



Topical treatments of *Saussurea costus* root and *Thuja orientalis* L. synergistically alleviate atopic dermatitis-like skin lesions by inhibiting protease-activated receptor-2 and NF-κB signaling in HaCaT cells and Nc/Nga mice



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ABSTRACT

Ethnopharmacological relevance: The root of *Saussurea costus* (*Aucklandia lappa* Decne, *Aucklandia* Radix, SC) and *Thuja orientalis* L. (TOL) have been traditionally used as anti-inflammatory agents in Korea. However, they have not been studied for the efficacy of atopic dermatitis (AD) treatment, a chronic inflammatory skin disease. We investigated the efficacy of topical applications with 1,3-butyleneglycol extracts of SC and TOL to alleviate the symptoms of AD.

Materials and methods: HaCaT cells and the dorsal skin of Nc/Nga mice had a local exposure of house mite extracts and 2,4-dinitrochlorobenzene (DNCB), respectively. After lesions developed, we topically applied 1,3-butylen glycol (vehicle; control), SC (30%), TOL (30%), or SC (15%)+TOL (15%) to the skin lesions for 5 weeks. The normal-control was not exposed to DNCB. The skin thickness, mast cell infiltration, serum immunoglobulin E (IgE) and IgG1 and gene expressions of interleukin (IL)-4, IL-13, and IFN-γ in the dorsal skin and HaCaT cells were measured.

Results: Chlorogenic acid ($129.6 \pm 10.2 \mu\text{g/g}$) for SC and catechin and apigenin (93.4 ± 13.2 and $16.9 \pm 1.3 \mu\text{g/g}$, respectively) for TOL were used as indicator compounds for the strength of the extracts. SC+TOL decreased the expression of protease-activated receptor-2 and ICAM-1 and the release of TNF-α and IL-6 in HaCaT cells activated by 3 μg/mL house mite extracts in comparison to either of SC or TOL alone. In Nc/Nga mice challenged with DNCB, SC+TOL synergistically attenuated clinical symptoms of AD such as erythema, hemorrhage, edema, excoriation and dryness in the dorsal skin better than either SC or TOL alone. Histological analysis of the dorsal skin also showed that SC+TOL treatment significantly and additively decreased the inflammatory cellular infiltrate, including mast cells and eosinophils in comparison to either of SC or TOL. SC+TOL also decreased serum IgE and IgG1 levels and the expression of IFN-γ, IL-4, and IL-13 mRNA in dorsal skin in DNCB-treated Nc/Nga mice.

Conclusion: SC+TOL relieved the symptoms of AD by reducing pro-inflammatory activity and over-activated immune responses. These data suggest that SC+TOL may be an effective alternative intervention for the management of AD.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease. The

prevalence of childhood AD has increased from 9.2% at in 1995 to 20.6% at in 2010, and has the greatest prevalence among chronic diseases especially in low-income countries (Nutten, 2015). Although

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the reason for its increase is not known, it may be related to allergens that induce inflammation and itching. This process is associated with autoimmune disease, and lifestyle changes including sanitation, and dietary patterns increases the prevalence of autoimmune diseases including AD (Darlenski et al., 2014). Since AD negatively influences the mental and physical growth of the children (Misery, 2011), parents are highly motivated to treat AD. Its exact causes remain unknown, but environmental factors lead to immunological abnormalities that contribute to the development of AD (Darlenski et al., 2014; Misery, 2011). Therefore, interventions that treat the fundamental causes of AD are not available. Current treatments mainly attempt reduce inflammation and itching by applying topical ointments and administering oral medications (Cury Martins et al., 2015). Topical corticosteroid cream has adverse effects such as skin atrophy and acne induction (Hengge et al., 2006). Tacrolimus and pimecrolimus, immunosuppressants, are also used as topical ointment and oral administration for the treatment of severe AD (Cury Martins et al., 2015; Huang and Xu, 2015). However, these topical ointments may also produce side effects such as transient local irritations and burning-tingling sensations (Tatlican et al., 2009).

