



Improvement of atopic dermatitis with topical application of *Spirodela polyrhiza*



Hye Ji Lee^a, Mi Hye Kim^a, You Yeon Choi^a, Eun Hye Kim^b, Jongki Hong^b, Kyuseok Kim^{c,*},
Woong Mo Yang^{a,*}

^a Department of Convergence Korean Medical Science, College of Korean Medicine and Institute of Korean Medicine, Kyung Hee University, Seoul 02447, Republic of Korea

^b College of Pharmacy, Kyung Hee University, Seoul 02447, Republic of Korea

^c Department of Ophthalmology, Otorhinolaryngology and Dermatology of Korean Medicine, College of Korean Medicine, Kyung Hee University, Seoul 02447, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: *Spirodela polyrhiza* has been used as a traditional remedy for the treatment of urticarial, acute nephritis, inflammation, as well as skin disease.

Aim of study: Atopic dermatitis (AD) is characterized hyperplasia of skin lesion and increase of serum immunoglobulin E (IgE) level. In this study, the topical effects of *S. polyrhiza* (SP) on 2, 4-dinitrochlorobenzene (DNCB)-induced AD mice model were investigated by several experiments.

Materials and methods: BALB/c mice were randomly divided into five groups as NOR, CON, DEX, SP 1, and SP 100 groups ($n=5$, respectively). To induce atopic dermatitis-like skin lesions, DNCB had been applied on shaved dorsal skin. SP was topically treated to DNCB-induced mice as 1 and 100 mg/mL concentrations. Histological changes were showed by hematoxylin and eosin (H&E) staining and the infiltration of mast cells was detected by toluidine blue staining. In addition, the level of IgE and each cytokines were measured and expressions of inflammatory signaling factors were analyzed by western blotting assay.

Results: SP treatment improved a hyperplasia of epidermis and dermis in DNCB-induced AD-like skin lesion. The infiltration of mast cells was also decreased by treatment of SP. In addition, SP reduced the level of IgE in serum and attenuated the secretion of cytokines such as interleukin (IL)-4, IL-6, and tumor necrosis factor (TNF)- α . Treatment of SP also inhibited the expressions of pro-inflammatory mediators including nuclear factor- κ B (NF- κ B), phosphor-I κ B- α , and mitogen-activated protein kinase (MAPK).

Conclusions: From these data, we propose that SP ameliorates AD via modulation of pro-inflammatory mediators. SP may have the potential to be used as an alternative for treatment of AD.

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1. Introduction

Atopic dermatitis (AD) is a common relapsing inflammatory skin disease (Galli et al., 2008). AD affects up to 18% of children and up to 5% of adults worldwide and it is continuously increasing (Tatyana et al., 2011). Clinical symptoms of AD include intense pruritus, dry skin, and skin hypersensitivity (Jonathan and Amy, 2003). Although the exact cause of AD is not completely explained, various genetic and environmental factors generating immunological abnormalities contribute to the pathogenesis and development of AD (Udompataikul and Limp-o-vart, 2012).

The general characteristics of AD patients are increased serum

immunoglobulin E (IgE), the number of mast cells, and infiltration of inflammatory cells (Stephen et al., 1976). In addition, the development of AD is related to skin barrier dysfunction and an imbalance between T-helper (Th) type 1 and Th type 2 cells in the immunological system (Haoli et al., 2009; Rebecca et al., 2012). Moreover, the expressions of several inflammatory mediators, including interleukin (IL)-4, IL-6, nuclear factor- κ B (NF- κ B), and mitogen-activated protein kinase (MAPK)s, are correlated with the severity of allergic inflammation of AD (Donald et al., 2004; Bradding et al., 1993).

Currently, treatment for AD commonly includes anti-inflammatory drugs such as corticosteroid, emollient, and immunosuppressive agents (James and Sheila, 2005). However, the long-term use of corticosteroids results in tachyphylaxis and drug resistance (Aoyama et al., 1995). In addition, the use of immunosuppressive drugs such as cyclosporine, tacrolimus and pimecrolimus has limited by systemic toxicity (Mark et al., 1998;

* Corresponding authors.

E-mail addresses: kmdkskim@khu.ac.kr (K. Kim),
wmyang@khu.ac.kr (W.M. Yang).

Nordwig and Thomas, 2003).

