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Sceptridium ternatum attenuates allergic contact dermatitis-like skin lesions by inhibiting T helper 2-type immune responses and inflammatory responses in a mouse model

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ABSTRACT

Background: *Sceptridium ternatum* (ST) is a medicinal herb used in folk remedies for the treatment of various disorders such as pertussis, allergic asthma, abdominalgia, diarrhea, and external use for wound healing. However, the biological and pharmacological activities of ST are not fully clarified besides anti-asthmatic effect.

Objective: We studied a *Sceptridium ternatum* ethanol extract (ST) with respect to its anti-inflammatory and immune regulatory activities in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, concanavalin A (conA)-stimulated BALB/c mice splenocytes, and a 2,4-dinitrochlorobenzene (DNCB)-induced allergic contact dermatitis (ACD) mouse model.

Methods: RAW 264.7 cells were pretreated with ST for 1 h and then stimulated with LPS. To determine the anti-inflammatory effects of ST, the production of nitric oxide (NO), interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) were measured using an enzyme-linked immunosorbent assay (ELISA). To determine its anti-allergic effects, splenocytes from BALB/c mice were incubated and stimulated with conA in the absence or presence of ST for 48 h. The production of IL-4 and interferon (IFN)- γ in culture supernatants were evaluated by ELISA. To test the effects of ST on ACD, 100 μ L of 1% DNCB was applied to the dorsal skin of BALB/c mice for 2 weeks, and ST was administered 2 h before DNCB application. The thicknesses of the epidermis and dermis were determined by skin histological analysis. Serum immunoglobulin (Ig) E levels, the production of IL-1 β , IL-4, and IL-6 in dorsal skin tissue, and T helper (Th) 2 cytokines production of CD4⁺ T cells were analyzed by ELISA. The expression of nuclear transcription factor- κ B (NF- κ B) both in vitro and in vivo was determined via immunoblotting.

Results: In RAW 264.7 cells, ST inhibited LPS-induced inflammation mediator production and NF- κ B expression. ST upregulated IFN- γ production and downregulated IL-4 production in conA-stimulated splenocytes. ST application reduced the thicknesses of the epidermis and dermis by decreasing serum IgE level and the expressions of IL-1 β , IL-4, IL-6, and NF- κ B in the dorsal skin of the DNCB-induced ACD model mice. Furthermore, ST treated group showed reduction of the Th2 cytokines production in activated CD4⁺ T cells.

Conclusion: These findings not only indicate that application of ST reduced skin thickening by regulating Th 2-type allergic responses and inhibiting expression of inflammatory mediators in a DNCB-induced ACD mouse model, but also suggest that *Sceptridium ternatum* is a natural option for the treatment of skin inflammation.

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1. Introduction

Allergic contact dermatitis (ACD) is a type of inflammatory skin disease, characterized by high levels of serum IgE, whose symptoms include edema, pruritus, redness, and warmth [1]. The pathogenesis of ACD is not understood, but is believed to be related to high levels of immunoglobulin (Ig) E and T helper 2

Abbreviations: ACD, allergic contact dermatitis; DNCB, 2,4-dinitrochlorobenzene; IgE, immunoglobulin E; IL-4, interleukin-4; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; NO, nitric oxide; ST, *Sceptridium ternatum* ethanol extract; TNF- α , tumor necrosis factor- α .

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(Th2)-type immune responses [2]. Repeated exposure to allergens can trigger excessive secretion of IgE resulting from Th2 cytokines such as interleukin (IL)-4, and the consequent secretion of inflammatory cytokines can provoke skin inflammation [1,3].

Inflammation is a biological response that protects organisms from harmful stimuli and initiates the healing process. In these processes, activated immune cells secrete many inflammatory mediators, including nitric oxide (NO) and cytokines [4]. NO is an important signaling molecule associated with inflammatory processes. Overproduction of NO can stimulate the expression of enzymes and other proteins involved in inflammatory reactions [5,6], and contribute to clinical cutaneous inflammatory responses [7]. Pro-inflammatory cytokines, including IL-1 β , IL-6, and tumor necrosis factor- α (TNF- α) can be initiators or mediators of inflammatory responses [8,9]. These inflammatory mediators promote a wide range of inflammatory responses, including skin thickening, edema, pruritus, redness, and warmth [10,11].

Sceptridium ternatum is a member of the ophioglossaceae, which are seedless vascular plants. Recently, wound healing activities of *Ophioglossum vulgatum*, another ophioglossaceae, have been reported [12,13]. However, the biological activities of *S. ternatum* have not been elucidated, except for an anti-asthmatic effect [14]. Thus, we investigated the effects of *S. ternatum* ethanolic extract (ST) on allergic skin inflammation. After confirming its anti-inflammatory activities in LPS-stimulated RAW 264.7 cells, we investigated its effects on the regulation of the Th1/Th2 response in concanavalin A (con A)-stimulated BALB/c mouse splenocytes, and on a 2, 4-dinitrochlorobenzene (DNCB)-induced allergic contact dermatitis murine model.

2. Methods

2.1. Materials and reagents

The entire *S. ternatum* plant was collected on Jeju Island, South Korea. Its botanical identity was authenticated by Y.P. Jang (Kyung Hee University, Seoul, South Korea). A voucher specimen of *S. ternatum* (KHUP-0519) was deposited in the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, South Korea). Methanol and formic acid were HPLC grade and were purchased from Duksan Pure Chemicals Co. (Ansan, South Korea). High purity nitrogen gas was provided by Shinyang Oxygen Co. (Seoul, South Korea).

2.2. Sample preparation for UPLC (ultra performance liquid chromatography) analysis

Dried whole *S. ternatum* plant (100 g) was extracted with 70% ethanol (1 L) by sonication for 2 h at 50 °C. The 70% ethanol extract of ST was filtered through filter paper (Hyundai Micro Co., Ltd., Korea) and the filtrate was concentrated in vacuo using a rotatory vacuum evaporator (EYELA, Japan) (yield: 18 g). The residue was dissolved in 1 mL of 30% methanol to yield a final concentration 30 mg/mL stock solution, and was filtered through a 0.2 μ m Acrodisc syringe filter (Pall Life Sciences, USA) before being injected into the UPLC apparatus.

