

Salvia plebeia Suppresses Atopic Dermatitis-Like Skin Lesions

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Abstract: *Salvia plebeia* R. Br. (Lamiaceae) has been used for folk medicines in Asian countries, including Korea and China, to treat skin inflammatory diseases and asthma. In this study, we investigated the effects of *S. plebeia* extract (SPE) on atopic dermatitis (AD)-like skin lesions and defined underlying mechanisms of action. We established an AD model in BALB/c mice by repeated local exposure of house dust mite extract (*Dermatophagoides farinae* extract, DFE) and 2,4-dinitrochlorobenzene (DNCB) to the ears. Repeated alternative treatment of DFE/DNCB caused AD-like skin lesions. The oral administration of SPE decreased AD symptoms based on ear thickness and histopathological analysis, in addition to serum IgE and IgG2a levels. SPE suppressed mast cell infiltration into the ear and serum histamine level. SPE inhibited Th1/Th2/Th17 phenotype CD4⁺ T lymphocytes expansion in the lymph node and the expression of Th1/Th2/Th17 cytokines in the ear tissue. To define the underlying mechanisms of action, the tumor necrosis factor (TNF)- α and interferon (IFN)- γ activated human keratinocytes (HaCaT) model was used. SPE significantly suppressed the expression of cytokines and chemokines through the down-regulation of

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mitogen-activated protein kinases, nuclear factor- κ B, and STAT1 in HaCaT cells. Taken together, our results suggest that SPE might be a candidate for the treatment of AD.

Keywords: *Salvia plebeia*; Atopic Dermatitis; Keratinocytes.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritus, eczema and cutaneous hyper-reactivity to environmental triggers, and is associated with elevated serum immunoglobulin (Ig) E levels (Leung *et al.*, 2004). *Dermatophagoides farinae*, a house dust mite, is a common environmental allergen associated with human AD. *D. farinae* extract (DFE) is known to contribute the pathogenesis of AD through the induction of both acute and chronic AD lesions (Kwon *et al.*, 2010; Dai *et al.*, 2011; Choi *et al.*, 2013). AD is characterized by the predominant infiltration of Th2 cells and the secretion of Th2-related cytokines such as, interleukin (IL)-4, IL-5, and IL-13 in the acute phase (Leung *et al.*, 2004). In addition, a progressive activation of Th2 and Th17, as well as Th1 cells, is characteristic of the chronic phase of AD (Gittler *et al.*, 2012). The activation of keratinocytes is a hallmark of the pathogenesis of acute and chronic stage of AD (Kaiko and Foster, 2011). The keratinocytes of AD patients exhibit a propensity to an exaggerated production of cytokines and chemokines, a phenomenon that can be relevant in promoting and maintaining inflammation (Girolomoni and Pastore, 2001). They are also an important source of thymic stromal lymphopoietin, which activates dendritic cells to prime naive T cells to produce IL-4 and IL-13 (Yoo *et al.*, 2005). Therefore, the suppression of keratinocyte activation remains a potential target for the treatment of AD (Trautmann *et al.*, 2001; Klunker *et al.*, 2003; Yoo *et al.*, 2005).

Corticosteroids and calcineurin inhibitors are the early phase therapy and provide rapid mitigation of AD symptoms (Guttman-Yassky *et al.*, 2011). However, it is well known that the long-term use of potent steroids and immune suppressors can cause various side effects. Recently, many natural materials have exhibited anti-inflammatory properties and have potential for treating skin inflammatory disorders such as AD. *Salvia plebeia* R. Br. (Lamiaceae) is an annual or biennial herb or mountainous herb used as a medicinal plant distributes in Korea, China, and Japan (Nugroho *et al.*, 2012). *S. plebeia* leaves are used as a folk medicine in the treatment of hepatitis, inflammation, hemorrhage, and stress (Lu and Foo, 2002). Its components contain 6-methoxyflavones (hispidulin, homoplantagin, nepetin, nepitrin), 6-hydroxyflavone (6-hydroxyluteolin), and rosmarinic acid (Nugroho *et al.*, 2012). *S. plebeia* extract (SPE) possesses certain pharmacological and clinical studies, including anti-oxidative, anti-oncotic, hepatoprotective and anti-inflammatory activities (Gu and Weng, 2001; Qu *et al.*, 2009; Nugroho *et al.*, 2012). In addition, SPE inhibits mast cell-mediated immediate-type allergic reactions (Shin and Kim, 2002). However, the role of SPE on AD has not been studied yet. Thus, based on the known anti-allergic and anti-inflammatory effects of *S. plebeia*, this study elucidates the effect of SPE on the AD-like skin lesions and defines the underlying mechanisms of these effects.

Materials and Methods

Animals

Six-week-old female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan). The animals were housed with 5–10 mice per cage in a laminar air flow room maintained at a temperature of $22 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 5\%$ throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Preparation of SPE and Reagents

The dried whole herb of *S. plebeia* was purchased from herbal store in Jeongeup, Korea. A voucher specimen (number WSP-11-114) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The dried material (30 kg) was ground and extracted with 95% ethanol for 2 weeks at room temperature. The ethanol extract was then concentrated with a rotary evaporator under reduced pressure to give a dried residue (2.6 kg). The ethanol extract was concentrated with a rotary evaporator under reduced pressure. In animal experiments, the dried residue was dissolved in 1% carboxymethyl cellulose in saline, whereas, in other experiments, it was dissolved in dimethyl sulfoxide (DMSO). *Dermatophagoides farinae* extract (DFE, Greer Laboratories, Lenoir, NC) was used as an antigen. All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. DFE was dissolved in phosphate-buffered saline containing 0.5% Tween 20. 2,4-dinitrochlorobenzene (DNCB, 1%) was dissolved in an acetone/olive oil (1:3) solution. Recombinant human tumor necrosis factor (TNF)- α and interferon (IFN)- γ were purchased from R&D systems (Minneapolis, MN).

Cell Culture and Viability

A human keratinocyte cell line, HaCaT, was maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin G, 100 $\mu\text{g/mL}$ streptomycin) at 37°C in 5% CO_2 . Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 24 h of treatment, MTT (5 mg/mL) was added into each well that contained a sample and incubated for 2 h. Isopropanol was added to dissolve the formazan crystals. Absorbance on each sample compared to that of the control, was calculated and expressed as percentage.

