

Schizonepeta tenuifolia Inhibits the Development of Atopic Dermatitis in Mice

You Yeon Choi,¹ Mi Hye Kim,¹ Jong-Hyun Kim,² Hyuk-Sang Jung,² Youngjoo Sohn,² Young Jin Choi,² Man Ki Hwang,² Sung-Hoon Kim,³ Jinju Kim⁴ and Woong Mo Yang^{1*}

¹Department of Prescriptionology, College of Oriental Medicine, Institute of Oriental Medicine, Kyung Hee University, Seoul, Korea

²Department of Anatomy, College of Oriental Medicine, Institute of Oriental Medicine, Kyung Hee University, Seoul, Korea

³Cancer Preventive Material Development Research Center, College of Oriental Medicine, Kyunghee University, Seoul, Korea

⁴Department of Oriental Physiology, College of Pharmacy, Kyung Hee University, Seoul, Korea

Historically, *Schizonepeta tenuifolia* (ST) has been used for the treatment of skin disorders, such as allergic dermatitis, eczema, and inflammatory diseases. In this study, we examined whether ST inhibited 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD) in BALB/c mice. In histopathological analyses of the epidermis and dermis, skin thickness was significantly increased in DNCB-induced mice as compared with normal group. Treatment with ST inhibited this inflammatory change and markedly suppressed the secretion of immunoglobulin E, tumor necrosis factor α , and interleukin 6 levels in the serum of DNCB-induced mice. In addition, ST treatment significantly restored the upregulation of proinflammatory factors, such as nuclear factor (NF)- κ B and mitogen-activated protein kinase expression. Taken together, due to its ability to suppress inflammatory factors and upregulate proinflammatory factors, ST may be useful as a therapeutic treatment for AD. ST extract application decreased both epidermis and dermis thickness in DNCB-induced mice. In serum, ST reduced immunoglobulin E, tumor necrosis factor, and interleukin 6 level. In addition, ST suppressed NF- κ B activation as well as the mitogen-activated protein kinase activities.

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Supporting information may be found in the online version of this article (Supplementary Material)

INTRODUCTION

Atopic dermatitis (AD) is a chronic and recurrent inflammatory skin disease accompanied by asthma or allergic rhinitis in many cases (Galli *et al.*, 2008). The prevalence of AD and allergic and irritant contact dermatitis has been increasing significantly in the general population (Ponyai *et al.*, 2007). Although the exact pathogenesis of AD is unclear, T-helper (Th) 1/2 cell dysregulation, skin barrier defects, and environmental factors, such as allergens and microbes, seem to play important roles in this disease (Navi *et al.*, 2007). Symptoms and characteristics of AD include pruritus, dry skin, erythema, scratching, and elevated levels of serum immunoglobulin E (IgE) (Siraganian, 2003). Skin lesions and edema are observed during the course of the disease, and the reparative process is evidenced histologically by the presence of fibrosis and hyperkeratosis (Zhang and Tinkle, 2000).

Treatment of AD generally includes steroids, anti-histamines, and immunosuppressive agents (Del and Friedlander, 2005). Additionally, a few compounds have been tested in clinical trials, and several specific kinase inhibitors have been developed as anti-inflammatory agents (Norris, 2005). However, serious side effects limit the use of these compounds for chronic skin

diseases. Therefore, using natural medicines in the treatment of AD might be more effective with relatively fewer side effects (Kimura *et al.*, 1986).

Schizonepeta tenuifolia (ST) belongs to the Labiatae family of plants and is widely used in China, Korea, and Japan (Yoo *et al.*, 2011). ST is commonly categorized in the mint family as a major herbal constituent included in treatments for skin inflammations and the common cold with fever and otitis media (Yamahara *et al.*, 1980). Several studies have shown that ST can alleviate symptoms of skin diseases. Its anti-inflammatory properties have been demonstrated in the human mast cell line HMC-1 (Yoo *et al.*, 2011), and ST has been shown to downregulate the expression levels of tumor necrosis factor (TNF)- α in a lipopolysaccharide-induced acute inflammation model (Kang *et al.*, 2010).