Spirodela polyrhiza (L.) Schleid (SP), also called 'greater duckweed', is a common pond plant mostly cultured in Korea, Japan, and China (Ezra and Dale, 1950). As a medicinal herb, SP has been known to treat urticarial, influenza, acute nephritis, inflammation, and skin disease (Xue et al., 2011; Kim et al., 2010). The previous studies have investigated the inhibitory effects of SP on the mast cell-mediated immediate hypersensitivity and therapeutic effects on inflammation (Kim and Ko, 2004; Seo et al., 2012). In addition, formula including SP has attenuated pruritus and inflammation (Park et al., 2015). Alkaloids of SP are reported to have down-regulatory effect on inflammatory mediators and pro-inflammatory cytokines (Shyni et al., 2015).

In this study, we investigated the effects of SP on 2,4-dinitrochlorobenzene (DNCB)-induced AD. To determine the effects of SP on AD, histologic analysis was conducted to observe cutaneous changes. Furthermore, several pro-inflammatory mediators such as cytokines, NF- κ B and MAPKs were detected to demonstrate the anti-inflammatory effects of SP.

2. Material and methods

2.1. Preparation of SP

The dried leaves of SP were purchased from Jungdo Pharm. INC (Seoul, Korea). The SP (30 g) was shaken in 300 mL of 70% ethanol for 24 h at room temperature (RT). The extract was filtered, concentrated in a rotary vacuum evaporator and lyophilized. The lyophilized powder (dry weight 1.7 g, yield: 5.67%) of SP was stored at -20°C until use. A voucher specimen was deposited at our laboratory.

The SP extract was identified to flavonoids including apigenin and luteolin by high-performance liquid chromatography diode array detector (HPLC-DAD, Agilent 1100 series). Chromatographic separation was achieved using the SHISEIDO CAPCELL PAK C18 ($250 \times 4.6 \text{ mm}^2$, $5 \mu\text{m}$). The mobile phase consisted of water and acetonitrile with 1.0 mL/min of flow rate at 35°C . The detection wavelength was 340 nm. The standard curves were calibrated by using the linear regression derived from the peak area. The concentrations of apigenin and luteolin in SP were $22.379 \mu\text{g/mL}$ (0.045%) and $3.408 \mu\text{g/mL}$ (0.007%).

2.2. Experimental design

The seven-week-old BALB/c female mice were purchased from RAON BIO (Yongin, Korea), and acclimatized for 1 week before start of experiment. All mice were provided free access to a standard chow diet and tap water. They were maintained at $22 \pm 2^{\circ}\text{C}$, with a relative humidity of $50 \pm 5\%$ and a 12 h light-dark cycle. All animal studies were performed in accordance with the National Institutes of Health guidelines and approved by the Committee on Animal Care at our institution (KHUASP (SE)-14-030).

The mice were randomly divided into five groups of five mice as follows: (i) NOR: normal control group with vehicle treatment; (ii) DNCB: negative control group, mice sensitized with DNCB; (iii) DEX: positive control group, mice sensitized with DNCB and treated with dexamethasone; (iv) SP 1: mice sensitized with DNCB and treated with 1 mg/mL SP; and (v) SP 100: mice sensitized with DNCB and treated with 100 mg/mL SP. On day 0, the dorsal skin region of mice in all groups was shaved with an electric razor for each experimental cutaneous application. During days 0–2, once daily application of 100 μL of 1% DNCB dissolved in acetone/olive oil (A/O, 1:4) was applied to the backs of mice in the DNCB, DEX, and SP groups. NOR group was treated vehicle (A/O). During days

3–7, mice were housed without any further treatment. After the first challenge, the barrier function of the skin and cuticle was removed by applying 100 μL of 4% sodium dodecyl sulfate (SDS). After a drying time of 3 h, NOR and DNCB groups were treated with the phosphate-buffered saline (PBS), and DEX group was treated 100 μL of 0.1% dexamethasone in PBS. SP 1 and SP 100 groups were treated with 100 mg/mL SP in PBS. Four hours later, the DNCB, DEX, and SP groups were treated topically with 0.5% DNCB in A/O. Mice were sacrificed on day 23 of the experiment. Skin tissues from the backs of mice were obtained and subjected to histological examination. Blood was collected in heparinized tubes from cardiac puncture.

