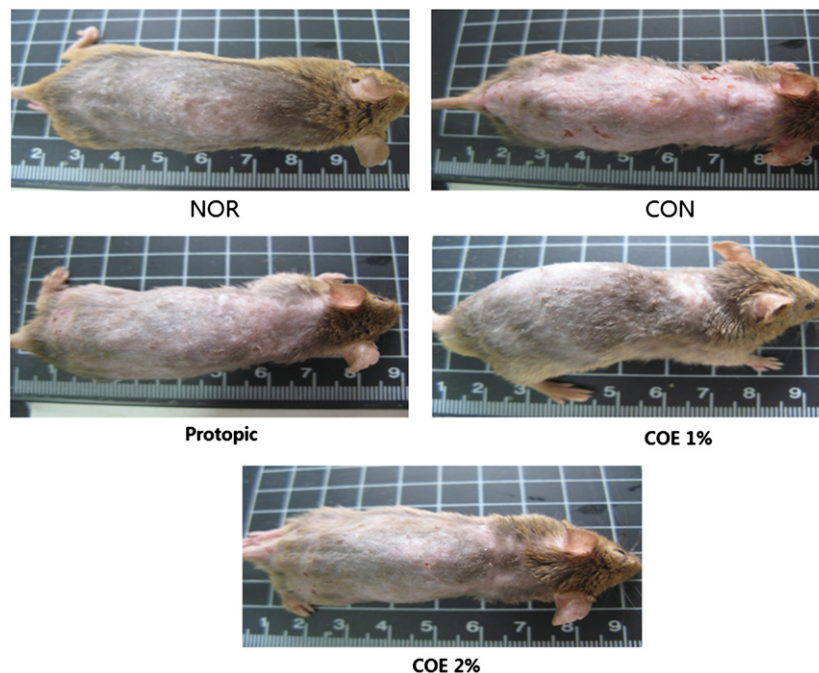


Fig. 2. Typical dorsal skin photos of NC/Nga mice with AD-like skin lesions that did or did not receive COE treatment. Photographs were taken on the 35th day after the first DfE application.