Induction of AD-Like Lesions in the Mouse Ear

The induction of AD-like lesions by DFE and DNCB was performed based on our previous research (Kwon *et al.*, 2010; Choi and Kim, 2013). A schematic experimental procedure is

described in Fig. S1A. Mice ($n = 5$) were divided into four groups, and the surfaces of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μ L of DNCB (1%) was painted on each ear and then 20 μ L of DFE (10 mg/mL) 4 days later. DFE/DNCB treatment was repeated once a week alternatively for 4 weeks. Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. After confirming an atopic condition by IgE level, SPE (20 or 100 mg/kg) was orally administered until the end of the 4 weeks induction. Ear thicknesses and clinical scores were measured 24 h after DFE or DNCB application with a dial thickness gauge (Mitutoyo Co., Tokyo, Japan).

On day 28, blood samples were collected by an orbital puncture. The plasma was stored at -70°C for further analysis. After blood collection, ears were removed and used for a histopathological analysis. Serum IgE and IgG2a levels were measured using an ELISA kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. The DFE-specific IgE level was indicated by the OD value.

Histological Observation

The ears were fixed with 10% formaldehyde and embedded in paraffin. Thin 5 μ m sections were stained with hematoxylin and eosin. Infiltrated lymphocytes, thickening of the epidermis, and fibrosis in the dermis were observed using microscope. For the measurement of mast cell infiltration, skin sections were stained with toluidine blue, and the number of mast cells in five sites chosen at random was counted. Eosinophils were counted blinded in 10 high-power fields at a magnification of $\times 400$. Dermal thickness was analyzed in H&E-stained sections viewed under a magnification of $\times 100$. Thickness was measured in five randomly selected fields from each sample.

Histamine Assay

Histamine content was measured through the *o*-phthalaldehyde spectrofluorometric procedure based on previous research (Bae *et al.*, 2011). The blood from the mice was centrifuged at 400 *g* for 10 min, and the serum was withdrawn to measure the histamine content. Fluorescent intensity was measured using 355 nm excitation and 450 nm filters and the fluorescence Spectrometer LS-50B (Perkin-Elmer, Norwalk, CT).

FACS

At the end of the experiment, mice were euthanized, and the auricular lymph node was collected from each mouse. Auricular lymph nodes were ground using 70 μ m nylon cell strainers (Falcon, Bedford, MA) to isolate single cells. Then the cells were stained using a mouse Th1/Th2/Th17 phenotyping kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions and the fluorescence intensity was detected by FACSCalibur (BD Biosciences, Oxford, UK).

Real-Time Polymerase Chain Reaction

For the detection of the expression of cytokines, quantitative real-time polymerase chain reaction (PCR) was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan) according to the manufacturer's protocol. At the end of *in vivo* experimental period, the ears were excised, and total RNA was isolated. HaCaT cells were pretreated with SPE for 1 h, and then stimulated with TNF- α (10 ng/mL) and IFN- γ (10 ng/mL) for 6 h. Total cellular RNA was isolated from cells (2×10^5 cells/24-well plates) as described in previous research (Choi *et al.*, 2013). Briefly, 2 μ L of cDNA (100 ng), 1 μ L of sense and antisense primer solution (0.4 μ M), 12.5 μ L of SYBR Premix Ex Taq (Takarabio Inc., Shiga, Japan), and 9.5 μ L of dH₂O were mixed together to obtain a final 25 μ L reaction mixture in each reaction tube. The conditions for PCR were similar to the ones of previous research (Choi *et al.*, 2013) except for the primer of human CCL22 (F 5'-AGG ACA GAG CAT GGC TCG CCT ACA GA-3', R 5'-TAA TGG CAG GGA GGT AGG GCT CCT GA-3'). The normalization and quantification of mRNA expression was performed using the TP850 software supplied by the manufacturer.

Nuclear Protein Extraction

HaCaT cells were pretreated with SPE for 1 h, and then stimulated with TNF- α (10 ng/mL) and IFN- γ (10 ng/mL) for 30 min. After stimulation, cells (2×10^6 cells/6-well plate) were washed in 1 ml of ice-cold PBS, centrifuged at 1200 g for 5 min, resuspended in 400 μ L of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 g for 30 s. After washing, pelleted nuclei were resuspended in 50 μ L of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000 g for 5 min at 4°C, and the supernatant was gathered.

Western Blot

Samples for Western blot were prepared as previously described (Lee *et al.*, 2011). Briefly, cells (2×10^6 cells/6-well plate) were stimulated for 20 min for MAPKs and STAT1, and 30 min for NF- κ B. Cells were then rinsed twice with ice-cold PBS, and total cell lysates were gathered in 200 μ L of lysis buffer. The lysates were spun in a micro-centrifuge for 20 min at 4°C, and the supernatant was collected. Proteins were electrophoresed using 8–12% SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were stained with reversible Ponceau S to ascertain equal loading of samples in the gel. The nucleus and cytosolic p65 NF- κ B, I κ B α was assayed using anti-NF- κ B (p65), anti-I κ B α antibodies (Santa Cruz Biotech., Santa Cruz, CA), respectively. The phosphorylation of ERK, p38, JNK, and STAT1 were determined using anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, and anti-phospho-STAT1 antibodies (Cell Signaling, Beverly, MA).

Immunodetection was done using supersignal west pico chemiluminescent substrate (Thermo scientific, Waltham, MA).

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA). Treatment effects were analyzed using one way analysis of variance followed by Dunnett's test. A value of $p < 0.05$ was used to indicate statistically significant differences.

Results

Effects of SPE on the Ear Thickness and Histopathological Observation

To investigate the efficacy of SPE on AD-like skin lesions, the DFE/DNCB-induced AD-like skin model was used. The experimental procedure of AD-like skin lesions was shown in Supplementary Fig. S1A. DFE/DNCB treatment for 4 weeks induced severe AD-like skin lesions (Fig. S1B). During the induction period, we measured ear swelling after 24 h of each induction. The tendency of ear swelling of each group was similar until 2 weeks. After 3 weeks of AD induction, the oral administration of SPE showed a significant reduction of ear thickness (Fig. 1).