Skin barrier dysfunction is a major characteristic of AD. Protease-activated receptor (PAR-2) is included in the G protein-coupled receptor subfamily and it is involved in epidermal permeability barrier function homeostasis (Steinhoff et al., 2003). PAR-2 is activated by proteases from allergens such as house mites and the activation by mites contributes to the delay of epidermal barrier recovery (Steinhoff et al., 2003). The activation of PAR-2 activates I κ B kinase (IKK)→nuclear factor-kappaB (NF- κ B) signaling, which induces inflammation by increasing Th2 cells acutely. As a result, the expression of IL-4, IL-5, and IL-13 mRNA and protein increases in AD lesions (Spergel et al., 1999). The increase of proinflammatory cytokines is associated with the exacerbation of AD. Therefore, increased expression or activity of PAR-2 may be a pathophysiological factor for AD that induces the cytokines in skin tissues.

Since corticosteroids as AD treatment have adverse effects, alternative medicines like herbal extracts need to be developed with potent efficacy and minimal side effects. Several herbal extracts including *Thuja orientalis* L. and *Saussurea lappa* root (*Aucklandia lappa* Decne, *Aucklandia* radix) have been traditionally used for alleviating various symptoms related to inflammation in Asian countries (Heo, 1613, 2005). *Thuja orientalis* L. of the family Cupressaceae contains flavonol derivatives such as quercetin-3-O- α -L-rhamnopyranoside and myricetin-3-O- α -L-rhamnopyranoside, as well as small amount of flavan compounds such as (+)-catechin and (+)-galocatechin and monoterpene constitutes such as α -thujone (Kupeli Akkol et al., 2015). *Thuja orientalis* L. decreases the production of reactive oxygen species and NF- κ B activation in HUVEC, along with tumor necrosis factor- α (TNF- α)-induced inflammation (Lee et al., 2010). *Thujae Orientalis* Semen also decreases the production of nitric oxide (NO), prostaglandin (PG)E₂ and IL-1 β in lipopolysaccharides (LPS)-stimulated BV-2 microglia (Jung et al., 2013). *Thuja orientalis* L. has been used to treat gout, rheumatism, diarrhea and chronic tracheitis (Fan et al., 2012). The root of *Saussurea lappa* is included in the family Asteraceae, and contains sesquiterpene (costunolide), sesquiterpene lactone (dehydrocostuslactone, alantolactone, isoalantolactone, saussurealactone), polyene alcohols, lignan, alkaloid and tannin (Jung et al., 1998; Kumar et al., 2014). The major components of *Saussurea lappa* root, costunolide and dehydrocostuslactone, have anti-microbial, ant-oxidant and anti-inflammatory activities (Jung et al., 1998). It has been demonstrated that the extracts of *Saussurea lappa* root alleviate inflammation induced by different inducers such as LPS, collagen, and house dust mites in various cells and tissues (Lim et al., 2014, 2015; Tag et al., 2016; Xu et al., 2016). Although *Saussurea lappa* root does not contain many flavonoids, much less than *Thuja orientalis* L., it does exert anti-inflammatory activities. The two herbs have different major components that suppress inflamma-

tion and they may have synergistic effects. However, their possible synergistic anti-inflammatory effects have not been studied.

Therefore, the hypothesis of the present study was that *Saussurea lappa* root and *Thuja orientalis* L. can synergistically alleviate the symptoms of AD in cell-based and animal studies. In this study, we examined the synergistic activity of extracts of *Saussurea lappa* root and *Thuja orientalis* L. against AD induced by butylene glycol in HaCat cells and induced by dust mite extracts and 2,4-dinitrochlorobenzene (DNCB) treatment in Nc/Nga mice, and the mechanisms for alleviating the symptoms were explored. HaCat cells are one of the cell lines used to mimic AD symptoms in response to allergens such as dust mites and DNCB by inducing immunological responses (Huang et al., 2016; Yang et al., 2015). Repeated application of dust mite extract and DNCB in mice have been reported to increase in serum IgE and T-helper (Th) 2 cytokines such as interleukin-4 (IL-4) and IL-13 at the chronic dermatitis site of mice (Kitagaki et al., 1995). These immunological changes are similar to those observed in AD patients (Leung et al., 2004).