2.3. UPLC-ESI-MS analysis

The UPLC instrument was an ACQUITY UPLC H-Class system running Empower software (Waters, Milford, MA, USA). The PDA detector was set to record between 210 and 450 nm. A Brownlee SPP C18 column (3.0 $\times$ 100 mm, 2.7 μ m) was selected for the UPLC study (PerkinElmer, Massachusetts, USA). The monitoring wavelength was set to 345 nm. The mobile phase consisted of methanol (A) and water (B) acidified with formic acid (0.1%). All solvents were filtered through a 0.2 μ m filter. The gradient program was: 0 min, 15% of solvent A; 35 min, 45% solvent A; 44 min, 100% solvent A; and 45 min, 15% solvent A, at a flow rate of 0.5 mL per min using a commercial splitter. The injection volume was 2 μ L. An AccuTOF[®] single-reflectron time-of-flight mass spectrometer was equipped with an ESI source (Electrospray ionization, JEOL, Peabody, MA, USA) and was operated with Mass Center system version 1.3.7b (JEOL, Peabody, MA, USA). In the positive ion mode, the typical values were set as follows: orifice 1 = 80 V, ring lens = 10 V, and orifice 2 = 5 V. The ion guide potential and detector voltage were set to 2000 V and 2300 V, respectively. ESI parameters were set as follows: needle electrode = 2000 V, nitrogen gas was used as a nebulizer, desolvating and their flow rate were 1 and 3 L/min, desolvating chamber temperature = 250 °C, and orifice 1 temperature = 80 °C. Mass scale calibration was accomplished with a YOKUDELNA calibration kit (JEOL, Peabody, MA, Japan) for accurate mass measurements. MS acquisition was set at a scan range of 150 to 1500 m/z.

2.4. Cell culture and cell viability assay

RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were

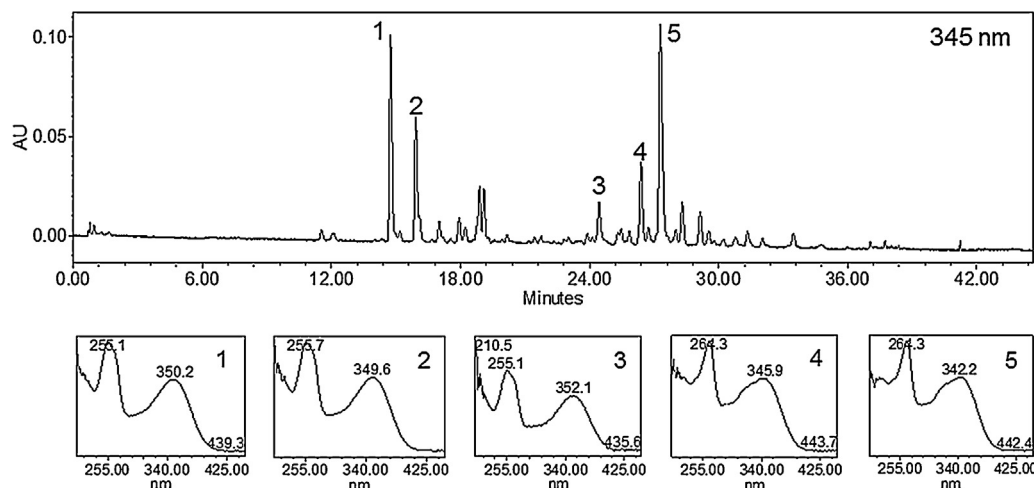


Fig. 1. UPLC chromatogram of ST and UV-vis absorption spectra of five peaks.

maintained in 100% humidity and 5% CO₂ at 37 °C in Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (P.S). The *S. ternatum* extract powder was dissolved 10 mg/ml in phosphate buffer saline (PBS) for 24 h, and filtered by 0.45 µm syringe filter (Millipore, MA, USA). The cytotoxicity of ST in RAW 264.7 cells was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates at a concentration of 1×10^5 cells/mL and incubated overnight. Thereafter, medium was replaced with FBS-free medium containing various concentrations of ST (0, 1, 10, 100 µg/mL). After 25 h of incubation, 50 µL of the MTT solution (2 mg/mL) was added to each well, followed by incubation for 4 h in a CO₂ incubator. Thereafter, 100 µL of DMSO was added to each well to dissolve formazan crystals, and optical density was measured at 570 nm using an ELISA reader.

2.5. Nitric oxide assay and enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were seeded in 12-well plates at a concentration of 5×10^5 cells/mL and incubated overnight. Culture medium was then replaced with FBS-free medium containing various

concentrations of ST (0, 1, 10, or 100 µg/mL). After 1 h, cells were stimulated with 1 µg/mL of LPS (Sigma, St. Louis, MO, USA) for 24 h. NO concentrations in the supernatant were measured by the Griess reaction using Griess reagent (Sigma). Levels of TNF-α, IL-6, and IL-1β in the supernatants were measured by ELISA, using a commercial kit (OptELA™ Kits; BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

2.6. Western blot assays

RAW 264.7 cells were seeded in 6-well plates at a concentration of 5×10^5 cells/mL and incubated overnight. Medium was then replaced with FBS-free medium containing various concentrations of ST (0, 1, 10, or 100 µg/mL). After 1 h, cells were stimulated with 1 µg/mL of LPS for 30 min and the supernatant was removed. Cell protein extracts were prepared by lysing cells with RIPA buffer. The lysates (20 µg/lane) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies (against β-actin and nuclear transcription factor-κB (NF-κB)) overnight, washed, and then incubated with secondary antibodies for 2 h. Protein bands were visualized using an enhanced chemiluminescence western blot analysis system (abClon, South Korea).