Histological analysis demonstrated that the administration of SPE markedly reduced hyperkeratosis, large ulcers, and acute inflammatory cell infiltration compared with AD-like skin lesions mice (Fig. 2A). Compared with AD mice, SPE significantly decreased DFE/DNCB-induced epidermal and dermal thickness (Fig. 2B) and infiltration of

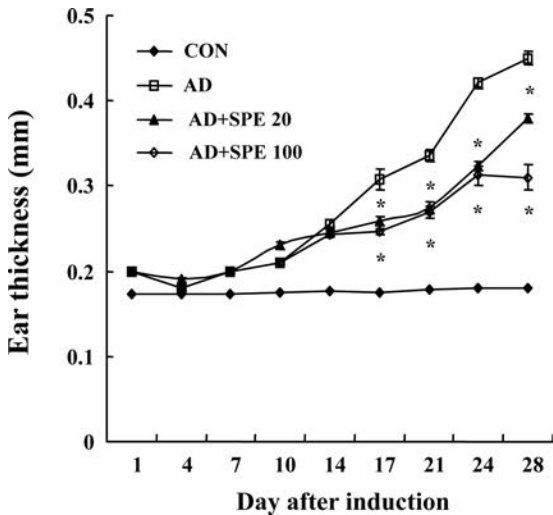


Figure 1. Ear thickness in AD mice. Ear thickness was measured 24 h after DFE or DNCB application with a dial thickness gauge. SPE was orally administered 20 or 100 mg/kg. Data are presented as mean $\pm$ SD of five determinations. * $p < 0.05$ significantly lower than AD.

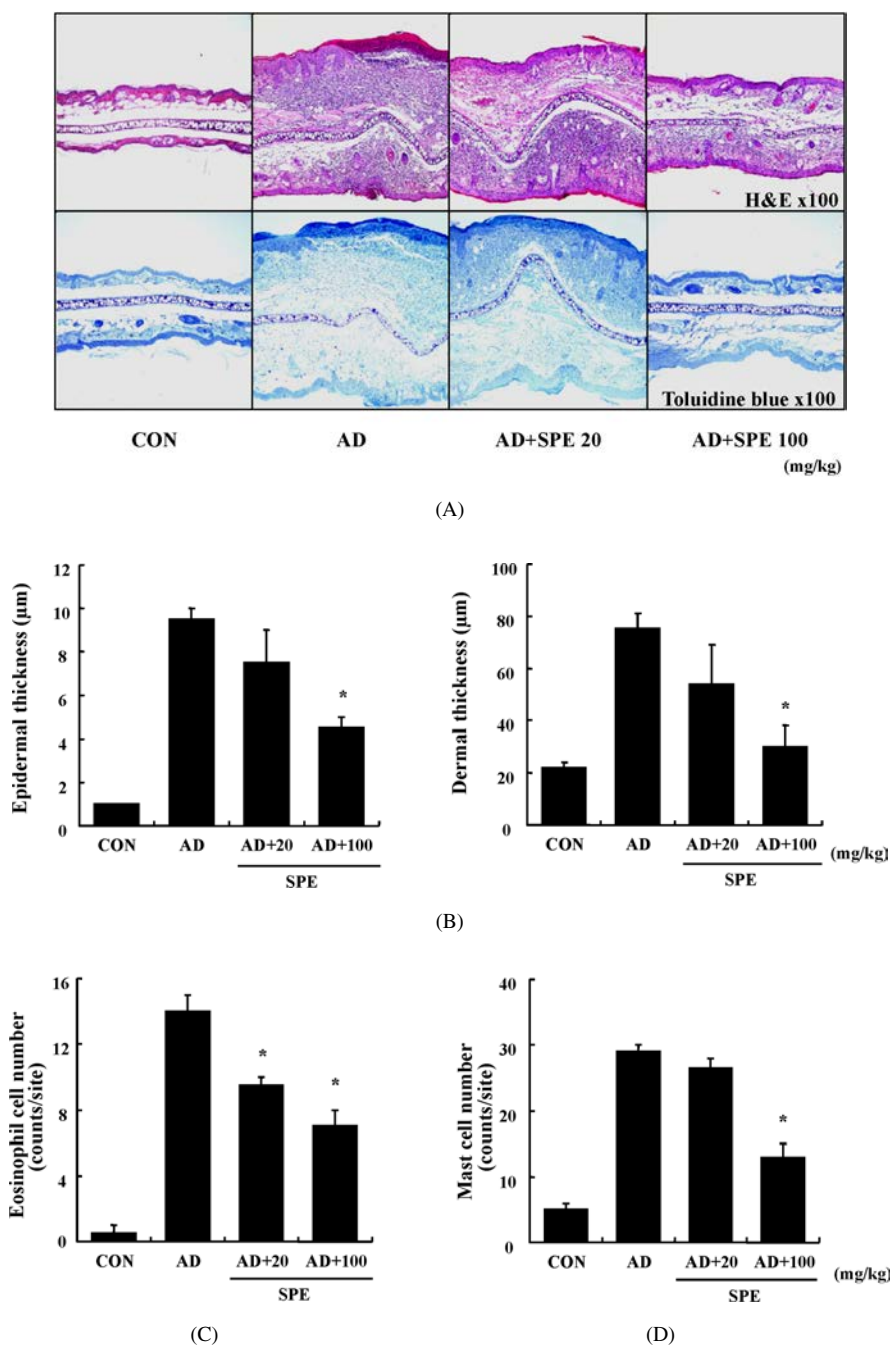


Figure 2. Histological analysis and cell infiltration in AD mice. (A) Representative photomicrographs of ear sections were stained with hematoxylin and eosin (original magnification $\times 100$) or toluidine blue (original magnification $\times 100$). (B) Epidermal and dermal thickness. (C) Numbers of eosinophils. (D) Numbers of mast cells. Data are presented as mean $\pm$ SD of five determinations. * $p < 0.05$ significantly lower than AD.