The 2,4-dinitrochlorobenzene (DNCB)-induced skin application mouse model has been reported as a reliable tool for demonstrating immunological and skin alterations similar to those observed in human AD (Jin *et al.*, 2009), including clinical features such as edema, hemorrhage, skin erosion, and upregulation of serum total IgE levels (Siraganian, 2003). In this study, we examined the effects of ST on the development of DNCB-induced AD in mice. We show that ST inhibited allergic inflammatory responses by suppressing the expression of total IgE, TNF- α , and interleukin 6 (IL-6) in serum. In addition, ST inhibited the expression levels of the proinflammatory cytokines nuclear factor- κ B (NF- κ B), and mitogen-activated protein kinases (MAPKs) in DNCB-induced mice.

* Correspondence to: Woong Mo Yang, M.D., Ph.D. Department of Prescriptionology, College of Oriental Medicine, Institute of Oriental Medicine, Kyung Hee University, Seoul 130-701, Korea.
E-mail: wmyang@khu.ac.kr

MATERIALS AND METHODS

Preparation of ST. Dried herb ST was purchased from Omni herb Inc. (Andong-si, Gyeongbuk, South Korea). ST extract was prepared by decocting 250 g of the dried herb with 5 L of boiling distilled water for 1 h 30 min. The filtrate was concentrated under reduced pressure and lyophilized. After filtration, an aqueous solution of the extract was concentrated in a rotary evaporator, freeze-dried for 3 days, and stored at 4 °C until use. The percentage yield was 7.0% w/w (dry weight 17.4 g). A voucher specimen (ST001) remains in our laboratory. Before each experiment, ST extract was dissolved in a phosphate buffered saline/olive oil (P/O) mixture (9:1) and vortexed for 2 min at room temperature.

Quantitative HPLC Analysis of standard materials to ST extract. ST was standardized based on the content of pulegone. Ten milligrams of ST was dissolved in 10 ml of methanol (HPLC reagent, J.T. Baker Co. Ltd, U.S.A.) and filtered through a 0.45- μ m syringe filter (polyvinylidene fluoride (PVDF), Milford, USA). The HPLC apparatus was a Gilson System equipped with a 234 Autosampler, a UV/VIS-155 detector, and a 321 HPLC Pump (Gilson, Seoul, Korea). A Luna 4.60 $\times$ 250 mm C18 reversed-phase column with 5- μ m particles (Phenomenex, CA, USA) was used. Chromatographic separation was carried out using an isocratic solvent with acetonitrile (HPLC grade, J.T. Baker Co. LTD., U.S.A) water (with 0.01% formic acid) in the ratio of 50:50 (v/v). The column eluent was monitored at UV 254 nm, following which all solvents were degassed with a micromembrane filter (PTFE, Advantec, Tokyo, Japan). Chromatography was performed at room temperature at a flow rate of 1.0 ml/min, and 10 μ l was analyzed for 30 min. The retention time of pulegone was 18.4 min. The standard curve was calibrated by using the linear regression derived from the peak area. The regression equation (correlation coefficient, R^2) of pulegone was $y = 23,174.1x - 85,935.5$ (0.99993), which exhibited good linearity. The content of pulegone in 1 g of ST water extract was 7.03 mg, which corresponds to 0.04898% of the ST.

Animal treatment. Female BALB/mice (aged 7 weeks) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained for 10 days in pathogen-free conditions before the start of the experiment. Mice were housed in an air-conditioned animal room with a 12-h light/dark cycle (08:00–20:00 light, 20:00–08:00 dark), a temperature of 23 ± 2 °C, and humidity of 58 ± 10 %. Mice were provided with a laboratory diet and water *ad libitum*. After the 10-day adaption period, mice were randomly divided into three groups of five mice as follows: (i) **Normal:** normal control group with vehicle treatment; (ii) **DNCB:** negative control group, mice sensitized with DNCB; and (iii) **DNCB + ST:** mice sensitized with DNCB and treated with ST. All animal procedures were performed in accordance with the National Institutes of Health guidelines and approved by the Committee on Animal Care at our institution. The dorsal skin region of mice in all groups was shaved with an electric razor in preparation for each experimental cutaneous application. During days 0–2, once daily applications of 100 μ l of 1% DNCB was applied to the backs of mice in the DNCB and DNCB + ST groups, and the normal control group received vehicle (acetone/olive oil (A/O) = 4:1). During