2.3. Histological analysis

The skin tissues from the backs of mice were fixed in 10% formalin. After fixing, specimens were dehydrated and embedded in paraffin. Embedded tissues were serially sectioned to a thickness of 4 μm for histological analysis. Hematoxylin and eosin (H&E) stained the dermal and epidermal thickness. Mast cells were stained with toluidine blue. The dermal and epidermal images were obtained using Lecia Application Suite (LAS) Microscope Software (Lecia Microsystems Inc., IL, USA) at a magnification of $\times 100$. Image of mast cells was obtained at a magnification of $\times 200$.

2.4. Measurement of serum total IgE levels

The blood samples were centrifuged at 14000g for 30 min and stored at -70°C until use. Total serum IgE levels were measured using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience, San Jose, CA, USA), according to the manufacturer's instructions. Plates were coated with capture antibody in 0.1 M sodium carbonate and incubated overnight at 4°C . Plates were washed with 1 M PBS contained 0.05% Tween 20 and subsequently blocked with 1 M PBS included 10% fetal bovine serum (FBS) for 1 h at RT. Standard antigens and samples were incubated to plates for 2 h at RT. After washing, biotin-conjugated anti-mouse antibody and streptavidin-horseradish peroxidase conjugate were added to plates for 1 h at RT. Finally, tetra methyl benzidine substrate solution was added for 30 min in the dark, and after which 2 N H_2SO_4 solution was added to stop the reaction. Absorbance was measured at 450 nm on an automated ELISA reader.

2.5. Measurement of inflammatory cytokines levels

The skin tissue (110 mg) was homogenized in 1 mL Tissue Protein Extraction Reagent (T-PER; Pierce, Rockford, IL, USA) buffer containing protease inhibitors (Roche, Idianapolis, IN USA) for measurement of IL-4, IL-6, and tumor necrosis factor (TNF)- α . Protein concentrations from dorsal skin homogenate were determined using Bradford assay. The concentrations of cytokines were quantified using a mouse TNF- α , IL-4, IL-6 ELISA kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. The cytokine levels were calculated as pg per mg total protein. The optical density was read at 450 nm using an ELISA reader (Molecular Devices, Downingtown, PA).

2.6. Determination of NF- κ B, phosphor-I κ B- α , and MAPKs

Proteins were prepared from dorsal skin tissue. The each skin tissue (110 mg) was homogenized in 1 mL RIPA buffer (Pierce, Rockford, IL, USA) containing protease inhibitors. Protein concentrations from dorsal skin homogenate were determined using the Bradford assay, and 30 μg proteins were denatured with

sodium dodecylsulfate (SDS) buffer. The denatured proteins were separated on 10% SDS polyacrylamide gel and transferred onto the poly-vinylidene fluoride membranes (Millipore, USA). Membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.5% Tween 20 (TBS-T) for 30 min at RT, and incubated overnight at 4 °C with primary antibodies (Anti-NF- κ B, -ERK1/2, -phosphor-ERK1/2, -p38, -phosphor-p38, -SAPK/JNK, -phosphor-SAPK/JNK and -phosphor-I κ B- α ; 1:1000 or 1:700 dilution in 5% BSA in TBS-T; Cell Signaling, USA). Anti-rabbit alkaline phosphatase-conjugated secondary antibodies (1:2000 diluted in TBS-T; Santa Cruz, CA, USA) were treated for 2 h at RT, and bound antibodies were visualized using an enhanced chemiluminescence detection reagent (Amersham Pharmacia, Piscataway, NJ, USA). β -actin was employed as an internal loading control. The relative band densities were determined using a computerized densitometry system.

2.7. Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests. In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. SP improved dermal skin hyperplasia

The histopathological features of the dorsal skin lesions are shown in Fig. 1. Following H&E staining, skin thickness of the epidermis and dermis in DNCB group were greater than those in normal group. Epidermal thickness was significantly diminished by SP treatment in a dose-dependent manner. Treatment of 100 mg/mL SP markedly reduced the thickness of dermis.

3.2. SP down-regulated the infiltration of mast cells and serum IgE levels

Toluidine blue staining showed that the number of mast cells into dermis was increased in DNCB group (Fig. 2A and B) compared with normal group. SP treatment significantly decreased the number of infiltrated mast cells in a dose-dependent manner. In addition, serum total IgE level was increased in DNCB group (Fig. 2C), as compared to normal group. The increase of serum IgE was markedly down-regulated by treatment of 100 mg/mL SP.