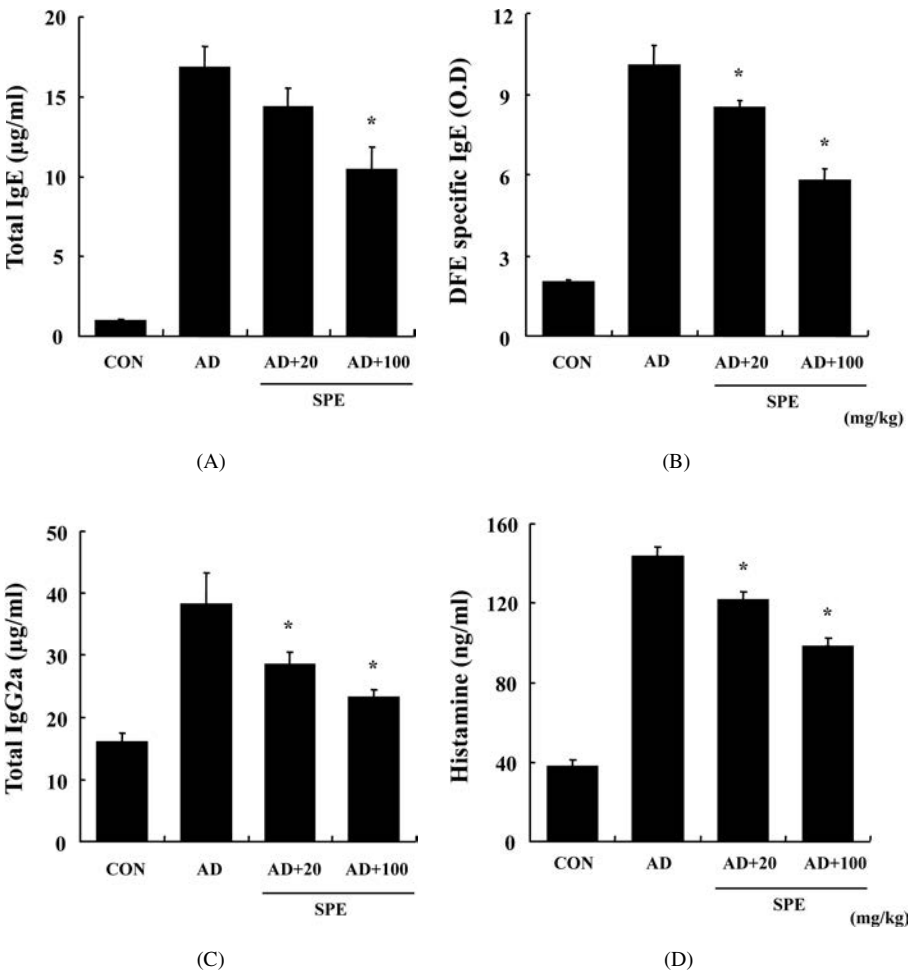


Figure 3. Serum levels of Ig and histamine in AD mice. Serum total IgE levels (A), DFE specific IgE levels (B) and serum total IgG2a (C) were measured by ELISA. (D) Serum histamine levels. The blood samples of vehicle, DFE/DNCB plus vehicle, and DFE/DNCB plus SPE (20, 100 mg/kg) groups were collected by orbital puncture at 28 days. Data are presented as mean $\pm$ SD of five determinations. * $p < 0.05$ significantly lower than AD.

eosinophils (Fig. 2C). Mast cell-derived histamine and inflammatory mediators contribute to the itching and inflammation in AD (Oyoshi *et al.*, 2009). Thus, the infiltration of mast cells in the AD site and the serum level of histamine were examined. SPE diminished the infiltration of mast cells (Fig. 2D) and serum histamine (Fig. 3D).

Effects of SPE on the Serum Ig and Expression of Cytokines

Cytokines have an effect on immunoglobulin isotype switching and expression (Spergel *et al.*, 1999). IL-4 and IFN- γ play an important role in isotype switching to IgE and IgG2a,

respectively (Bieber, 2010). We previously described the significant increase of serum Ig and cytokine levels in DFE/DNCB-induced AD model (Choi *et al.*, 2013). To discriminate the role of SPE on the Th1 or Th2 response, we examined serum levels of IgE (total and DFE specific) and IgG2a. Compared with the AD group, total IgE, DFE-specific IgE, and IgG2a were markedly reduced in the serum of the SPE administrated mice (Figs. 3A–3C).

AD pathogenesis is characterized as a biphasic T cell-mediated disease; although a Th2 predominates in the acute phase, a Th2 to Th1 switch promotes disease chronicity (Leung *et al.*, 2004; Bieber, 2010). Recently, IL-17 was implicated in the pathogenesis of AD. CD4⁺ T cells that orchestrate immune responses can be divided into Th1, Th2, and Th17 cells, based on the cytokines they primarily produce (for example, IFN- γ , IL-4, and IL-17, for Th1, Th2, and Th17 cells, respectively) (Reiner, 2007). We investigated whether the frequency of Th1, Th2, and Th17-phenotype CD4⁺ T lymphocyte in AD mice with SPE differed from that in AD mice. After 28 days, we identified the population of CD4⁺ Th1 (IFN- γ ⁺), Th2 (IL-4⁺), and Th17 (IL-17A⁺) cells in auricular lymph node by FACS analysis. In the AD group, the CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17⁺ populations

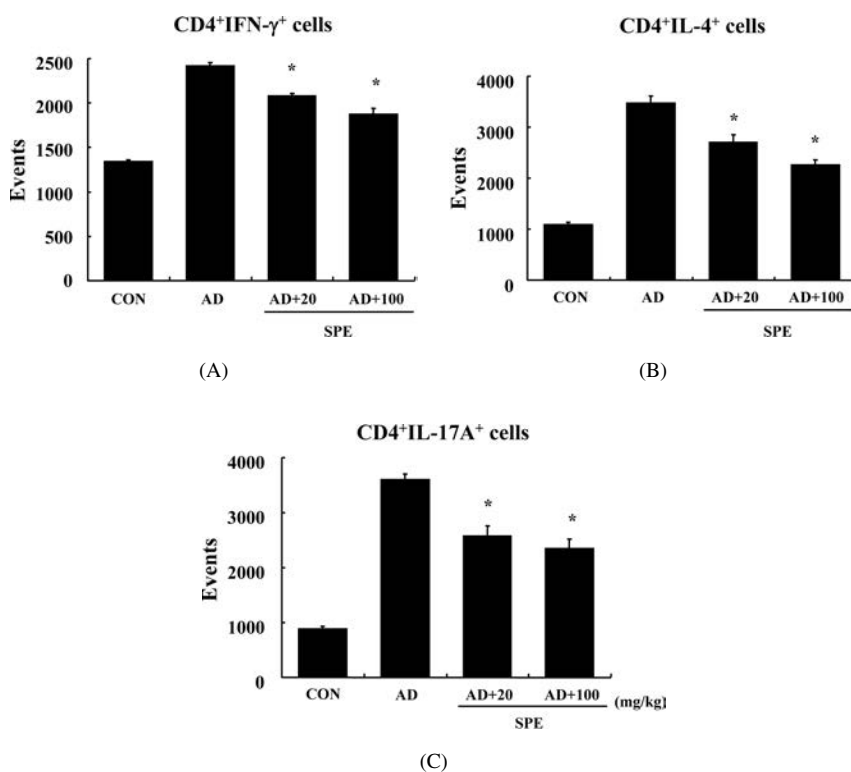


Figure 4. Phenotypes of T lymphocytes in the lymph node of AD mice. The auricular lymph node was collected from each mouse. From the isolated single cells, numbers of CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17A⁺ were detected by FACSCalibur. Data are presented as mean $\pm$ SD of five determinations. * p < 0.05 significantly lower than AD.