days 3–7, mice were housed for 5 days without any further treatment. After the first challenge, the barrier function of the skin and cuticle was removed by applying 100 μ l of 4% sodium dodecyl sulfate (SDS). After a drying time of 3 h, Normal and DNCB groups were treated with the P/O mixture (9:1), and the DNCB + ST group was treated with 1% ST in P/O (9:1). Four hours later, the DNCB and DNCB + ST groups were treated topically with 0.5% DNCB in A/O (4:1). The normal control group received vehicle alone (A/O, 4:1). Mice were sacrificed on day 17 of the experiment. Skin tissues from the backs of mice were excised and subjected to histological examination, and blood was collected in heparinized tubes from cardiac puncture.

Adaptation	DNCB treatment	No Treatment	SDS + ST + DNCB treatment	Sacrifice
For 10 day	0–2 Day	3–7 Day	8–16 Day	On 17

Histological analysis. Mid-sagittal skin specimens (1 $\times$ 0.5 cm) were obtained and fixed in 10% formaldehyde. Fixed tissues were embedded in paraffin for 24 h and serially sectioned to a thickness of 4 μ m for histological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) and examined for general morphology. To assess epidermal and dermal hyperplasia in all three groups, the thicknesses of the epidermis and dermis were measured using the Leica Application Suite (LAS; Leica Microsystems, Buffalo Grove, IL). Digital images were obtained at a magnification of 100 $\times$.

Measurement of serum total IgE. Serum samples were obtained from centrifuged blood (14,000 $\times$ g, 30 min) from sacrificed mice and stored at -70 °C until use. Total serum IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. KT-401; Kamiya Biomedical, Seattle, WA), following the manufacturer's instructions. Serum IgE levels were measured by determining the absorbance at 450 nm using an ELISA reader (Molecular Devices, Downingtown, PA).

Determination of cytokine levels (TNF- α and IL-6). The levels of TNF- α and IL-6 in the serum samples were measured using a Milliplex[®] Map Mouse Cytokine/Chemokine kit (Millipore, Billerica, MA), according to the manufacturer's instructions. In addition, a Bio-Plex[™] 200 (Bio-Rad, Hercules, CA) was used to determine cytokine levels (TNF- α and IL-6) in the serum samples. The multiplexed cytokines were analyzed using Bio-Plex Manager ver. 4.1.1 (Bio-Rad), and the data were analyzed in triplicate.

Determination of NF- κ B and MAPKs. Western blot analysis was used to detect activated extracellular signal-regulated kinases (ERKs), NF- κ B, c-Jun amino-terminal kinases (JNKs), and p38 MAPK (Cell Signaling, USA). To prepare samples for analysis, 10% of each frozen tissue homogenate sample ($n = 5$ in each group) was incubated on ice for 15 min in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors cocktail (Roche, Indianapolis, IN). The resultant homogenate was centrifuged at 10,000 $\times$ g for 30 min at 4 °C, and the supernatant was carefully removed. This process

was repeated four times. Protein concentration was measured using the Bradford method, and 50- μ g protein was denatured with SDS buffer. The denatured protein was separated on a 15% polyacrylamide gel and transferred onto a PVDF membrane. Non-specific labeling was blocked with a Tris-buffered saline and Tween 20 (TBS-T) buffer containing 5% skim milk for 2 h. The blots were incubated with one of the primary antibodies (anti-NF- κ B, -ERK, -JNK, or -p38 MAPK; Cell Signaling, USA, 1:800 dilutions in TBS-T) overnight. Anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:2000 dilutions in TBS-T; Santa Cruz, CA, USA) was used to detect the target proteins. After incubation for 2 h at room temperature, blots were visualized using an enhanced chemiluminescence detection reagent (Amersham Pharmacia, Piscataway, NJ, USA). β -actin was used as an internal loading control. The relative band density was calculated with respect to the β -actin blot developed under similar conditions using a computerized densitometry system.