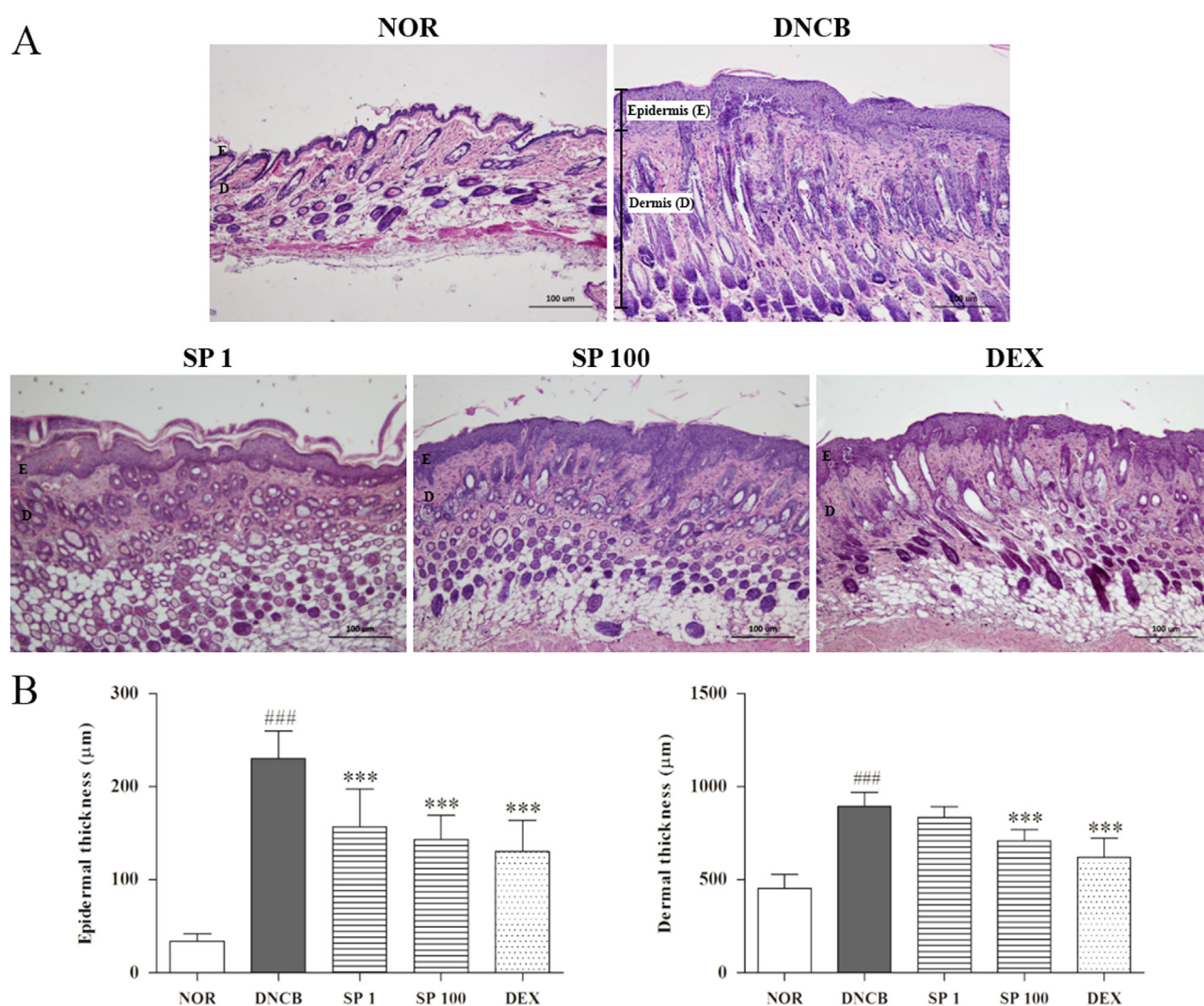


Fig. 1. Improvement of epidermal and dermal hyperplasia by treatment of SP. Histopathological findings by H&E staining of skin section ($n=5$, magnification: $\times 100$). Results are represented as mean $\pm$ SEM. ### indicates that the mean differs significantly between the NOR group and DNCB group ($p < 0.001$). *** indicates that the mean differs significantly between the DEX or SP and DNCB group ($p < 0.001$).