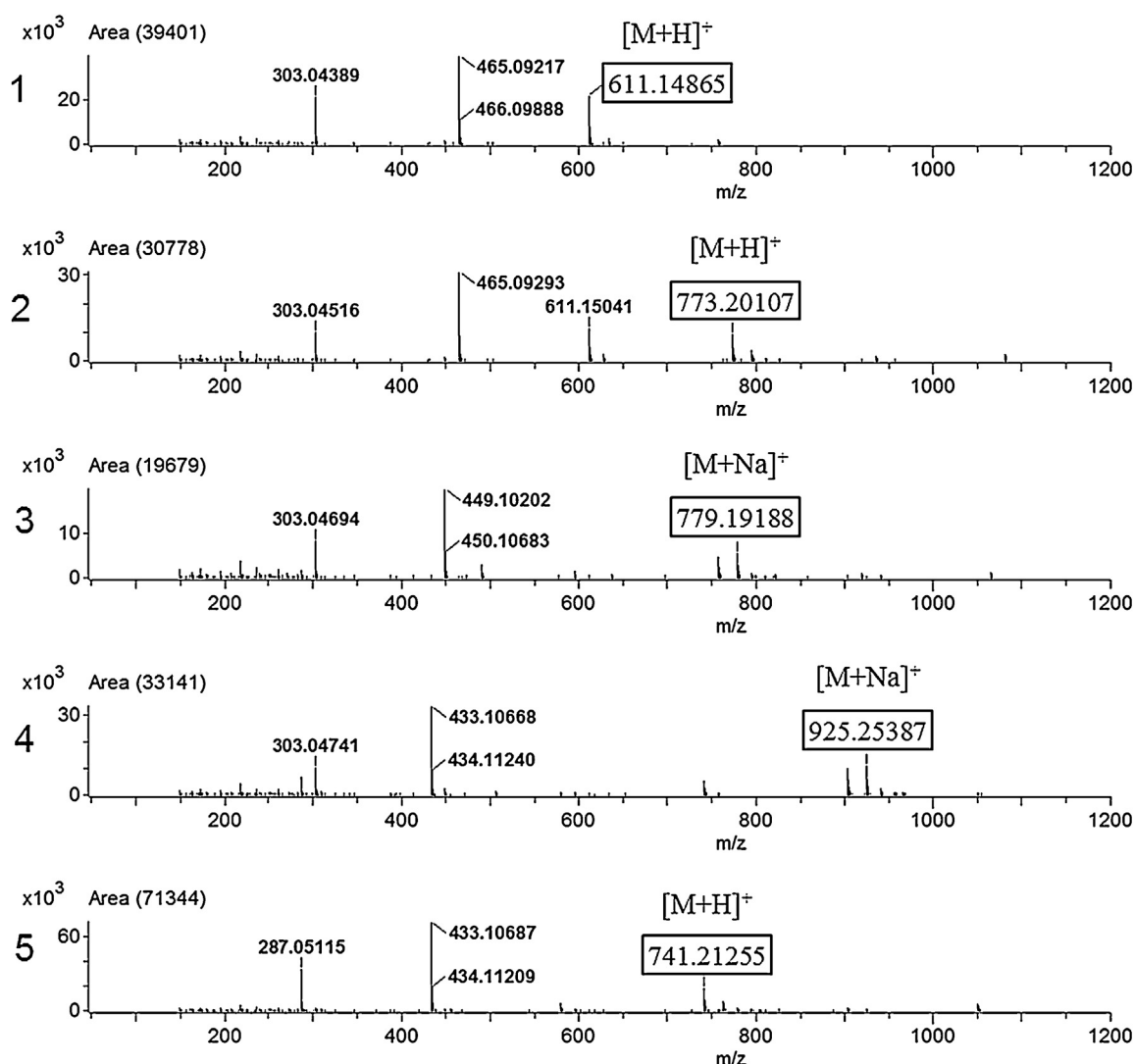


Fig. 2. ESI-MS spectra of five peaks from ST.

Table 1
The observed and calculated mass numbers of *S. ternatum* 70% ethanol extract.

Peak No.	Rt (min)	Theoretical mass [M + H] ⁺	Observed mass [M + H] ⁺	Mass difference (mmu)	Identification
1	14.74	611.16121	611.14865	−12.56	Quercetin 3-O-α-L-rhamnosyl-7-O-β-D-glucoside
2	15.92	773.21403	773.20107	−12.96	Quercetin 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-β-D-glucoside
5	27.28	741.22420	741.21255	−11.65	Kaempferol 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-L-rhamnoside
Peak No.	Rt (min)	Theoretical mass [M + Na] ⁺	Observed mass [M + Na] ⁺	Mass difference (mmu)	Identification
3	24.44	779.20106	779.19188	−9.18	Quercetin 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-L-rhamnoside
4	26.40	925.25897	925.25387	−5.10	Kaempferol 3-O-(2,3-di-O-β-D-glucosyl)-α-L-rhamnoside-7-O-α-L-rhamnoside

2.7. Animals

Female BALB/c mice (aged 6 weeks) were purchased from Nara Bio Inc. (Korea). Animals were housed under pathogen-free conditions and were fed a laboratory diet. The experimental protocols used in this study complied with the guidelines of the Animal Care and Ethics Committee of Kyung Hee University (KHUASP (SE)-13-048).

2.8. Preparation of splenocytes and IL-4 and IFN-γ measurement

The spleens of mice were removed and were pressed in RPMI 1640 using glass slides (Hyclone, UT, USA). The cell suspensions were filtered through a 70 μm cell strainer (BD Falcon, MA, USA) and were centrifuged at 1500 rpm for 5 min. After removing the supernatant, the pellet was incubated with RBC lysis buffer (Sigma-Aldrich, MO, USA) for 1 min. The cells were then washed three times and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS (Hyclone, UT, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 2 mM 2-mercaptoethanol. Cells were seeded in 96-well plates at a concentration of 5×10^5 cells/mL and incubated for 48 h with 100 μg/mL ST. The cell viability was determined by MTT assay. We then examined the effect of ST on the production of IL-4 and IFN-γ in conA (Sigma-Aldrich, MO, USA)-stimulated mouse splenocytes. Cells were seeded in 24-well plates at a concentration of 1×10^6 cells/mL and incubated with 2 μg/mL conA in the absence or presence of 100 μg/mL ST for 48 h. Cytokine levels in the supernatants were measured by ELISA [15].

2.9. Induction of atopic dermatitis-like lesions

Mice were randomly divided into three groups ($n=5$): (1) Normal group: normal mice without any treatment, (2) DNCB + PBS group: DNCB sensitization without drug treatment, and (3) DNCB+ST group: DNCB sensitization with 1% ST treatment. Mice were sensitized by topically applying 100 μL of 1% DNCB (in 4:1 acetone/olive oil) to their shaved dorsal skin on days 1–3. Four days later, the mice were resensitized by applying 100 μL of 0.5% DNCB to dorsal skin each day for 2 weeks. The barrier function of skin was eliminated by applying 100 μL of 4% sodium dodecyl sulfate (SDS). 100 μL of 1% ST was applied to the dorsal skin of the mice 2 h before DNCB application, every day for 2 weeks. Mice were sacrificed on day 21 of the experiment [16].

2.10. Histological analysis

Dorsal skin samples were fixed in 10% formaldehyde fixing solution for 24 h. The tissues were embedded in paraffin and sectioned at a thickness of 4 μm. Sections were stained with hematoxylin and eosin solution to assess epidermal and dermal hyperplasia. The thicknesses of the epidermis and dermis were evaluated using Leica Application Suite Microscope Software (Leica Microsystems Inc., IL, USA) (100× magnification).