2. Materials and method

2.1. Preparation of extracts

The root of *Saussurea lappa* (*Aucklandia* radix) and leaves of *Thuja orientalis* L. were purchased in Kyung-Dong Herb market (Seoul, Korea) in 2011 and identified by Dr. Byung Seob Ko (Korean Herbal Medicine Institute, Daejeon, Korea) and Dr. Young Seung Joo (Department of Herbiology, Woosuk University, Wanju, Korea). Voucher specimens were deposited at the herbarium of Department of Food & Nutrition, Hoseo University (No. 2014-05 and No. 2014-06, respectively). Since 1,3-butylene glycol is one of major solvents for cosmetic usages, it was used as the solvent to make the herbal treatments. *Saussurea lappa* root and *Thuja orientalis* L. were extracted with 3.3 L of 1,3-butylene glycol (a good solvent for making skin lotion) in an ultrasonic extractor at room temperature for 12 h, and filtered. The filtrates were centrifuged at 450 $\times$ g and the supernatants were used for topical application and each extract was abbreviated as SC and TOL. The final concentration of each extract was 30%. The herbal extracts did not induce skin irritation such as rash without DNCB pretreatment in the preliminary study.

2.2. UPLC-MS/MS analysis

The components of SC and TOL were extracted with methanol and the methanol extracts were lyophilized. The powder was dissolved in methanol and their components were determined using an Acquity UPLC system (Waters, Miliford, MA, USA) with an Acquity UPLC BEH C18 column (2.1 mm $\times$ 100 mm, 1.7 μ m). The mass spectrometer was a Waters Xevo TQ triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) mode. MassLynx 4.1 (Waters) software was used for data processing. The mobile phase included 10 mM ammonium formate and 0.1% formic acid aqueous solution (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a gradient elution program was performed: 0–2 min, 90–90% solvent A; 2–3 min, 90–0% solvent A; 3–5 min, 0–0% solvent A; 5–5.5 min, 0–90% solvent A. The flow rate was set at 0.35 mL/min. Column temperature was kept at 40 $^{\circ}$ C and the total run time was 7 min. The auto-sampler was conditioned at 4 $^{\circ}$ C and the injection volume was 5 μ L. An LC-MS/MS system was operated in negative ESI mode and scanned using multiple reaction monitoring (MRM) mode. The voltage of the capillary, cone and collision energy was set at 2.5 kV, 20 V and 15 V, respectively. The gas flow for desolvation and cone was 800 and 50 L/h. The source temperature and desolvation gas temperature was 150 and 400 $^{\circ}$ C, respectively.

2.3. Cell culture

HaCaT cells, a human immortalized keratinocyte cell line, were cultured as described previously (Park et al., 2015). HaCaT cells were cultured with RPMI 1640 supplemented with 10% FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin in a humidified atmosphere of 5% CO₂/95% air at 37 °C. The cells were treated with house dust mite extracts (0.3 and 3 µg/mL, HM-0.3 and HM-3, respectively) and 10 nM DNCB for 24 h and cDNA was synthesized from the cells. The mRNA expressions of PAR-2, intercellular Adhesion Molecule 1 (ICAM-1) and IL-4 were measured with realtime PCR as described below.

The cells were treated with DMSO (control), SC (20 µg/mL), TOL (20 µg/mL) or SC (10 µg/mL)+TOL (10 µg/mL) at 30 min before inducing inflammation with house dust mite extracts (3 µg/mL), which induced a state similar to AD. Normal-controls were treated with of 1,3-butylene glycol without dust mite extracts in HaCaT cells. After 24 h treatment, cells were harvested and media was collected and assayed for TNF-α and IL-4 with ELISA kits. The cells were harvested to synthesize cDNA and mRNA of TNF-α and IL-4 was measured with realtime PCR as described below.