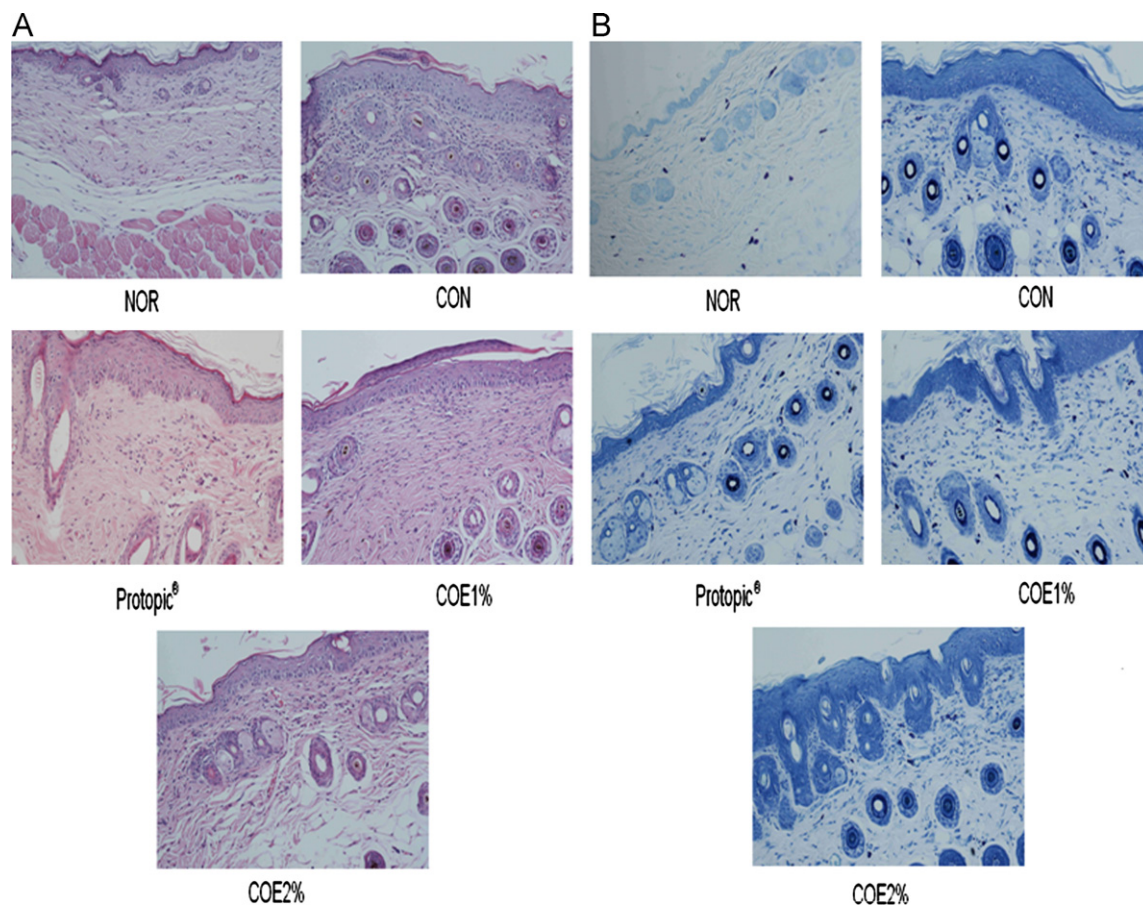


Fig. 4. (A) Representative images of H&E stained dorsal skin section from NC/Nga mice with AD-like skin lesions. (B) Representative images of toluidine-blue stained dorsal skin section from NC/Nga mice with AD-like skin lesions. Original magnification 200 $\times$.

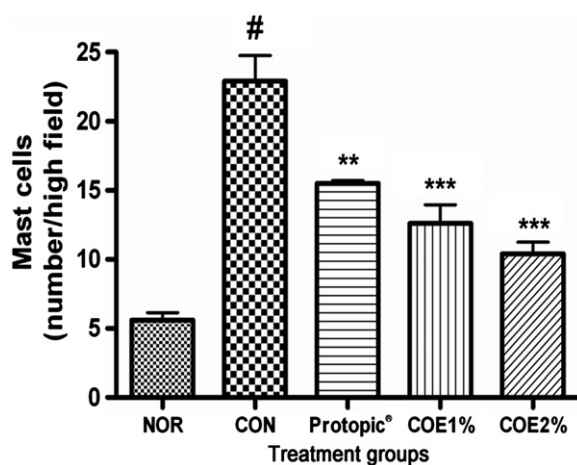


Fig. 5. Effect of COE on the number of mast cells in the dorsal skin of NC/Nga mice with AD-like skin lesions. Data are presented as means $\pm$ SD ($n=6$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control; # $p < 0.05$ control vs. normal.

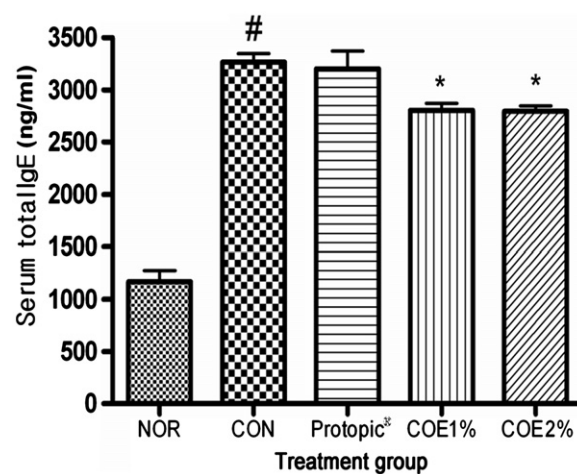


Fig. 6. Effect of COE on serum total IgE production of NC/Nga mice with AD-like skin lesions. Data are presented as means $\pm$ SD ($n=6$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control; # $p < 0.05$ control vs. normal.

4. Discussion

The negative side effects of steroidal and nonsteroidal AD drugs create the need to develop new drugs that have novel modes of action and do not produce considerable side effects.

Medicinal herb-based agents with a transcriptional mode of action, good efficacy, and low risk of side effects are promising substances for the treatment and prevention of AD.