2.11. Measurement of Th2 cytokines production in vitro

CD4⁺ T cells were isolated from mice lymph nodes, and were purified using a MACS separation column (Qiagen, Germantown,

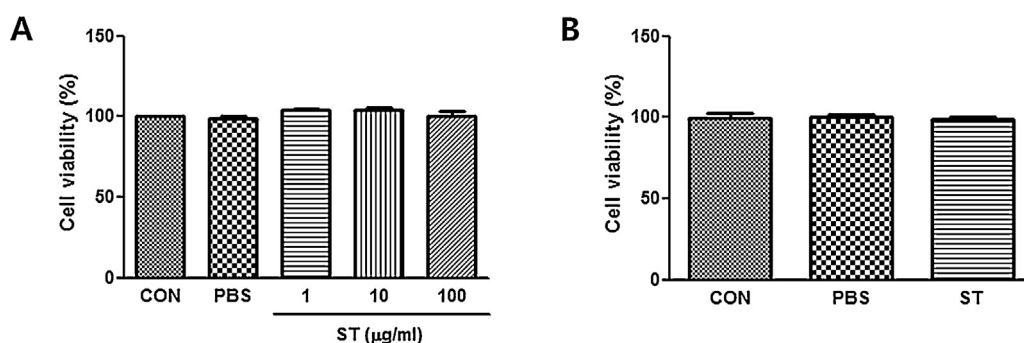


Fig. 3. Effects of ST on cell viability in RAW 264.7 macrophages and mouse splenocytes. RAW cells were incubated with 10 μL/mL of phosphate buffer saline (PBS) and various concentrations (1, 10, 100 μg/mL) of ST for 25 h (A), and mouse splenocytes were treated with 10 μL/mL of PBS and 100 μg/mL of ST for 48 h (B). Cytotoxicity was measured by MTT assay. Values are the means ± SEM of three experiments performed in triplicate.

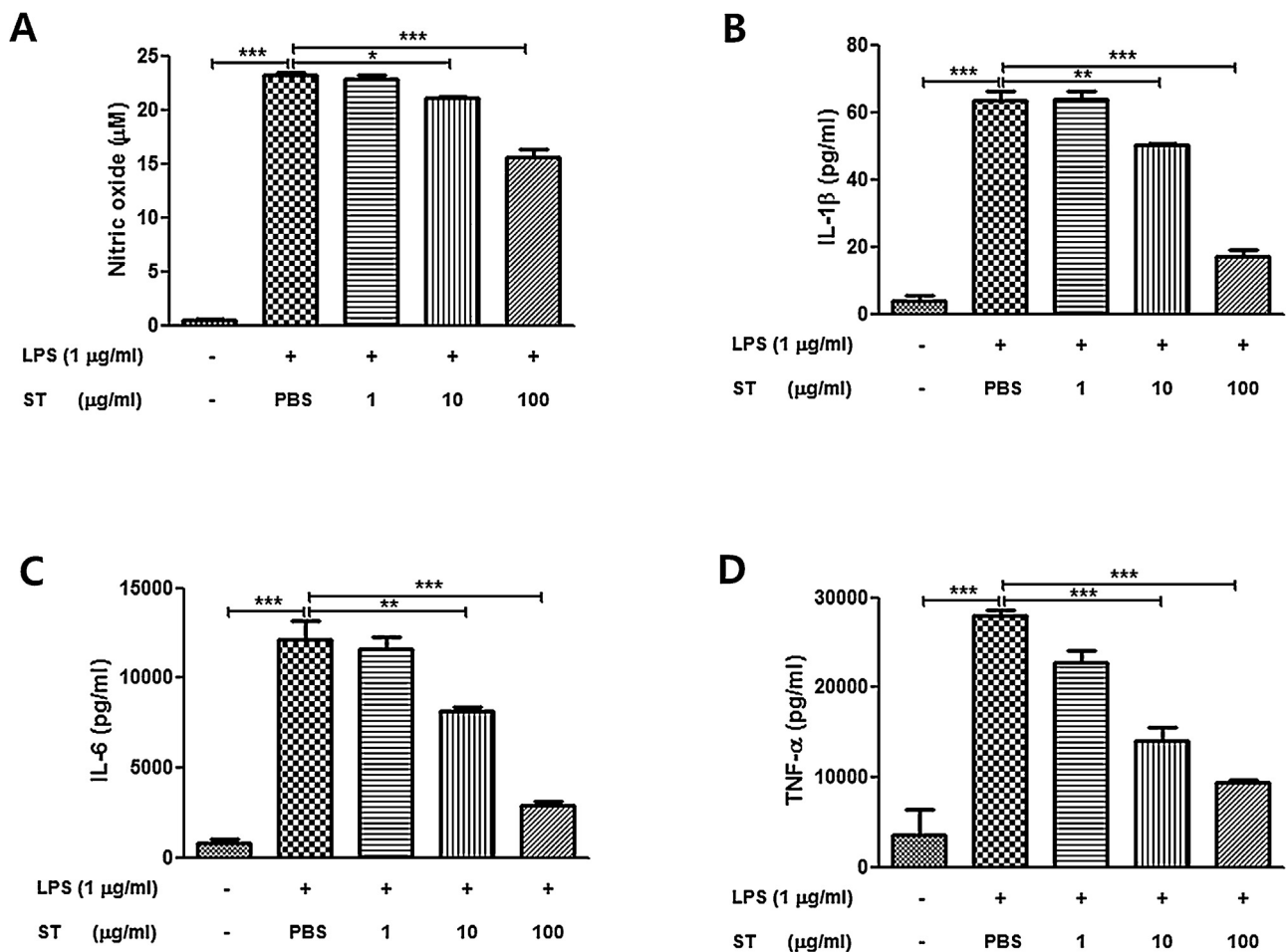


Fig. 4. Effects of ST on the productions of nitric oxide (NO) and cytokines in LPS-stimulated RAW 264.7 cells. Cells were pretreated with PBS or ST (1, 10, 100 μg/mL) for 1 h, thereafter exposed to 1 μg/mL LPS for 24 h. The amount of NO (A), IL-1β (B), IL-6 (C) and TNF-α (D) in medium was measured by using NO assay and ELISA. Data represents the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. LPS stimulation + PBS.

MD, USA) according to the manufacturer's instructions. The purified CD4⁺ T cells (1×10^6 cells/mL) were stimulated with plate bound anti-CD3 antibody (BD Pharmingen, San Diego, CA, USA) (5 μg/mL) and the soluble form of purified anti-CD28 antibody (BD Pharmingen) (2 μg/mL) in RPMI-1640 supplemented with 10% FBS, 50 μM of β-mercaptoethanol, 2 mM of glutamine, and 1% P.S for 48 h. The amount of IL-4 and IL-13 production in the culture supernatant was measured by ELISA.

2.12. Serum and tissue protein analysis

Serum levels of IgE were measured by ELISA. To analyze cytokines in skin tissue, tissue proteins were extracted using T-PER tissue protein extraction reagent containing a protease inhibitor cocktail. Total protein concentrations were measured using the Bradford assay, and levels of IL-1β, IL-4, and IL-6 in the dorsal skin were measured by ELISA. NF-κB expression in the skin tissue was detected by western blot.