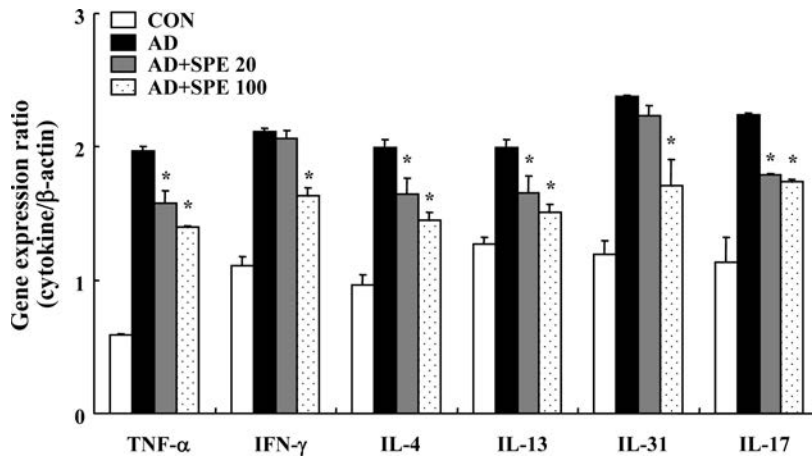


Figure 5. Expression of cytokines in the ear of AD mice. Ears were excised, and total RNA was isolated. The quantitative real-time PCR was performed as described in the Materials and Methods. SPE was orally administered 20 or 100 mg/kg. Data are presented as mean $\pm$ SD of five determinations. * $p < 0.05$ significantly lower than AD.

were increased (Figs. 4A–4C and Fig. S3). SPE significantly inhibited the CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17⁺ population compared with AD group. Next, we examined the effect of SPE on the expression of AD-related inflammatory cytokines from the ear tissue by the real-time PCR. All cytokines were up-regulated in AD, and SPE suppressed the expression of all Th1 (TNF- α , IFN- γ), Th2 (IL-4, IL-13 and IL-31), and Th17 (IL-17) cytokines (Fig. 5). These results suggest that SPE suppresses expression of Th1, Th2, and Th17 cytokines in AD-like skin lesions.

Effects of SPE on the Activation of Keratinocytes

After the observation of the reducing effect of SPE on the AD-like skin lesions *in vivo* models, keratinocytes model was used to clarify the biological function and molecular mechanism of SPE. We first tested the cytotoxicity of SPE. Human keratinocytes cell line (HaCaT) was exposed to various amounts of SPE for 24 h. SPE did not show cytotoxicity up to 100 μ g/mL in keratinocytes (Fig. S2). Tacrolimus (FK 506), a clinically used medication for the systemic therapy of AD, was used as a positive control. Keratinocytes have a similar effect on immune responses during the development of skin disorders such as AD-like skin lesions (Guttman-Yassky *et al.*, 2011). Therefore, TNF- α /IFN- γ -induced CCL17 and CCL22 in keratinocytes are considered as the most important role for the pathogenesis of AD (Vestergaard *et al.*, 2000). To investigate the effect of SPE on the pro-inflammatory cytokines and chemokines, HaCaT cells were pretreated with SPE for 1 h and stimulated with TNF- α /IFN- γ for 6 h. The results of real-time PCR showed that SPE inhibited TNF- α /IFN- γ -induced gene expression of TNF- α , IL-1 β , IL-6, CCL17, and CCL22 in HaCaT cells (Fig. 6). These results suggest that SPE restored the inflammatory

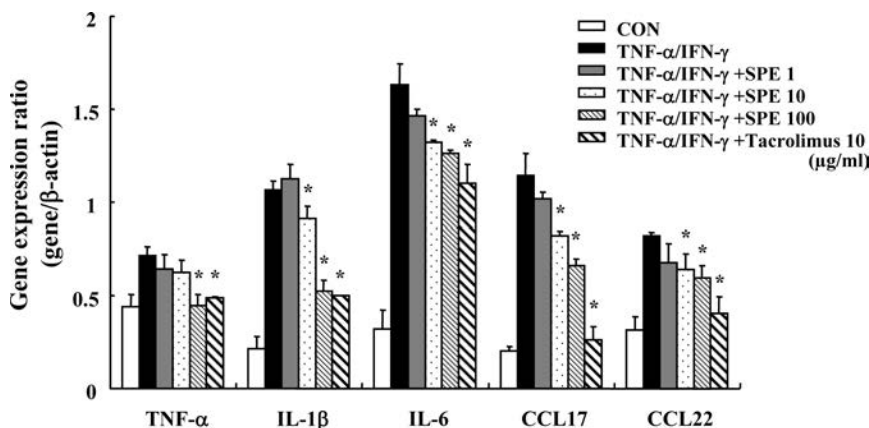


Figure 6. Expression of cytokines and chemokines in TNF- α /IFN- γ -stimulated HaCaT cells. Cells were pretreated with SPE (1–100 μ g/mL) or tacrolimus (10 μ g/mL) 1 h before the stimulation with TNF- α (10 ng/mL) and IFN- γ (10 ng/mL) during 6 h. The expression levels of cytokines and chemokines were determined by the real-time PCR. Data are presented as mean $\pm$ SD. * p < 0.05 significantly lower than stimulated TNF- α /IFN- γ positive control.

response by down-regulating the pro-inflammatory cytokines and chemokines in AD-like skin lesions.

Next, the regulatory effect of SPE on the expression of pro-inflammatory cytokines and chemokines was examined. Previous studies have reported that NF- κ B and JAK/STAT signaling pathways contribute to production of pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and chemokines (CCL17 and CCL22) in TNF- α /IFN- γ -induced HaCaT cells (Kim *et al.*, 2011; Kwon *et al.*, 2012). In addition, the MAPKs signaling cascades are involved in the activation of NF- κ B (Dhar *et al.*, 2002). To find out the mechanism responsible for the inhibitory effect of SPE on the expression of cytokine and chemokine, we investigated the effect of SPE on the TNF- α /IFN- γ -induced MAPKs, STAT1, and NF- κ B. As shown in Fig. 7, the treatment of TNF- α /IFN- γ showed the activation of MAPKs, STAT1, and NF- κ B. SPE (100 μ g/mL) decreased TNF- α /IFN- γ -induced phosphorylation of MAPKs (p38 and JNK), STAT1 and the nuclear translocation of p65 NF- κ B following the degradation of I κ B α . However, SPE did not alter the activation of ERK. These results suggest that SPE inhibits TNF- α /IFN- γ -induced expression of pro-inflammatory cytokines and chemokines through the down-regulation of MAPKs (p38 and JNK), STAT1, and NF- κ B in keratinocytes.