The NC/Nga mouse model is considered one of the most valuable mouse models for representing human AD (Gao et al., 2004). AD-like

Table 2

Effect of COE on pro-inflammatory cytokine (IL-1 β , IL-6, TNF- α) production-quantitative ELISA-based infrared (IR) assay of mouse dorsal skin tissue harvested at the end of the study.

group	IL-1 β (pg/mg)	IL-6 (pg/mg)	TNF- α (pg/mg)
NOR	107.6 $\pm$ 2.9	13.0 $\pm$ 1.3	56.4 $\pm$ 5.3 ^a
CON	378.8 $\pm$ 46.9 [‡]	42.0 $\pm$ 6.1 [‡]	202.3 $\pm$ 16.9 [‡]
Protopic [®]	180.8 $\pm$ 20.8***	26.3 $\pm$ 4.0***	123.8 $\pm$ 7.4***
COE1%	327.7 $\pm$ 18.2*	37.0 $\pm$ 3.5	180.9 $\pm$ 10.9*
COE2%	319.9 $\pm$ 15.5*	33.1 $\pm$ 2.4*	172.8 $\pm$ 9.9**

^a Data are presented as means $\pm$ SD ($n=6$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ vs. control.

[‡] $p < 0.05$ control vs. normal.

Table 3

Effect of COE on Th2-type cytokine (IL-4, IL-5, IL-13) production-quantitative ELISA-based infrared (IR) assay of mouse dorsal skin tissue harvested at the end of the study.

group	IL-4 (pg/mg)	IL-5 (pg/mg)	IL-13 (pg/mg)
NOR	29.4 $\pm$ 5.1	14.4 $\pm$ 2.5	36.4 $\pm$ 3.0
CON	180.4 $\pm$ 15.5 [#]	93.8 $\pm$ 8.5 [#]	307.3 $\pm$ 34.8 [#]
Protopic [®]	79.0 $\pm$ 7.6***	79.5 $\pm$ 5.7***	97.2 $\pm$ 8.6***
COE1%	159.8 $\pm$ 7.1*	88.6 $\pm$ 6.8	200.5 $\pm$ 28.7***
COE2%	156.5 $\pm$ 7.2**	81.5 $\pm$ 5.7*	178.2 $\pm$ 26.7***

[†]Data are presented as means $\pm$ SD ($n=6$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$ vs. control.

[#] $p < 0.05$ control vs. normal.

skin lesions in NC/Nga mice closely resemble those of human AD and show various grades of signs and symptoms such as erythema and hemorrhage, followed by edema, superficial erosion, deep excoriation, alopecia, and dryness of the skin. In addition, IgE hyperproduction in the serum, severe itching behavior, and invasion of inflammatory cells into the skin have been observed in NC/Nga mice with AD-like skin lesions (Shiohara et al., 2004).

In this study, the anti-allergic effects of COE on NC/Nga mice were evaluated following application of a mite antigen DfE ointment to the backs of NC/Nga mice. The dermatitis scores increased rapidly and became significant within 1 week of the first application of DfE ointment. Repeated topical application of DfE ointment induced AD-like skin lesions, with itching, skin dryness, mild erythema, edema, and hemorrhage within 2 weeks. Interestingly, the dermatitis scores remained high for at least 2 weeks after the last application of DfE ointment on the 19th day. Even though the severity of AD in humans might differ from those in the mouse model, in this study, as in previous studies, these clinical signs and symptoms of the NC/Nga mice were very similar to those of human AD (Matsuoka et al., 2003; Yamamoto et al., 2007). From the 21st day, application of COE to the dorsal skin of mice reduced skin symptoms and mouse dermatitis scores (Fig. 3). In the COE groups, the dermatitis scores had significantly decreased by the 35th day, as compared to the scores in the CON group (Fig. 3). The dermatitis score reduction ratios were COE1%, 42.9%; COE2%, 44.4%; Protopic[®], 81.0%. This result indicates that COE suppressed the AD skin symptoms induced by DfE in NC/Nga model mice (Fig. 2).

Histologically, acute eczematous lesions in AD patients exhibit hypertrophy and hyperkeratosis of the epidermis, intracellular edema, and infiltration of inflammatory cells (Kawakami et al.,

2009). In this study, histological analysis clearly revealed that the application of COE inhibited skin inflammation in NC/Nga mice. Hypertrophy and hyperkeratosis of the epidermis, intracellular edema, and infiltration of inflammatory cells, including mast cells in the dermis were observed in the CON group, whereas these changes were diminished in the COE1%, COE2%, and Protopic[®] groups (Fig. 4).