2.13. Statistical analysis

Statistical analysis of the data was carried out using Prism 4 software (GraphPad Software Inc., CA, USA). Data are presented as mean ± standard error of the mean (SEM), and multiple comparisons were performed using one-way ANOVA. Results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of phytochemicals by UPLC-ESI-MS

In order to establish a standard chromatogram for the 70% ethanol extract of ST, UPLC analysis was performed with a gradient elution system. The optimal chromatographic profile was established. The identification of major peaks of chromatogram was performed by UPLC-ESI-MS (Figs. 1 and 2). The retention time, observed mass, mass difference, and proposed compounds for five peaks are listed in Table 1. Based on mass numbers and UV-vis spectra, all five peaks were identified as flavonoid glycosides [17]. Although flavonoid glycosides from ST had complex sugar linkage structures, the only aglycone structures found were those of kaempferol and quercetin. Consequently, we focused on the aglycone ions at m/z 303 (quercetin) and 287 (kaempferol). The mass spectrum of peak 1 showed protonated molecular ions of m/z 611 and 465 and an aglycone ion peak of 303, corresponding to quercetin 3-O-α-L-rhamnosyl-7-O-β-D-glucoside. Peak 2 showed protonated molecular ions of m/z 773, 611, and 465, and an aglycone ion peak of 303, corresponding to quercetin 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-β-D-glucoside. Peak 5 showed protonated molecular ions of m/z 741 and 433 and an aglycone ion peak of 287, corresponding to kaempferol 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-L-rhamnoside. Peak 3 showed a sodiated molecular ion of m/z 779, a protonated

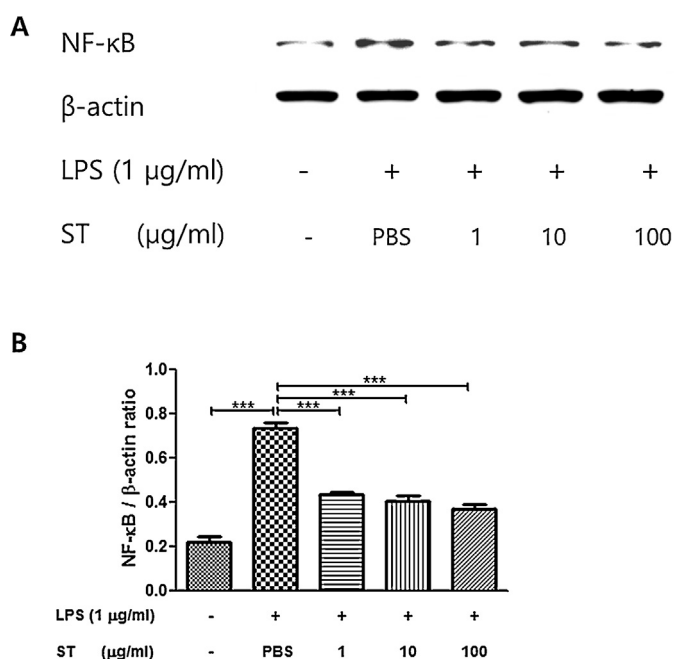


Fig. 5. Effects of ST on NF-κB expression in LPS-stimulated RAW 264.7 cells. Cells were pretreated with PBS or ST (1, 10, 100 μg/mL) for 1 h, and then exposed to 1 μg/mL LPS for 30 min. Levels of NF-κB expression were analyzed by western blot assay (A). Relative NF-κB protein levels were quantified using the ImageJ software (<http://imagej.nih.gov/ij/>), and normalized to the internal control (β-actin) protein levels (B). Data represents the mean ± SEM. ****p* < 0.001 vs. LPS stimulation + PBS.

fragmented ion of *m/z* 449, and an aglycone ion peak of 303, corresponding to quercetin 3-*O*-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-*O*-α-L-rhamnoside. Peak 4 showed a sodiated molecular ion of *m/z* 925, a protonated fragmented ion of *m/z* 433, and an aglycone ion peak of 287, corresponding to kaempferol 3-*O*-(2,3-di-*O*-β-D-glucosyl)-α-L-rhamnoside-7-*O*-α-L-rhamnoside. These results confirmed the botanical identity of the plant and provided chemical fingerprints for its constituents.

3.2. ST reduced the production of NO and inflammatory cytokines in LPS-stimulated RAW 264.7 cells.

Cells were treated with ST (0, 1, 10, or 100 μg/mL) for 25 h, and a MTT assay was performed. ST was demonstrated to have no cytotoxicity up to a concentration of 100 μg/mL (Fig. 3A). The amount of NO, a marker of the inflammatory response, was measured using Griess reagent. Relative to the negative control (unstimulated cells), the positive control, stimulated with LPS, had significantly increased NO levels. Compared to the positive control,

the cell groups treated with 10 μg/mL and 100 μg/mL of ST had 9% and 33% reductions in NO production, respectively (Fig. 4A). Levels of IL-1β, IL-6, and TNF-α were measured by ELISA using a commercial kit. Similarly, relative to the negative control, the positive control had significantly increased levels of IL-1β, IL-6, and TNF-α. Compared to the positive control, the cell groups treated with 10 μg/mL and 100 μg/mL of ST had 21% and 73% reductions in IL-1β production, respectively (Fig. 4B). The cells treated with ST (1, 10, or 100 μg/mL) displayed significant dose-dependent decreases in their levels of IL-6 (4%, 33%, and 76%, respectively) and TNF-α (19%, 50%, and 66%, respectively) (Fig. 4C and D).

3.3. ST inhibited NF-κB expression in LPS-stimulated RAW 264.7 cells.

The expression of NF-κB was measured by western blot analysis. As shown in Fig. 5, compared to the negative control, cells stimulated with LPS increased their level of NF-κB expression. Compared with LPS-stimulated cells, ST treatment (1, 10, or 100 μg/mL) significantly reduced expression of NF-κB in a dose-dependent manner (41%, 45%, and 51%), respectively.

3.4. ST upregulated IFN-γ production and downregulated IL-4 production in mouse splenocytes.

Mouse splenocytes were incubated with 100 μg/mL of ST for 48 h, and we confirmed that ST does not have cytotoxicity by a MTT assay (Fig. 3B). And after, to investigate the effects of ST on the production of IFN-γ and IL-4, splenocytes were stimulated with conA in the absence or presence of ST for 48 h. The amounts of IFN-γ and IL-4 were measured using ELISA. Compared with conA only-stimulated cells, the addition of 100 μg/mL of ST significantly increased IFN-γ production (81%) and decreased IL-4 production (19%) (Fig. 6).

3.5. ST reduced hyperplasia of the epidermis and dermis in the DNCB-induced mouse model.

Skin thicknesses were evaluated to determine the effects of ST on hyperplasia of the epidermis and dermis. Compared to the dorsal skin of the normal group, the dorsal skin of mice treated with DNCB demonstrated epidermal and dermal hyperplasia. ST administration to DNCB-treated mice significantly reduced the thickness of the epidermis, by up to 64%, and the thickness of the dermis, by up to 38% (Fig. 7).