Statistical analysis. Differences between the treatment groups were examined using a one-way analysis of variance and Student's *t*-tests. All data are presented as the mean $\pm$ standard error. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Effects of ST extract on skin lesions and histological changes. We developed DNCB-induced AD-like skin lesions in mice and examined the effect of ST on skin

inflammation. Photographs of typical specimens stained with H&E are shown in Fig. 1. Skin thicknesses of the epidermis and dermis measured in the DNCB group were significantly greater (69.44% and 61.28%, respectively) than that measured in the normal control group. In contrast, both skin thickening and hyperplasia of the epidermis and dermis in the DNCB+ST group were markedly decreased (38.15% and 42.37%, respectively) as compared with the DNCB group. Histological analysis revealed that ST treatment inhibited DNCB-induced AD-like skin thickening and hyperkeratosis symptoms of inflammation (Fig. 1).

Effects of ST on serum levels of IgE, TNF- α , and IL-6.

The serum levels of total serum IgE, TNF- α , and IL-6 were measured to assess the effects of ST on DNCB-induced mice. At day 17, the total serum IgE levels were significantly increased in the DNCB-induced group (approximately 85.72%) as compared with the normal control group, whereas serum IgE levels in the DNCB+ST treatment group declined significantly (approximately 46.26%; Fig. 2) as compared with the DNCB-induced group. Similarly, serum levels of TNF- α and IL-6 were increased by 42.37% and 73.64%, respectively, in the DNCB-induced group as compared with the normal control group. In contrast, the levels of TNF- α and IL-6 decreased by 41.97% and 70.42%, respectively, in the DNCB+ST group as compared with the DNCB-induced group. These results demonstrate that AD-like immune alterations were successfully induced, and that topical ST treatment on the DNCB-induced skin inhibited the increase in total serum levels of IgE, TNF- α , and IL-6.

Effects of ST on NF- κ B. The effects of ST treatment on protein expression levels of NF- κ B in the dorsal skin of