Allergens may enter skin that has a defective barrier function and they are picked up by Langerhans cells (LCs) through the antigen-specific IgE that is bound to these cells via an IgE receptor. Antigen-loaded LCs migrate to lymph nodes and stimulate naive CD4⁺ T cells (Th0) to differentiate into Th2 cells, which, in turn promote differentiation of B cells into IgE-producing plasma cells. The level of serum total IgE is associated with the severity of AD and affected by abnormalities of the skin barrier, a key feature of AD (Liu et al., 2011). In this study, COE groups had significantly lower serum total IgE levels (Fig. 6) and infiltration of mast cells (Fig. 4) than controls. This means that COE can suppress serum total IgE and mast cells, leading to the inhibition of the immediate phase of allergic inflammation.

Pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 are known to be important accessory molecules in T-cell activation (Schreiber et al., 1992). TNF- α is involved in systemic inflammation, and is a member of a group of cytokines that stimulate the acute phase reaction. TNF- α can induce the release of IL-1 β and IL-6 which, in turn, enhance the sensitivity of histiocytes to TNF- α . TNF- α is an endogenous pyrogen which can cause fever, and stimulate endothelial cells and leukocytes to release a series of inflammatory mediators, such as NO and oxyradical. NO can promote TNF- α production in mouse macrophages (Murray et al., 1992). Thus, the inhibition of the excessive production of TNF- α , IL-1 β , and IL-6 can be employed as criteria to evaluate the anti-inflammatory effects of medicinal herbs (Pae et al., 2003). In this study, pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α were significantly lower in the skin lesions of NC/Nga mice treated with COE than in controls (for IL-1 β : COE1%, $p < 0.05$ vs. control and COE2%, $p < 0.05$ vs. control; for IL-6: COE1% was not significant and COE2%, $p < 0.05$ vs. control; for TNF- α : COE1%, $p < 0.05$ vs. control and COE2%, $p < 0.01$ vs. control; Table 2). These data indicate that COE suppressed systemic inflammation in NC/Nga model mice by inhibiting the production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6). This is consistent with previous reports (An et al., 2002; Pae et al., 2003; Cho et al., 2007).

Dysregulated Th1 and Th2 responses that are characterized by Th2-dominant allergic inflammation are thought to be central to the pathology of diseases such as AD (Grewe et al., 1998). In particular, Th2 immune responses mediated by IL-4, IL-5, and IL-13 are important in the pathogenesis of atopic disorders (Brandt, Sivaprasad, 2011). IL-4 and IL-13 primarily regulate IgE synthesis by B cells. IL-5 plays an important role in eosinophil infiltration. In fact, the correlation between the elevation of serum total IgE and the production of Th2 cytokines has been previously reported (Matsuda et al., 1997). In this study, there was less IL-4, IL-13, and IL-5 in the skin lesions of NC/Nga mice treated with COE than in controls (for IL-4: COE1%, $p < 0.05$ and COE2%, $p < 0.01$; for IL-13: COE1%, $p < 0.001$ and COE2%, $p < 0.001$; for IL-5: COE2%, $p < 0.05$; Table 3). These data indicate that COE suppressed DfE-induced dermatitis in NC/Nga model mice by suppressing the Th2 cell response.

TSLP is produced in large amounts by keratinocytes in acute and chronic AD lesions. TSLP converts blood DCs into antigen-presenting cells that elicit 'pro-allergic' T cells. Pro-allergic T cells are CD4⁺ T cells that produce substantial amounts of the typical Th2 cytokines, IL-4, IL-5 and IL-13, and secrete large quantities of the inflammatory cytokine TNF- α (Soumelis et al., 2002). In addition, LCs secrete the Th2-attracting chemokine TARC/CCL17 in response to TSLP (Ebner et al., 2007).