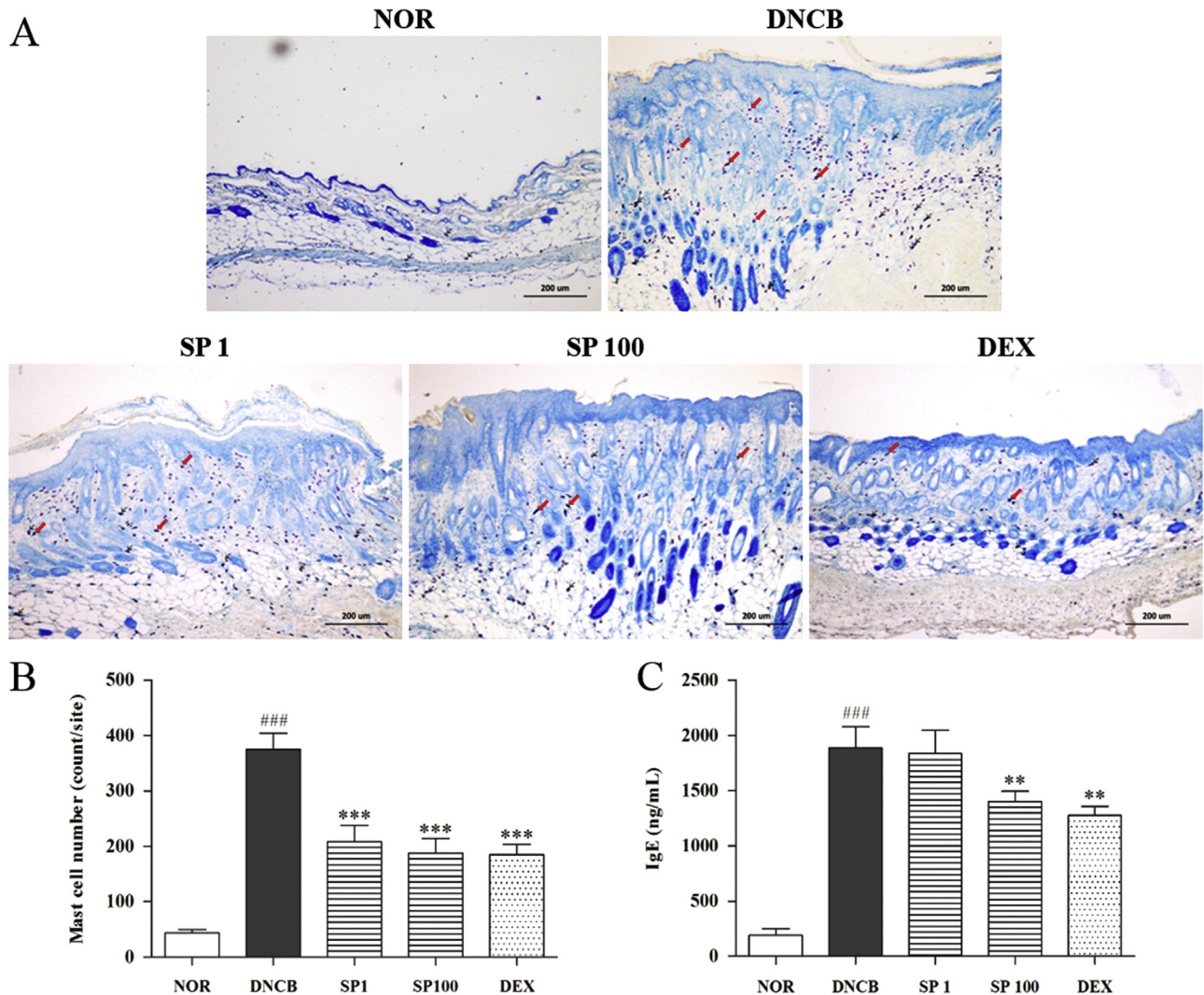


Fig. 2. Inhibition of mast cells infiltration (A and B) and serum IgE production (C) by SP treatment. Mast cells findings by toluidine blue staining of skin sections (magnification: $\times 200$). Red arrows mark the stained mast cells. Each data are represented as mean $\pm$ SEM. ### indicates that the mean differs significantly between the NOR group and DNCB group ($p < 0.001$). ** and *** indicate that the mean differs significantly between the DEX or SP and DNCB group ($p < 0.01$, and $p < 0.001$, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. SP inhibited the production of pro-inflammatory cytokines

In skin lesions, levels of IL-4, IL-6, and TNF- α were elevated in DNCB group compared to normal group (77.49%, 95.42%, 79.91%; Fig. 3). Pro-inflammatory cytokines levels including IL-4, IL-6, and TNF- α were reduced in SP treated group as compared with DNCB group. IL-4 and IL-6 were markedly diminished only at the high concentration of SP (31.35% and 21.77% respectively). The production of TNF- α in SP 1 and SP 100 groups was significantly

decreased by SP treatment in a dose-dependent manner (20.57% and 39.77%).