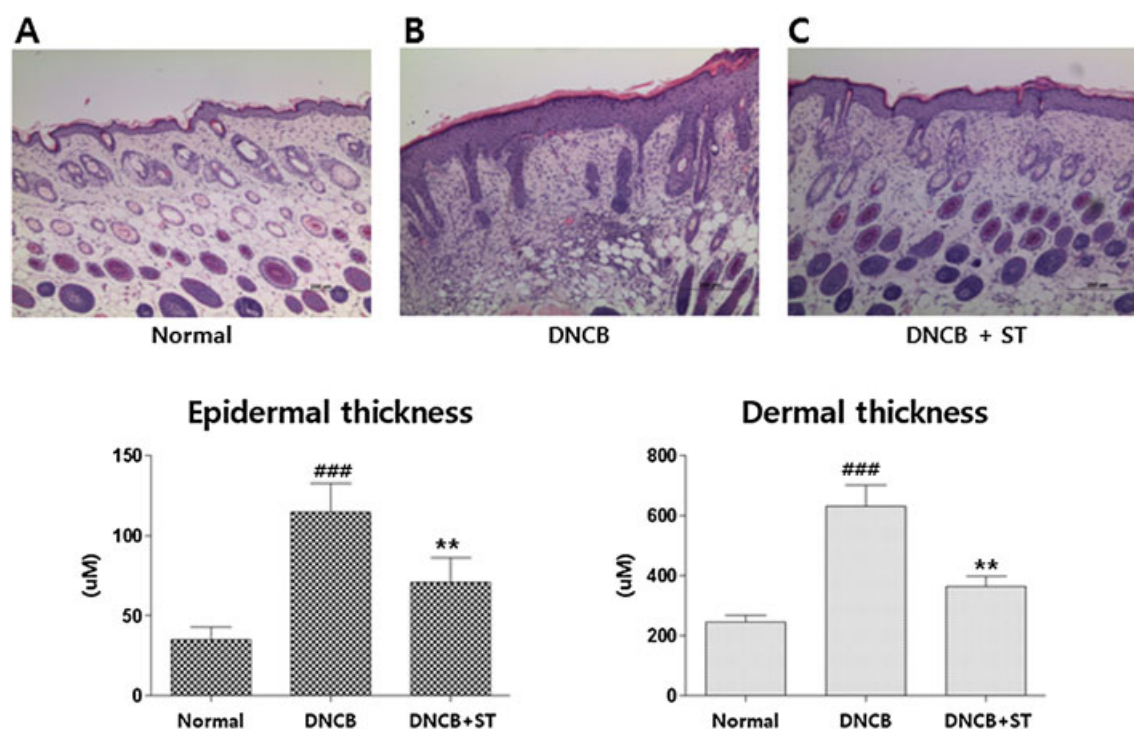


Figure 1. Reduced hyperplasia after ST treatment in epidermis and dermis of sensitized with DNCB in mice. Histopathological findings by H&E staining of skin sections ($n = 5$, magnification: $\times 100$). The skin thickness of DNCB group was significantly increased compared to the normal control group. DNCB + ST group significantly decreased markedly thickening and hyperplasia all of the epidermis and dermis compared to DNCB group. Each data represents the mean $\pm$ SEM. * indicates significance ($^{***}p < 0.001$) for the difference between normal control group and DNCB group. * indicates ($^{**}p < 0.01$) significantly different from DNCB group. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

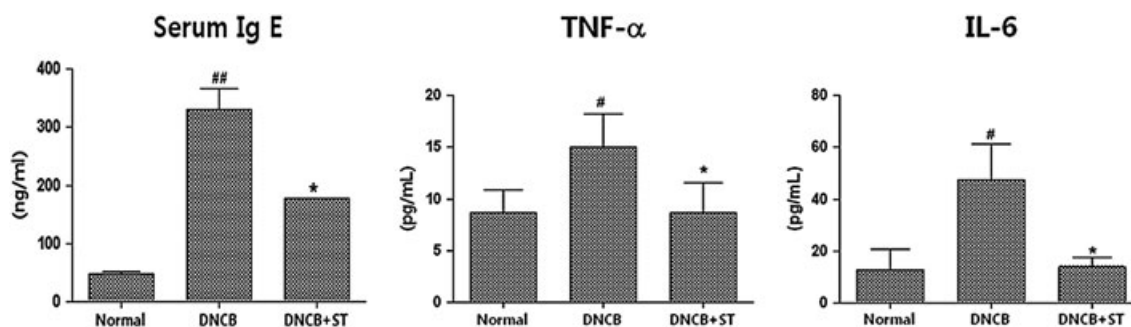


Figure 2. Suppression of IgE, TNF- α , and IL-6 level by ST treatment. The elevated level of IgE, TNF- α , and IL-6 by DNCB was significantly restored in DNCB + ST group. Each data represents the mean $\pm$ SEM. # indicates significance ($^{\#}p < 0.5$, $^{\#\#}p < 0.01$) for the difference between normal control group and DNCB group. * indicates ($^*p < 0.05$) significantly different from DNCB group.

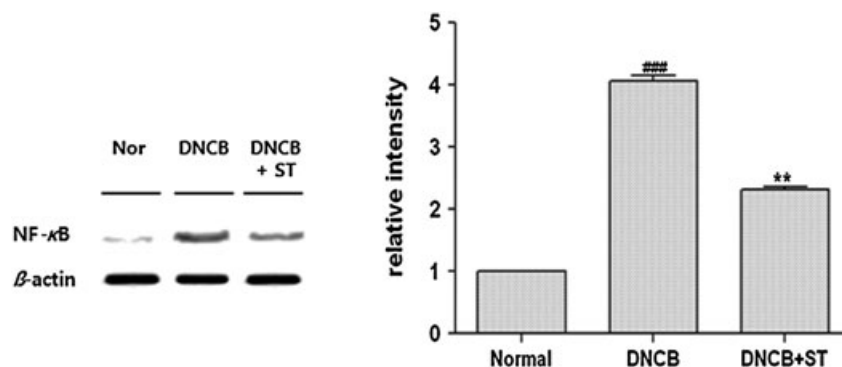


Figure 3. Downregulation of NF- κ B expression by ST. NF- κ B activation was significantly decreased in the dorsal skin of ST-treated group by 38.43%, as comparison with DNCB group ($p < 0.01$). Each data represents the mean $\pm$ SEM. ### indicates significance ($p < 0.001$) for the difference between normal control group and DNCB group. ** indicates ($p < 0.01$) significantly different from DNCB group.