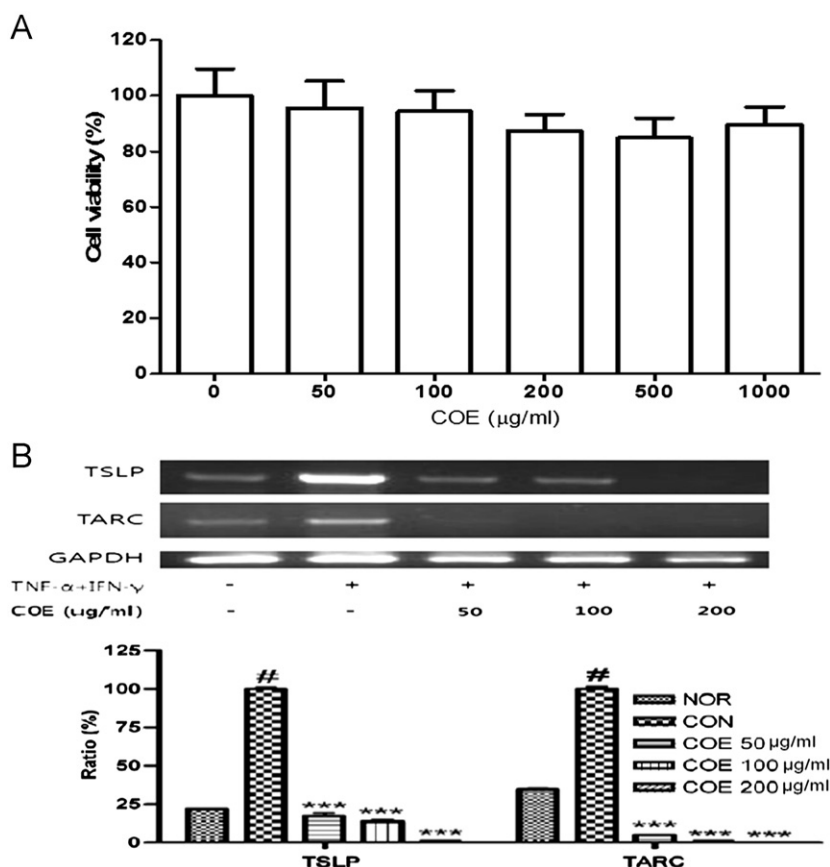


Fig. 7. Effect of COE on TSLP and TARC mRNA expression in HaCaT cells treated with IFN- γ /TNF- α . (A) Effect of COE on cell viability. (B) Effect of COE on TSLP and TARC mRNA expression in HaCaT cells. Data are presented as means $\pm$ SD ($n=3$). Data were analyzed using one-way analysis of variance followed by Dunnett's post-hoc test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control; # $p < 0.05$ control vs. normal.

TARC is overproduced in epidermal keratinocytes (Shimada et al., 2004), and is believed to attract Th2 cells into the skin from circulating blood. This may trigger or exacerbate the inflammatory process of AD (Nakatani et al., 2001). RANTES (CCL5) is chemotactic for Th2 lymphocytes, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites. RANTES was first identified in a search for genes expressed “late” (3–5 days) after T cell activation. A significant correlation was found between RANTES levels and both serum total IgE levels and eosinophil numbers in patients with AD (Kaburagi et al., 2001). Therefore, these chemokines, TARC and RANTES, may be important mediators that induce AD via the local accumulation of inflammatory cells. In this study, stimulation of HaCaT cells with IFN- γ and TNF- α significantly increased production of TSLP and TARC. The levels of TSLP and TARC were significantly inhibited in a dose-dependent manner by treatment with COE (Fig. 7). This is consistent with earlier reports (Yang, 2009).

In summary, we demonstrated that COE inhibited the AD-like skin lesions in NC/Nga mice that were induced by DFE. COE significantly reduced dermatitis scores by alleviation of clinical severity, infiltration of inflammatory cells, and serum total IgE levels. COE significantly inhibited pro-inflammatory cytokines, Th2 cytokines, Th2 chemokine TARC, and pro-Th2 cytokine TSLP in the AD-like skin lesions in NC/Nga mice. This means that COE suppressed the differentiation of naive CD4⁺ T cells (Th0) into Th2 cells, and reduced systematic inflammation. These results demonstrate that *C. ovata* stem bark may be a useful external medicine for treatment of AD. Further investigation is necessary to determine appropriate COE dosage and to evaluate the safety of this medicinal herb.

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