2.4. Animals

Twenty male 6-week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan), and maintained under conventional conditions with a 12 h light/12 h dark cycle, room temperature of 22–23 °C and humidity of 55 ± 15%. The mice had free access to food and water. All surgical and experimental procedures were performed according to the guidelines of the Animal Care and Use Review Committee at Hoseo University, Korea.

2.5. Induction of AD-like skin lesion

Mice were anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg body weight) and the animal's back hair and right ear were shaved. At the first day after shaving, 1% DNCB in acetone/olive oil (3:1) was applied to the dorsal skin and then 0.2% DNCB was applied 3 days a week for 5 weeks (Park et al., 2015). Repeated application of DNCB onto the dorsal region causes apparent AD in NC/Nga mice (Kim et al., 2014; Park et al., 2015). Normal-control mice were applied with the same volume of acetone/olive oil solution without DNCB in the same manner and they did not develop AD-like skin lesions.

Animals with induced AD-like skin lesions were divided into four groups of 10 mice each: 1,3-butylene glycol (BG; control), 30% SC, 30% TOL, or 15% SC+15% TOL twice a day. These groups were then received topical applications to the dorsal skin with 200 µL of their respective 30% herbal extracts on a daily basis for 5 weeks. The DNCB and herbal extracts were applied at least 3 h apart. Mice in the normal-control group were treated with 1,3-butylene glycol instead of herbal extracts. Thus, total number of groups in the present study were 5: control, SC, TOL, SC+TOL and normal-control.

2.6. Evaluation of skin lesion

The relative severity of AD was evaluated weekly with a microscope using the following scoring procedure (18): 1) erythema and hemorrhage, 2) edema, 3) erosion (excoriation), and 4) scaling (dryness). In each score, 0 was defined as exhibiting no symptoms, 1 as mild symptoms, 2 as moderate symptoms, and 3 as severe symptoms. One investigator scored apparent AD symptoms with blinding to the treatments throughout the experiment to minimize technique variations.

2.7. Measurement of serum IgE and TNF-α levels

Total serum IgE and IgG1 were quantified using an enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The serum concentrations of the cytokines, tumor necrosis factor-α (TNF-α) and IL-4 were also quantified using a mouse cytokine enzyme immunoassay kit (R & D Systems, Minneapolis, MN, USA) and Biotrak ELISA system (Amersham Biosciences, Piscataway, NJ), respectively.

2.8. Measurement of cytokine production

Cytokine levels were determined by ELISA. Dorsal back samples (n = 10) of the regions with induced AD were homogenized in RIPA buffer containing a protease inhibitor cocktail (Merck, Darmstadt, Germany). Homogenates were then centrifuged at 12,000×g for 20 min at 4 °C to obtain the supernatant. Cytokine levels of IL-1β, IL-4, and TNF-α were analyzed using the Biotrak ELISA system (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Cytokine levels were normalized to protein concentrations using a protein assay kit and calculated as pg per mg total protein.

2.9. Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

The dorsal skin tissues were collected in five randomly selected mice from each group at the end of the treatment period. HaCaT cells were harvested after treatment. Total RNA was separated from the skin tissues and cells using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Invitrogen, Rockville, MD, USA). The cDNA was synthesized with the mixture of equal amounts of total RNA, superscript III reverse transcriptase and high fidelity Taq DNA polymerase by polymerase chain reaction (PCR). The cDNA was mixed with the primers of the genes of interest and sybergreen mix, and the expressions of the genes of interest were analyzed using a realtime PCR machine (BioRad Laboratories, Hercules, CA, USA). The primers for PAR-2, ICAM-1, TNF-α, IL-4, IFN-γ, IL-13, and β-actin were given in previous studies (Park et al., 2015; Tripathi et al., 2014). The gene expression levels in unknown samples were quantitated using the comparative cycle of threshold (CT) method (Livak and Schmittgen, 2001).