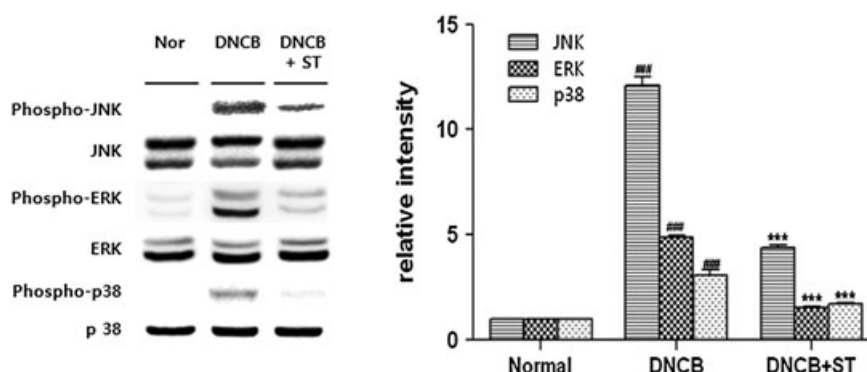


Figure 4. Suppression of MAP kinases expression by ST. MAP kinases degradation was significantly suppressed by ST. ST treatment significantly suppressed the expression of the JNK, ERK, and p38 MAPK about 63.60%, 68.48%, 43.89% compared to DNCB treatment group, respectively. Each data represents the mean $\pm$ SEM. ### indicates significance ($p < 0.001$) for the difference between normal control group and DNCB group. *** indicates ($p < 0.001$) significantly different from DNCB group.

DNCB-induced mice were examined by Western blot analysis. NF- κ B is a transcription factor that regulates immune responses stimulated by proinflammatory agents. Expression of the active form of NF- κ B in the dorsal skin of DNCB-induced mice was 73% higher than that expressed in the normal control group ($P < 0.001$; Fig. 3). NF- κ B expression was inhibited by ST treatment in DNCB-induced mice, as demonstrated by 38.43% lower compared to DNCB group ($P < 0.01$; Fig. 3).

Effects of ST on MAPKs. Phosphorylations of several MAPKs were significantly increased in DNCB-induced mice compared to normal group. Similar to the NF- κ B expression results, ST treatment

significantly suppressed the expression of the JNK, ERK, and p38 MAPK by a 63.60, 68.48, and 43.89% reduction, respectively, compared to those expressed in the DNCB-induced mice ($P < 0.001$; Fig. 4). Taken together, these results clearly indicated that ST exerts anti-inflammatory effects.

DISCUSSION AND CONCLUSION

In this study, we investigated the effects of topical ST on IgE levels and the production of proinflammatory cytokines, such as TNF- α and IL-6 in DNCB-induced AD

mouse model. Histological analysis revealed that ST treatment inhibited inflammation symptoms in DNCB-induced AD-like skin lesions. Whereas the DNCB-induced group developed dermatitis, a marked decrease in thickening and hyperplasia of the epidermis and dermis was detected in the DNCB + ST group.

IgE functions as one of the most important molecules leading to the development of atopic allergy. IgE production is upregulated by Th2 cytokines, such as IL-4 and IL-6 (Friedmann, 2006). These cytokines enhance B cell proliferation and induce immunoglobulin class switching for IgE production (Chan *et al.*, 2001). Because both IgE and Th2 cytokines play central roles as inducers of allergic disease, the suppressive effect of ST on IgE expression and IL-6 from Th2 cytokines was evaluated. The results show that serum IgE in the ST-treated group of DNCB-induced mice was strongly downregulated as compared with the DNCB control group, and IL-6 expression was also decreased by ST application. TNF- α is a Th1 cytokine that has a wide range of actions in inflammation, infection, and immunity (Prussin and Metcalfe, 2003). In particular, TNF- α is known to be excessively abundant during allergic reactions (Boislevy *et al.*, 2004). In this study, we found that dermal application of ST extracts resulted in the downregulation of TNF- α , suggesting that ST extract may influence Th2 cell activation as well as Th1 cell activation.