3.6. ST suppressed Th2 cytokines production in lymph nodes of DNCB-stimulated mice.

CD4⁺ T cells from draining lymph nodes in the mice were stimulated with anti-CD3 antibody + anti-CD28 antibody for 48 h.

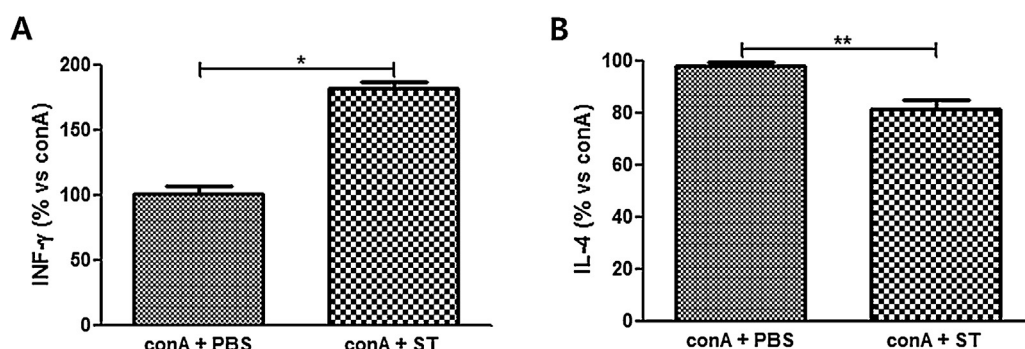


Fig. 6. Effects of ST on the production of cytokines in mouse splenocytes. Mouse splenocytes were stimulated with conA in the absence or presence of ST 48 h. The amount of (A) IFN-γ and (B) IL-4 was measured by using the ELISA. Data represents the mean ± SEM. **p* < 0.05 and ***p* < 0.01 vs. conA stimulation + PBS.

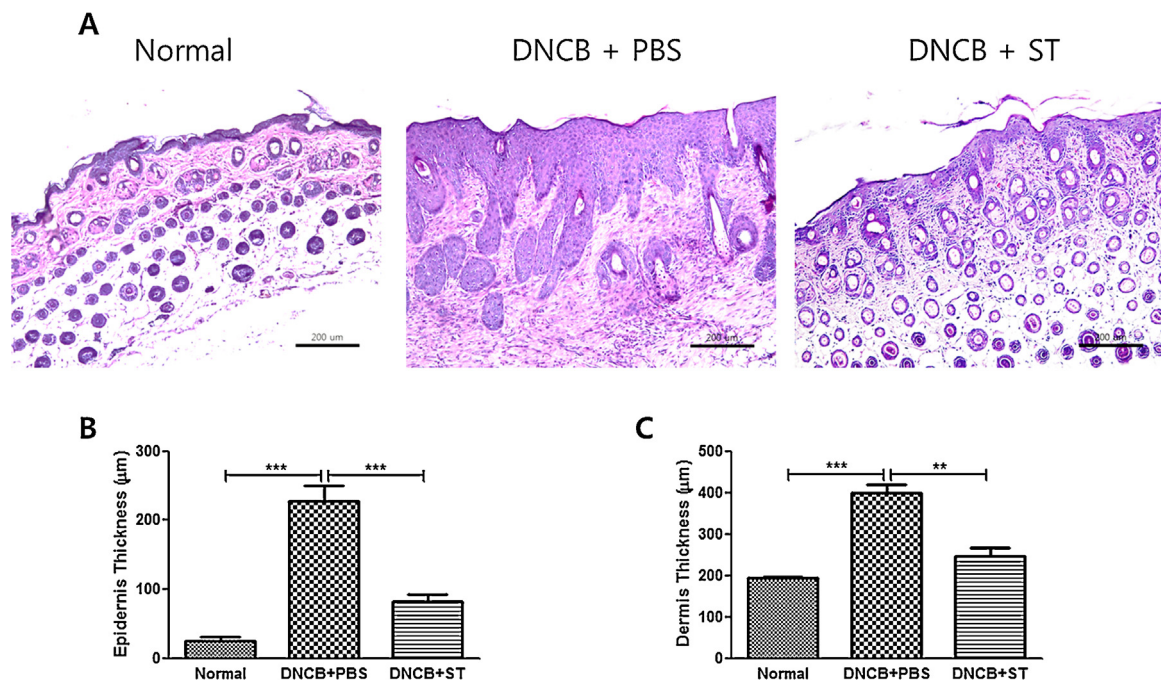
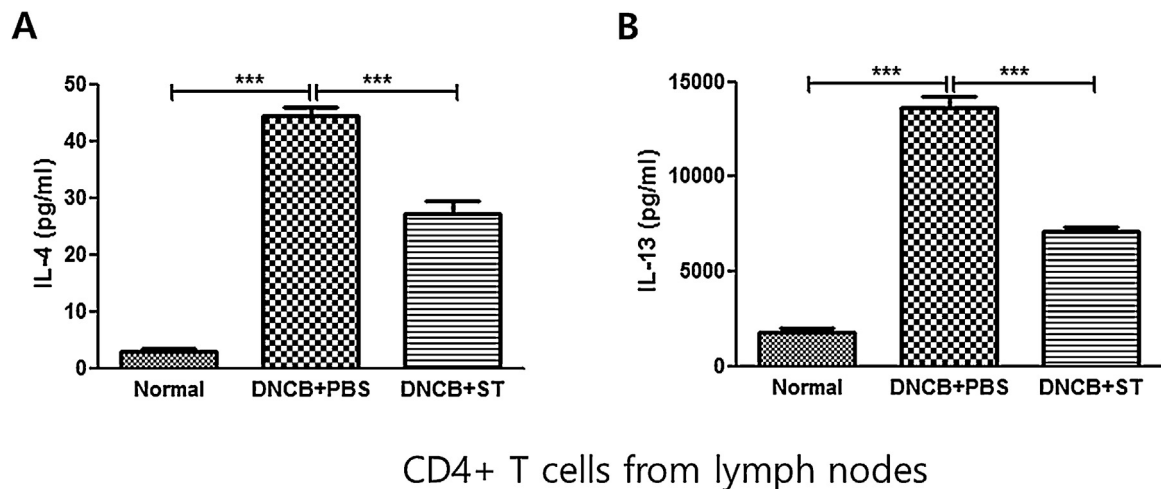


Fig. 7. Effects of ST on the pathological change: (A) Skin tissue sections with H&E staining (magnification $\times 100$); (B–C) Evaluation of epidermis and dermis thicknesses by digital image analysis in H&E stained sections. Data represents the mean $\pm$ SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. DNCB + PBS group.