3.4. SP attenuated the expressions of NF- κ B, phosphor-I κ B- α , and MAPKs proteins

In dorsal skin of DNCB-induced AD mice, the expressions of NF- κ B and phosphorylation of I κ B- α were higher than NOR group (Fig. 4A). In SP 100 group, the expression of NF- κ B and

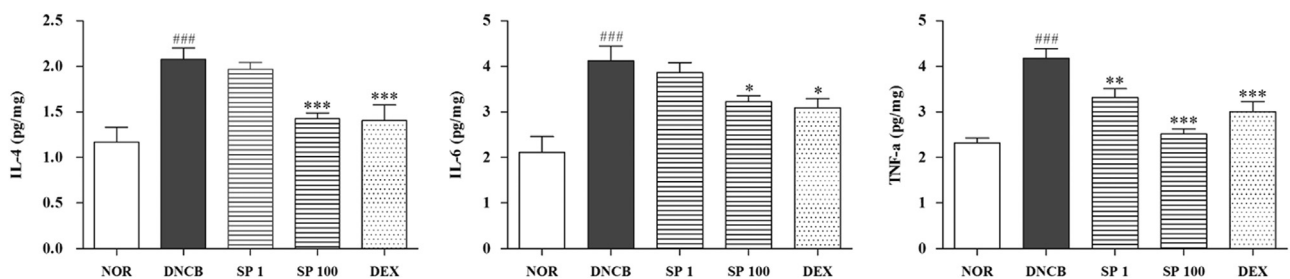


Fig. 3. Suppression of IL-4, IL-6, and TNF- α levels by SP in skin lesion. Results are represented as mean $\pm$ SEM. ### indicates that the mean differs significantly between the NOR group and DNCB group ($p < 0.001$). * indicates that the mean differs significantly between the DEX or SP and DNCB group ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, respectively).