NF- κ B and MAPKs are activated by several stimuli commonly found in inflammation diseases (Kim *et al.*, 2008). NF- κ B is a transcription factor that controls the expression of genes involved in apoptosis and inflammation (Wuertz *et al.*, 2012). Activation of NF- κ B plays a key role in inflammatory skin processes (Smahi *et al.*, 2002). In addition, TNF- α gene expression is known to be regulated by NF- κ B induction (Boislevy *et al.*, 2004). We confirmed that ST extract inhibited activation of NF- κ B,

and the inhibition of NF- κ B might lead to the downregulation of TNF- α expression.

The MAPK signaling cascade plays an essential role in the initiation of inflammatory responses, and several recent studies have reported that suppression of MAPKs may reduce the number of mast cells involved in the inflammatory response (Kim *et al.*, 2008). p38 is activated in immune cells by inflammatory cytokines and plays an important role in activation of the immune response (Kim and Shin, 2009). Our results showed that DNCB-induced phosphorylation of JNK, ERK1/2, and p38 was significantly suppressed by ST treatment. Therefore, it seems that the inhibition of JNK, ERK1/2, and p38 activity by ST in AD may synergize with the inhibition of NF- κ B activation.

In conclusion, we demonstrated that ST extract on skin tissues exert anti-inflammatory effects against DNCB-induced AD. The results of this study suggest that ST extract reduces the allergic responses by decreasing the expression of proinflammatory cytokines as well as crucial factors of inflammation, such as NF- κ B and MAPKs. The results of this study suggest that ST should be considered as an alternative treatment for allergic contact dermatitis.

Acknowledgement

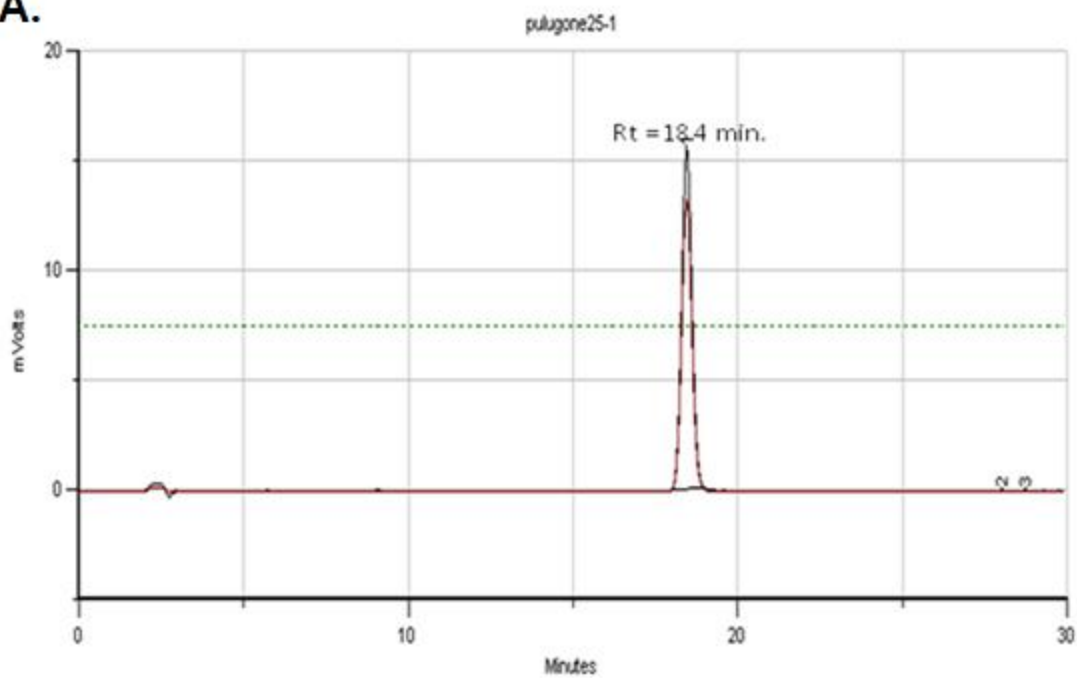
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Conflict of Interest

The authors have declared that there is no conflict of interest.

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