Discussion

Phytochemical studies on *S. plebeia* demonstrated that it included flavonoids, lignans, diterpenoids, homoplantagin, luteolin, aliphatic compounds, and caffeic acid (Jin *et al.*, 2008). Several pharmacological and clinical studies have demonstrated that *S. plebeia* possesses different biological activities, such as antimicrobial, hepatoprotective, antitumor, anti-inflammation and anti-allergic activity (Gu and Weng, 2001; Shin and Kim, 2002;

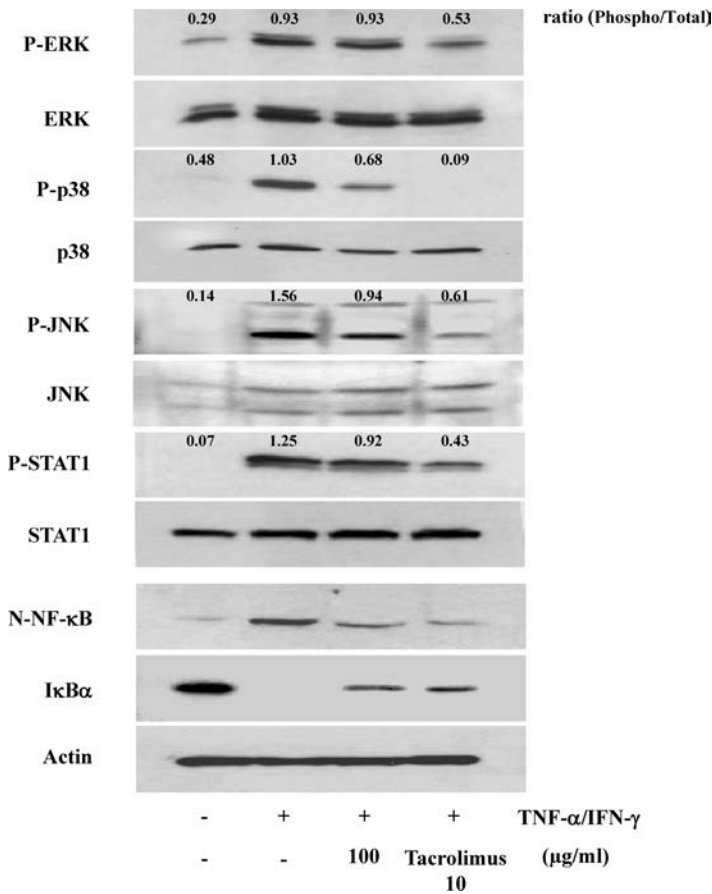


Figure 7. Activation of MAPKs, STAT1 and NF-κB in TNF-α/IFN-γ-stimulated HaCaT cells. Cells were pre-treated with SPE (100 μg/mL) or tacrolimus (10 μg/mL) 1 h before the stimulation with TNF-α (10 ng/mL) and IFN-γ (10 ng/mL) 20 min for MAPKs and STAT1, and 30 min for NF-κB. The phosphorylation of MAPKs and STAT1, the degradation of IκBα and the nuclear translocation of NF-κB were analyzed by Western blot. The experiment shown is representative of three independent experiments. The ratios of Western blot bands were measured using the ImageJ software. Actin was used as a loading control. N-NF-κB, nucleus NF-κB.

Qu *et al.*, 2009). Based on the known anti-allergic and anti-inflammatory effects of *S. plebeia*, we examined the role of *S. plebeia* on AD-like skin lesions using *in vivo* and *in vitro* models.

To investigate AD-like skin lesions, we adopted a mouse model of AD-like skin lesions inflammation through epicutaneous sensitization with house dust mite, a common allergen associated with human AD. We previously reported that this mouse model exhibits phenotypes reminiscent of both acute and chronic AD lesions, including fibrosis, epidermal hyperplasia, spongiosis, and infiltration of inflammatory cells (eosinophils and mast cells) (Choi and Kim, 2013; Choi *et al.*, 2013). In the present report, we confirmed that mice epicutaneously sensitized with DFE/DNCB exhibited ear swelling and redness, hyperplasia

and dysregulated differentiation of the epidermis, and infiltration of dermal inflammatory cells. Oral administration of SPE mitigated typical and histological changes, such as severe ear thickness, dysregulated differentiation of the epidermis and ulcers, dermal and epidermal thickness, epidermal hyperplasia and infiltration of inflammatory cells (eosinophils and mast cells). Mast cells are key effector cells in patients with IgE (FcεRI)-bearing immediate allergic disorders (Oka *et al.*, 2013). The activation of mast cells leads to the release of mediators, such as cytokines, proteases, and histamine. Histamine mainly induces pruritogen and edema and is likely to be a significant mediator in patients with AD (Kawakami *et al.*, 2009). In addition, serum histamine levels were reported to be significantly higher in patients with AD than in normal human skin (Greaves, 2005). In our results, the oral administration of SPE reduced serum histamine and pathogenesis of the skin lesions in AD. Previous studies reported that the oral administration of SPE inhibited the histamine release from rat peritoneal mast cells (Shin and Kim, 2002). These accumulated reports support our results showing the diminution of histamine release in AD. According to these results, we speculate that SPE contributes to the suppression of histamine secretion of AD, being linked with pruritus and edema through reduction of the mast cells.

In AD patients, increased total IgE and IgE specific to environmental allergens can be detected (Schmid-Grendelmeier *et al.*, 2011). Th2 cell activation elevates the expression of IL-4 and IL-13 which are known to induce isotype switching to IgE synthesis (Dokmeci and Herrick, 2008). The level of IL-31 is also related with Th2 cells in the skin of AD (Neis *et al.*, 2006). IL-31 is abruptly up-regulated in acute disease, potentially reflecting its role as an itch mediator in patients with AD (Gittler *et al.*, 2012). In addition, it is known that IFN-γ drives the production of IgG2a (Jin *et al.*, 2009). Previously, the percentage of Th17 cells in the peripheral blood was higher in patients with AD, and IL-17⁺ cells infiltrated in the papillary dermis of patients with atopic eczema more markedly in acute lesions than in chronic lesions (Koga *et al.*, 2008). IL-17 has been suggested to have important roles in skin immunity (Kolls *et al.*, 2008). In our AD model, Th1/Th2/Th17-type cytokines were up-regulated in mouse ear skin lesions and draining lymph nodes. Consequently, SPE markedly inhibited the expression of Th1 (IFN-γ), Th2 (IL-4, IL-13, and IL-31) and Th17 (IL-17) cytokines, and the serum level of IgE and IgG2a. These results suggest that SPE had a controlling effect on the AD immune reaction for Th1, Th2, and Th17. In addition, these results imply that SPE reduces both acute and chronic AD symptoms.