2.10. Histological analysis

At the end of the experiments, the dorsal skin samples were taken 24 h after final herbal administration and fixed in 10% buffered neutral formaldehyde and embedded in paraffin wax. Histological sections were 6 µm thick and were stained with haematoxylin and eosin and used to score the lesions in the dermal tissues. The sections were also stained with 0.5% toluidine blue for determining the number of mast cells. The cell counts and the skin lesion scorings were performed in two sections randomly selected from six consecutive microscopic fields at 400X magnification.

2.11. Statistical analysis

Statistical analysis was performed using SAS software and all results expressed as mean ± standard deviation. The biological and metabolic effects of SC, TOL, SC+TOL, vehicle (a negative control) and positive control were compared by one-way ANOVA. Significant differences in the main effects among the groups were identified by Tukey's test at $P < 0.05$. The significance of differences between the mice with and without AD like skin lesions were determined by two-sample *t*-test.

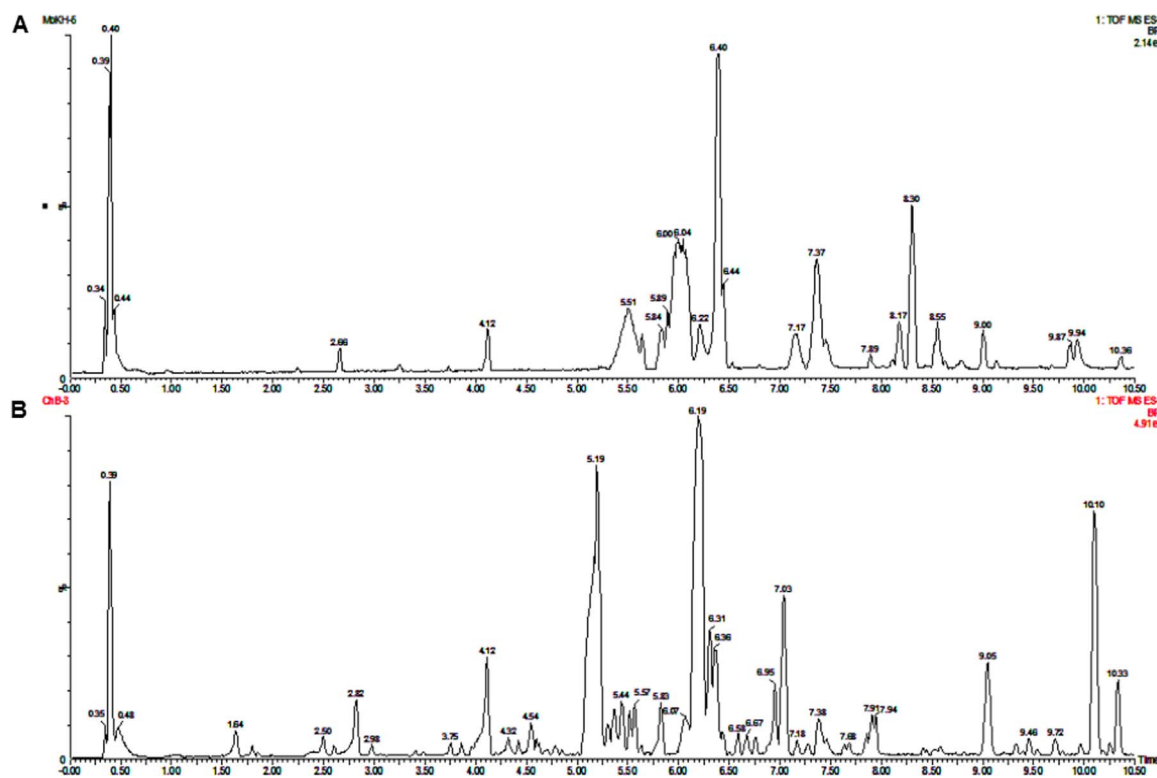


Fig. 1. Chromatograms of extracts of *Saussurea lappa* root (A) and *Thuja orientalis* L. leaves (B).