CD4+ T cells from lymph nodes

Fig. 8. Effects of ST on Th2 cytokines in activated CD4⁺ T cells from lymph nodes of DNCB-stimulated mice. Draining lymph node CD4⁺ T cells were stimulated with plate bound anti-CD3 antibody and the soluble form of purified anti-CD28 antibody for 48 h. The amount of IL-4 (A) and IL-13 (B) in the culture supernatant was measured by using the ELISA. Data represents the mean $\pm$ SEM. $^{***}p \leq 0.001$ vs. DNCB + PBS group.

The amount of IL-4 and IL-13 in the supernatant was measured using ELISA. As shown in Fig. 8, compared to the normal group, the DNCB+PBS group had increased IL-4 and IL-13 production. Compared with the DNCB+PBS group, the ST-treated group showed a significant decrease in IL-4 (37%) and IL-13 level (48%).

3.7. ST reduced serum IgE levels and the production of cytokines in dorsal skin tissue.

IgE levels in serum and the amounts of IL-4, IL-1 β , and IL-6 in dorsal skin tissue were measured using ELISA. Compared with the normal group, the DNCB+PBS group had increased IgE serum levels. As shown in Fig. 9A, the ST-treated group showed a significant decrease in serum IgE (19%). Furthermore, we confirmed that DNCB application to the dorsal skin of mice

significantly increased IL-4, IL-1 β , and IL-6 production by comparing their levels to those in the normal group. We also observed that ST administration to the dorsal skin of DNCB-treated mice significantly reduced IL-4 (19%), IL-1 β (64%), and IL-6 (55%) expression relative to levels seen in the DNCB+PBS group (Figs. 9B–D).

3.8. ST inhibited NF- κ B expression in the DNCB-treated mouse model.

The expression of NF- κ B in DNCB-treated dorsal skin tissue was evaluated by western blot analysis. As shown in Fig. 10, NF- κ B expression was increased in the dorsal skin of mice treated with DNCB relative to levels in the dorsal skin of the untreated group. ST administration to DNCB-treated mice significantly reduced their NF- κ B expression (55%).

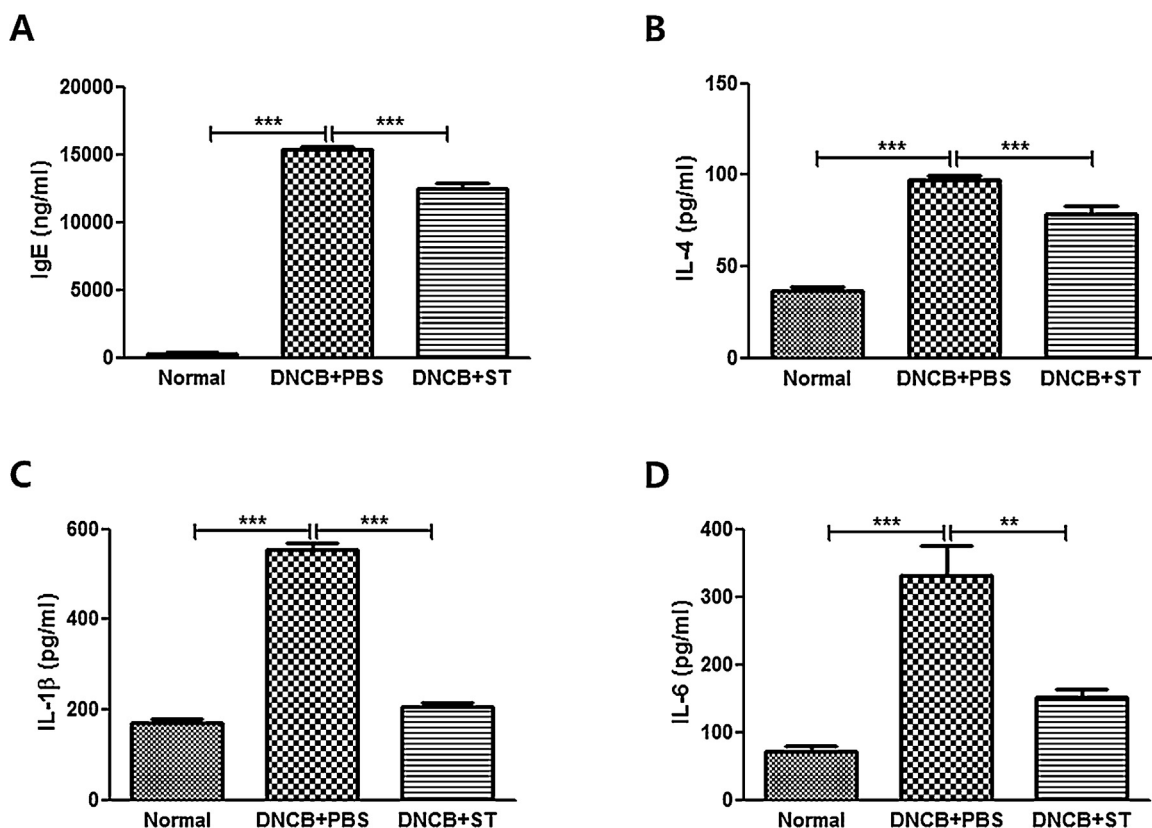


Fig. 9. Effects of ST on production of cytokines in DNCB-induced atopic contact dermatitis mice. Serum IgE level (A) and the amount of IL-4 (B), IL-1β (C) and IL-6 (D) in dorsal skin tissue were measured by using the ELISA. Data represents the mean ± SEM. ** $p < 0.01$ and *** $p < 0.001$ vs. DNCB + PBS group.

4. Discussion

Our study began by performing a screening test in macrophage cell line in order to find a medicinal plant with anti-inflammatory activity. Among 60 screened herbs, ST was found to have anti-inflammatory activity. As can be seen in our results, the anti-inflammatory activity of ST was prominent. Expecting the association between ST's anti-inflammatory effect and Th response, we started the following study.

Ophioglossaceae, seedless vascular plants, is a medicinal herb that is used for folk remedies of pertussis, allergic asthma, abdominalgia, and diarrhea. Yuan et al. reported that ST regulated the imbalance of Th1/Th2 responses in a mouse asthma model [14]. Clericuzio et al. reported on the wound-healing activities of flavonoid oligoglycosides [13] and galactoglycerolipids [12] from *Ophioglossum vulgatum*. Despite the many reported therapeutic properties of ophioglossaceae, current data on the molecular activities of the ophioglossaceae are inadequate. Specifically, the characterization of *S. ternatum* is insufficient except for its anti-asthmatic effect. Thus, in the present study, we investigated the anti-inflammatory activity of *S. ternatum*, which grows naturally on Jeju Island of Korea, with respect to allergic contact cutaneous inflammation.