In AD condition, keratinocytes secrete a unique profile of chemokines and cytokines after exposure to proinflammatory cytokines (Akdis *et al.*, 2006). TNF-α and IFN-γ induce cytokines and chemokines for AD in keratinocytes, and this experimental model has been widely used to mimic AD environment *in vitro* (Leung and Bieber, 2003; Leung *et al.*, 2004). Several studies have reported that CCL17 and CCL22 are over-expressed in the serum levels of AD patients and that the seriousness of AD was markedly correlated with these chemokine levels (Kakinuma *et al.*, 2002; Shimada *et al.*, 2004; Jahnz-Rozyk *et al.*, 2005). CCL17 and CCL22 are Th2 chemokines, and these chemokines are thought to play a key role in the pathogenesis of AD (Kwon *et al.*, 2012). Tacrolimus (FK506) is widely

used and is effective as an immunosuppressive medication in the treatment of AD. Previous research reported that tacrolimus inhibited the TNF- α /IFN- γ -induced CCL22 production in human keratinocytes (Xiao *et al.*, 2003). In our results, SPE treatment suppressed TNF- α /IFN- γ -induced TNF- α , IL-1 β , IL-6, CCL17, and CCL22. Accordingly, these results confirm the inhibitory effect of SPE in an AD mouse model.

NF- κ B and STAT1 in the cytoplasm translocate into the nucleus, where they participate in the expression of proinflammatory genes (Kim *et al.*, 2012; Kwon *et al.*, 2012). CCL17 and CCL22 promoters contain NF- κ B and STAT1-binding sequences, and these transcription factors may mediate the transcription of genes (Kanda *et al.*, 2007; Kwon *et al.*, 2012). In this study, we demonstrated that SPE inhibited the signaling pathways leading to activation of both NF- κ B and STAT1. SPE inhibited the degradation of I κ B α and the nuclear translocation of NF- κ B. SPE also suppressed STAT1 phosphorylation in HaCaT cells. These results demonstrate that SPE exerts its inhibitory effect on CCL17 and CCL22 through the reduction of both NF- κ B and STAT1. MAPK

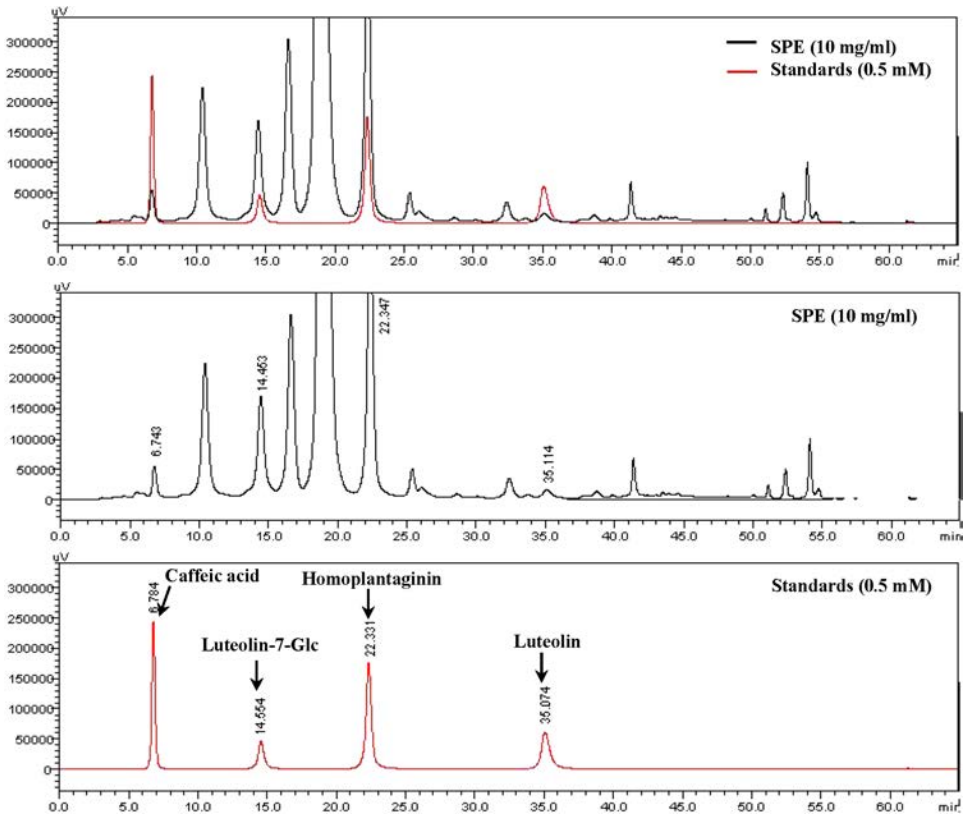


Figure 8. HPLC profiling of ethanol extract of *S. plebeia*. HPLC analysis was performed on Phenomenox Gemini C18 column (4.6 $\times$ 250 mm), wavelength: 342 nm, solvent gradient: 45 min (MeOH, 0.5% acetic acid in H₂O). The peaks were confirmed by the addition of standard caffeic acid, luteolin-7-glucoside, homoplantagin and luteolin in SPE.

cascade is also an important signaling pathway in immune responses and cross talks with NF- κ B to regulate inflammation signaling (Arbabi and Maier, 2002). Our previous studies showed that TNF- α /IFN- γ -induced keratinocytes showed activation of three types of MAPKs: ERK, JNK, and p38 (Choi and Kim, 2013). It has been reported that IFN- γ induces the p38 MAPK mediation of the activation of NF- κ B and STAT1 in keratinocytes (Kwon *et al.*, 2012). TNF- α induces the activation of various signaling molecules including ERK, JNK, and p38 MAPKs in many cell types (Takada *et al.*, 2004). In the present study, we observed that SPE significantly inhibited the activation of p38 and JNK in HaCaT cells but not ERK. This study provides evidence that the suppressive effect of SPE on the TNF- α /IFN- γ -induced expression of pro-inflammatory cytokines, CCL17 and CCL22 are mediated by blocking p38, JNK, NF- κ B, and STAT1 pathways in keratinocytes. Thereby, our results indicate that SPE may reduce AD symptoms by suppressing CCL17 and CCL22.