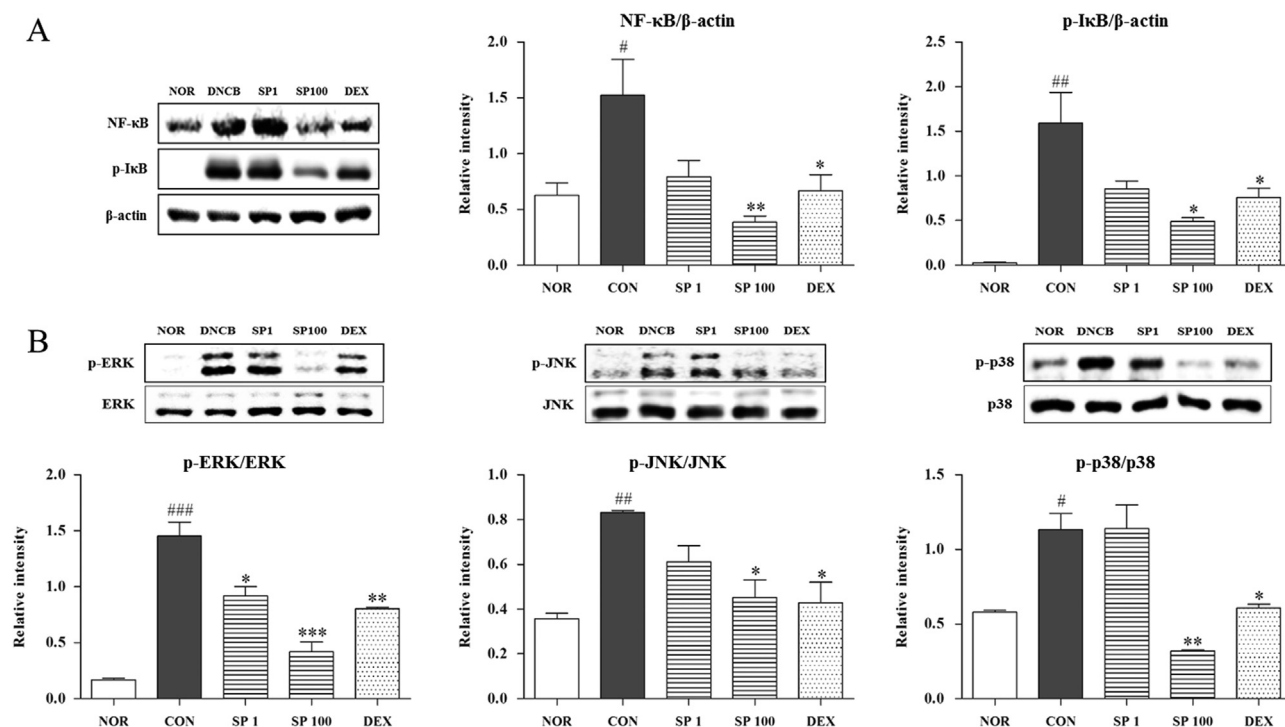


Fig. 4. Downregulation of NF-κB and p-IκB expression (A) and phosphorylation of ERK1/2, SAPK/JNK, and p38 MAP kinases (B) by SP treatment. Each data are represented as mean $\pm$ SEM. # indicates that the mean differs significantly between the NOR group and DNCB group (# p < 0.05, ## p < 0.01, and ### p < 0.001, respectively). * indicates that the mean differs significantly between the DEX or SP and DNCB group (* p < 0.05, ** p < 0.01, and *** p < 0.001, respectively).

phosphorylation of IκB-α were lower than DEX group, as compared with DNCB group. In addition, phosphorylation of ERK1/2, SAPK/JNK, and p38 MAPKs was increased in AD-induced mice, compared to normal group (Fig. 4B). The phosphorylation of ERK1/2 was dose-dependently suppressed in both SP 1 and SP 100 groups, as compared with DNCB group. The phosphorylation of SAPK/JNK and p38 were inhibited in SP 100 group.

4. Discussion

Most patients with AD are pathologically characterized by hyperplasia of skin lesion (Stephen et al., 1976). Skin hyperplasia provides a basis for supplying more pro-inflammatory mediators, which lead to abnormal skin barrier function (Kazuhiko et al., 2015; Hong et al., 2014). In this study, histological analysis showed the changes of epidermal and dermal thickness. The thickness of epidermis was diminished by SP treatment in a dose-dependent manner. Dermal thickness was markedly reduced in SP 100 group. These results suggested that treatment of SP to DNCB-induced AD mice affected in ease of skin hyperplasia.

In addition, most AD patients present the increase of mast cells which play a major role in hypersensitivity allergic reactions. The increased mast cells in allergic responses are related with the release of inflammatory mediators including histamine and cytokines (Kawa, 2012; Shyni et al., 2015). Toluidine blue staining results showed that SP treatment dose-dependently reduced the number of mast cells in dermis. The majority of AD patients have also elevated serum IgE level, which is mediated to mast cell activation (Shyni et al., 2015). IgE secreted from mature B lymphocytes promotes the differentiation of mast cells, which lead to release inflammatory mediators contributed to the development of AD (Kelly et al., 2010; Eric and Umasundari, 2011). In our results, the application of 100 mg/mL SP significantly diminished serum IgE concentration. These results suggested that the mast cells were reduced by decrease of serum IgE by SP treatment.

Pro-inflammatory cytokines including IL-4, IL-6, and TNF-α are known to be important accessory molecules in AD skin lesion (Schreiber et al., 1992). The release of IL-4 secreted from Th2 cells primarily regulates IgE hyper-production which promotes differentiation of mast cell (Peter, 2006; Kim et al., 2014; Lawrence et al., 2001). In addition, TNF-α expression is known to be excessively abundant during allergic reactions such as and associated the release of IL-6 (Fanny et al., 2004; Yang et al., 2013). IL-6 is a multifunctional cytokine produced by various cell types such as macrophages, T and B cells (Toshio, 2010; Turksen et al., 1992; Fanny et al., 2004). The production of IL-6 stimulated the synthesis of acute phase protein which leads to disruption of skin barrier function (Giuliano et al., 1988; Kim et al., 2014). In this experiment, the levels of IL-4 and IL-6 were markedly declined by treatment of 100 mg/mL SP in DNCB-induced AD mice. TNF-α was significantly diminished by SP treatment in a dose-dependent manner. Accordingly, these results demonstrated that SP treatment decreases inflammatory cytokines such as IL-4, IL-6, and TNF-α, resulting in the attenuations of secretion of IgE and destruction of skin barrier function.