In this study, the 70% ethanol extract of ST was analyzed to establish the standard chromatogram and identified major phytochemicals by UPLC-ESI-MS analysis. The following five flavonoid glycosides were identified which exhibit considerable cure of skin inflammation: quercetin 3-O-α-L-rhamnosyl-7-O-β-D-glucoside, quercetin 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-β-D-glucoside, kaempferol 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-

7-O-α-L-rhamnoside, quercetin 3-O-β-D-glucosyl-(1 → 2)-α-L-rhamnoside-7-O-α-L-rhamnoside and kaempferol 3-O-(2,3-di-O-β-D-glucosyl)-α-L-rhamnoside-7-O-α-L-rhamnoside.

In the inflammation processes, activated macrophage which is one of the inflammatory cells secretes many pro-inflammatory mediators [4,5]. Production of NO and inflammatory cytokines is regulated by NF-κB activation [18]. The expression of NF-κB and subsequent production of the inflammatory mediators promote cutaneous inflammatory responses, such as itchiness and skin thickening [10,11]. In the in vitro study, we confirmed that ST significantly inhibited the production of pro-inflammatory mediators and the expression of NF-κB, a transcription factor that regulates production of inflammatory mediators, in LPS-stimulated RAW 264.7 cells.

We then investigated the effect of ST on the production of IL-4 and IFN-γ in conA-stimulated mouse splenocytes. Allergic inflammatory skin diseases are associated with Th2 responses. IL-4 promotes Th2 immune responses and inhibits Th1 immune responses [19], while IFN-γ regulates Th2 immune responses [20]. Our results show that ST upregulates IFN-γ production and downregulates IL-4 production. This result suggested the strong probability that ST might regulate allergic disorders related to Th2 immune responses, such as atopic dermatitis, in a mouse model.

Accordingly, we induced ACD in mice by applying DNCB [21], and then analyzed the clinical effects of ST on the skin of the mice. ACD is a chronic inflammatory skin disease caused by Th2 dominant hypersensitivity reaction. Exposure to allergens leads CD4⁺ T cells to differentiate into Th2 cells, and the Th2 lymphocytes produce cytokines, such as IL-4 and IL-13, which

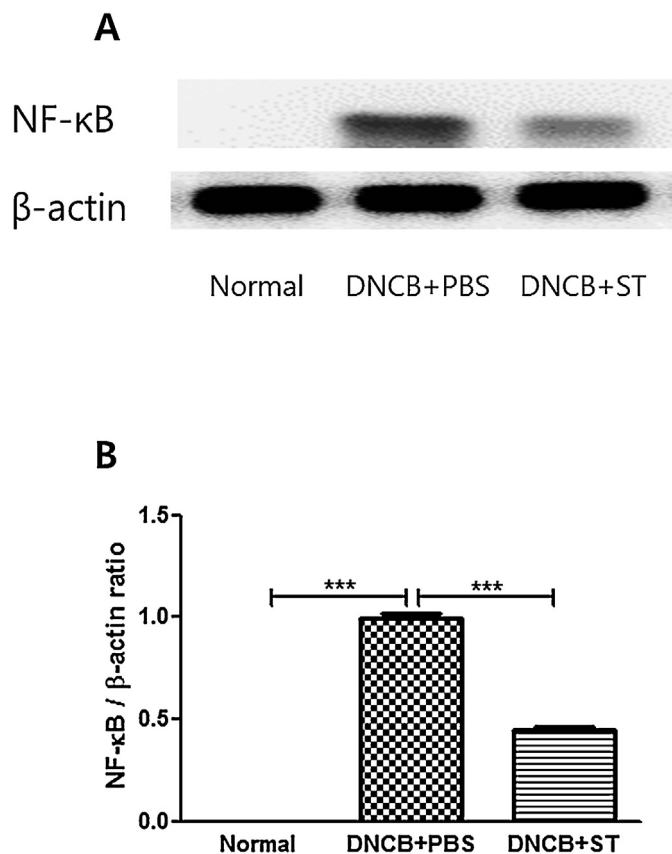


Fig. 10. Effects of ST on the NF-κB expression in dorsal skin tissue. (A) Level of NF-κB expression was analyzed by western blot assay. (B) Evaluation of NF-κB level by digital image analysis. Data represents the mean $\pm$ SEM. *** $p < 0.001$ vs. DNCB + PBS group.

promote an increase in IgE synthesis [22,23]. The excessive secretion of IgE induces the subsequent production of cytokines that cause numerous inflammatory diseases, such as skin diseases and allergic inflammation [24].

Inflammatory cytokines play various roles in skin inflammation. IL-1 β in inflammatory skin lesions aggravates skin inflammatory disorders, leading to hyperplasia [25,26]. IL-6 is a pro-inflammatory cytokine that plays crucial roles in the early phase of inflammation and stimulates keratinocyte proliferation, causing skin thickening [27]. Both cytokines are known to promote Th2 responses and amplify IgE production [28–30]. NF-κB is an important transcription factor for these cytokines, which are involved in immune responses. NF-κB promotes inflammatory responses, causing psoriasis and skin hyperplasia by participating in the production of cytokines [18,31,32].

Repeated topical application of DNCB induced hyperplasia of the epidermis and dermis in BALB/c mice, while ST significantly reduced the resultant skin thickness. In the production of Th2 cytokines in the activated CD4⁺ T cells from lymph nodes of DNCB-applied mice, we identified that ST treatment suppressed DNCB-induced excessive secretion of IL-4 and IL-13. Serum IgE levels and the amounts of IL-1 β , IL-4, and IL-6 in the dorsal skin were measured. We confirmed that DNCB application to the dorsal skin of BALB/c mice significantly increased IgE levels in the serum and production of IL-1 β , IL-4, and IL-6 in the dorsal skin, and that ST treatment significantly reduced the expression thereof. Furthermore, we investigated the expression of NF-κB in dorsal skin tissue, and found that DNCB application to the dorsal skin of mice significantly increased NF-κB expression, while ST treatment significantly reduced NF-κB expression.

In conclusion, this study confirmed that ST reduces levels of inflammatory mediators such as NO, as well as pro-inflammatory cytokines, via inhibition of NF-κB expression in LPS-stimulated RAW 264.7 cells, and that ST upregulates IFN- γ production and downregulates IL-4 production in conA-stimulated mouse splenocytes. Furthermore, this study demonstrated that ST reduced skin hyperplasia caused by allergic inflammatory responses by regulating serum IgE levels and production of cytokines related to Th2 and inflammatory responses.

Conflict of interest

The authors have no conflict of interest to declare.

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