The active component of SPE responsible for the suppression of AD symptoms and the molecular targets of this effect are not elucidated yet. In this study, the HPLC profile of SPE indicated that caffeic acid, homoplantagin, luteolin and luteolin-7-glucoside are the major components of SPE (Fig. 8). It was reported that caffeic acid attenuates mast cell-mediated immediate allergic reaction (Nader, 2013). In addition, homoplantagin, luteolin and luteolin-7-glucoside showed beneficial biological effects such as anti-inflammation, anti-allergy and hepatoprotection (Qu *et al.*, 2009; Iwaoka *et al.*, 2010). Therefore, we assumed that these components might be responsible for the inhibitory effects of SPE on AD-like skin lesions. However, a more detailed study should be implemented.

In this study, we demonstrate that the oral administration of SPE suppressed the development of AD-like skin lesions *in vivo* and *in vitro* models. SPE inhibited the important cytokines and chemokines for AD through the reduction of MAPKs (p38 and JNK), NF- κ B, and STAT1 in keratinocytes. We provide evidence that SPE could be a potential therapeutic candidate for AD. Our data imply that SPE can be used as a useful pharmacological agent or food supplement.

Acknowledgments

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Supplementary Information

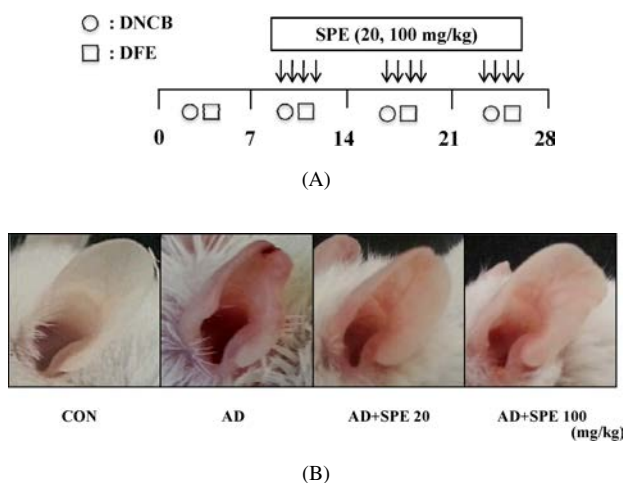


Figure S1. Experimental design and photograph in AD mice. (A) Experimental design for the induction of AD. One week after the first boost with 1% DNCB (20 μ L/ear), DFE (10 mg/mL, 20 μ L/ear), and DNCB were alternatively applied to both ears once a week for 4 weeks. After 1 week of induction, SPE (20, 100 mg/kg) was orally administered every day for 3 weeks ($n = 5$). (B) Representative photographed example of the effects of SPE on DFE/DNCB-induced AD-like skin lesions in the ear.

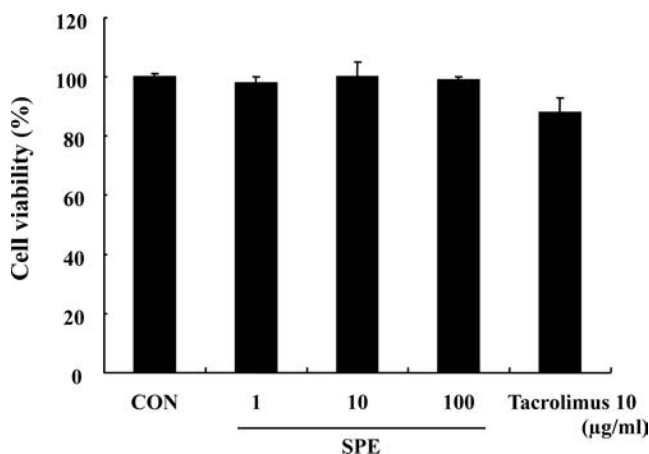


Figure S2. Effects of SPE on cell viability of HaCaT cell. The cell viability was determined using the MTT assay. Data are presented as mean $\pm$ SD of five determinations.

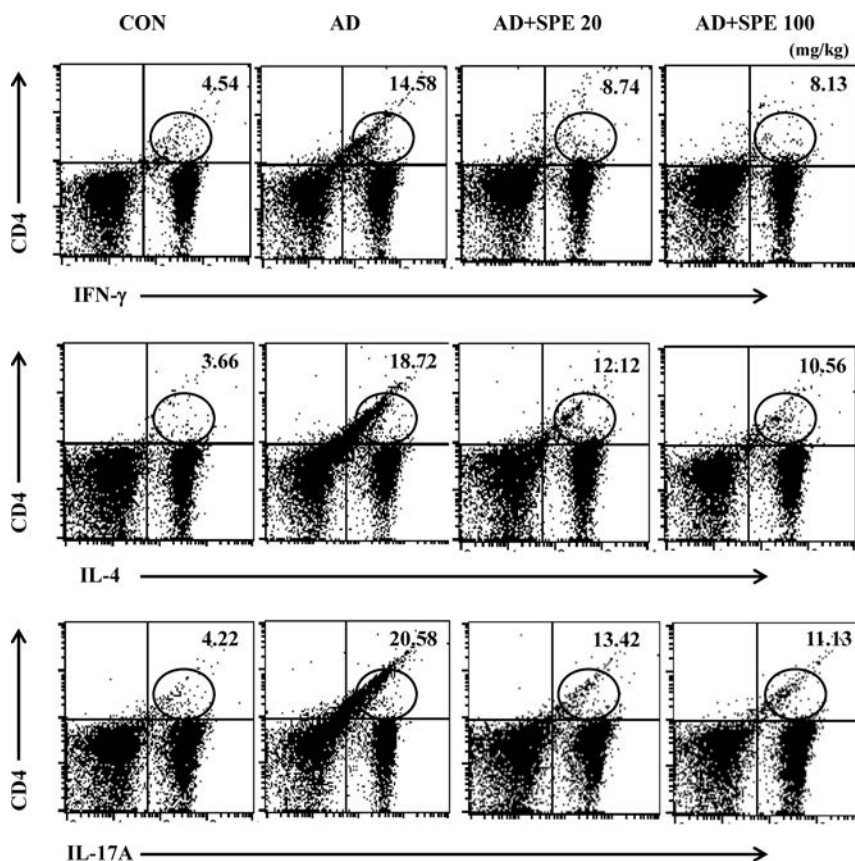


Figure S3. Phenotypes of T lymphocytes in the lymph node of AD mice. Percentages of CD4⁺ T cells expressing IFN- γ , IL-4 and IL-17A were analyzed by FACSCalibur. The upper right quadrant represents CD4⁺IFN- γ ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17A⁺ cells.