Additionally, NF-κB is an important transcription factor that controls the expression of genes involved in apoptosis and inflammation (Turksen et al., 1992). NF-κB in cytoplasm translocate into the nucleus, where it participates in the expression of many pro-inflammatory genes (Fanny et al., 2004). IκB-α is the major ubiquitous cytoplasmic inhibitor that is critical for regulating the rapid transient nuclear induction of NF-κB (Giuliano et al., 1988; Karin et al., 2012; Margit et al., 2002). Our results showed that activation of NF-κB and phosphorylation of IκB-α were significantly inhibited by the treatment of 100 mg/mL SP demonstrating that NF-κB and p-IκB-α expression levels in the skin of SP-treated mice were reduced, indicating that SP blocked the translocation of NF-κB to the nucleus and the phosphorylation of IκB-α. These results are consistent with previous study which reported SP treatment inhibited cytokines and NF-κB expressions in LPS-induced raw264.7 cells (Seo et al., 2012).

Another important signaling related to inflammation is mitogen activated-protein kinase (MAPKs) pathway, which cascade plays an essential role in the initiation of inflammatory responses (Kisseleva et al., 2002; Kim et al., 2008). ERK1/2, SAPK/JNK, and p38 MAP kinases are activated in immune cells by pro-inflammatory cytokines such as TNF- α (Kim and Shin, 2009; Kim et al., 2011). These results showed that the DNCB-induced phosphorylation of ERK1/2, SAPK/JNK, and p38 were decreased by SP treatment. Especially, p-ERK1/2 and p-p38 were significantly reduced in SP 100 group, compared to DNCB group.

In conclusion, SP significantly diminished epidermal hyperplasia, infiltration of mast cells into dermis, and level of IgE in dorsal skin in a dose-dependent manner. Furthermore, treatment of 100 mg/mL SP markedly inhibited expression levels of several mediators as well as crucial factors of inflammation including IL-4, IL-6, NF- κ B, p-I κ B- α , and MAPKs. These results suggest that SP might be considered as an alternative treatment for improvement of AD.

Acknowledgment

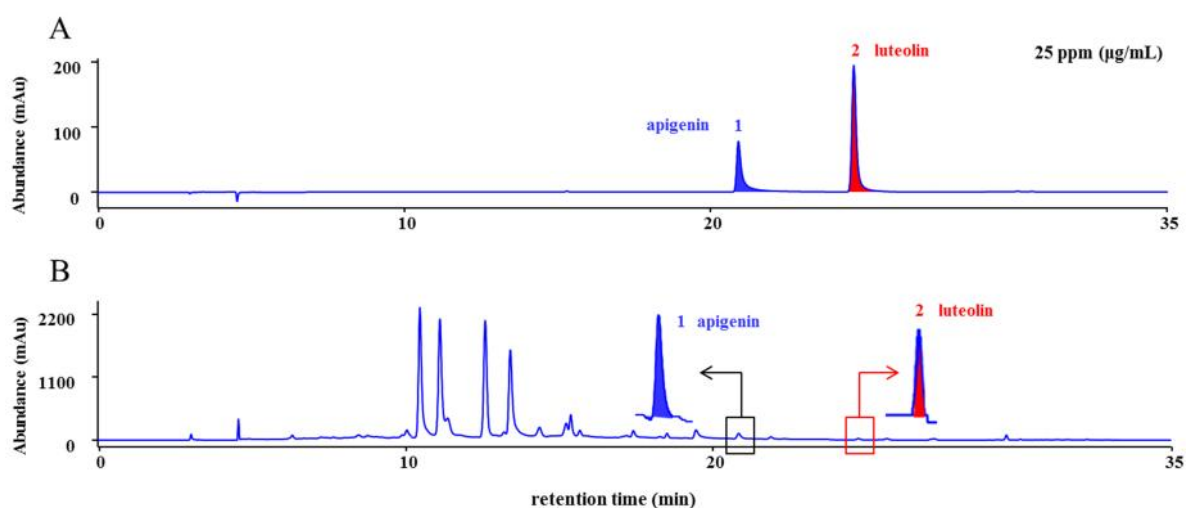
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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2016.01.010>.

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Phytochemical analyses of standards materials on SP extract by HPLC. HPLC chromatograms (A) presented standard peaks of apigenin and luteolin. Chromatograms (B) showed the peaks of apigenin and luteolin in SP extract. The peaks for SP corresponded to standard compound, which are components of *Spirodela polyrhiza*.