3. Results

3.1. The contents of SC and TOL components

The chromatograms of SC and TOL presented in Fig. 1 and the contents of each of the components were given in Table 1. SC and TOL contained organic acids and flavonoids. TOL contained much more flavonoids than SC. Indicator compounds were used to identify and standardize the strength of the extracts; chlorogenic acid was used as the indicator compound for SC and catechin and apigenin were used as the indicator compounds for TOL. SC and TOL contained substantial amounts of the assigned indicator compounds and they did not include

the indicator compound of the other herbs.

3.2. HM decreased mRNA expression of PAR-2, ICAM-1 and IL-6 in HaCaT cells

HM increased the expression of PAR-2 in a dose-dependent manner that subsequently elevated the release of ICAM-1 and IL-6. ICAM-1 and IL-6 induced over-activation of immune responses and inflammation, resulting in the development of AD. The cells treated with HM-3 exhibited similar expressions of PAR-2, ICAM-1 and IL-6 as much as 200 $\mu\text{g/mL}$ DNCB treatment (Fig. 2A).

SC and TOL decreased the release of TNF- α and IL-6 in HaCaT cells

Table 1

The components of *Saussurea costus* root and *Thuja orientalis* Linne.

Unit (ug/g)	RT (min)	Precursor ion (m/z)	Product ion (m/z)	<i>Saussurea costus</i> root (n=3)	<i>Thuja orientalis</i> Linne (n=3)
Quinic acid	0.4	191	85	128.0 $\pm$ 20.1	904.5 $\pm$ 15.2
Malic acid	0.42	133.1	115.1	442.0 $\pm$ 14.1	129.5 $\pm$ 0.72
Galic acid	0.92	169.1	107.2	0.40 $\pm$ 0.01	1.28 $\pm$ 0.19
Genesitic acid	1.57	152.80	109.0	5.45 $\pm$ 2.21	13.4 $\pm$ 0.44
Protocatechuic acid	1.84	153.1	81.1	0.31 $\pm$ 0.08	0.80 $\pm$ 0.03
Hydroxybenzoic acid	2.29	137.1	93	0.30 $\pm$ 0.01	1.65 $\pm$ 0.65
Chlorogenic acid	2.77	353.2	85.1	129.6 $\pm$ 10.2	0.67 $\pm$ 0.27
Caffeic acid	3.04	179.1	107.2	5.49 $\pm$ 0.35	0.58 $\pm$ 0.15
Epicatechin	3.76	289.2	109.1	23.5 $\pm$ 3.08	256.8 $\pm$ 17.6
Catechin	3.79	289.2	109.1	1.47 $\pm$ 0.11	93.4 $\pm$ 13.2
Epigallocatechin gallate	3.88	457.2	169.2	0.55 $\pm$ 0.11	0.24 $\pm$ 0
Coumaric acid	4.03	162.7	119.3	7.13 $\pm$ 0.17	1.50 $\pm$ 0.03
Rutin	5.38	609.3	271.4	0.27 $\pm$ 0.01	0 $\pm$ 0
Epicatechin gallate	5.44	441.2	169.1	0.19 $\pm$ 0.01	0.13 $\pm$ 0.01
Quercetin-3-Glucoside	5.52	463.3	179.2	0.30 $\pm$ 0.05	66.9 $\pm$ 0.63
Kaempferol-3-Glucoside	6.22	447.3	255.3	0.15 $\pm$ 0.10	5.28 $\pm$ 0.84
Naringin	6.53	579.3	151.3	0 $\pm$ 0	0.61 $\pm$ 0.04
Hesperidin	6.82	609.4	301.5	0.78 $\pm$ 0.03	0.46 $\pm$ 0.01
Quercetin	7.96	301.4	151.1	0 $\pm$ 0	5.19 $\pm$ 0.10
Luteolin	8.02	285.1	151.1	0.16 $\pm$ 0.02	6.40 $\pm$ 0.71
Apigenin	9.16	269.2	117.1	0 $\pm$ 0	16.9 $\pm$ 1.26
Isorhamnetin	9.7	315.1	151.0	0.57 $\pm$ 0.09	2.68 $\pm$ 0.34

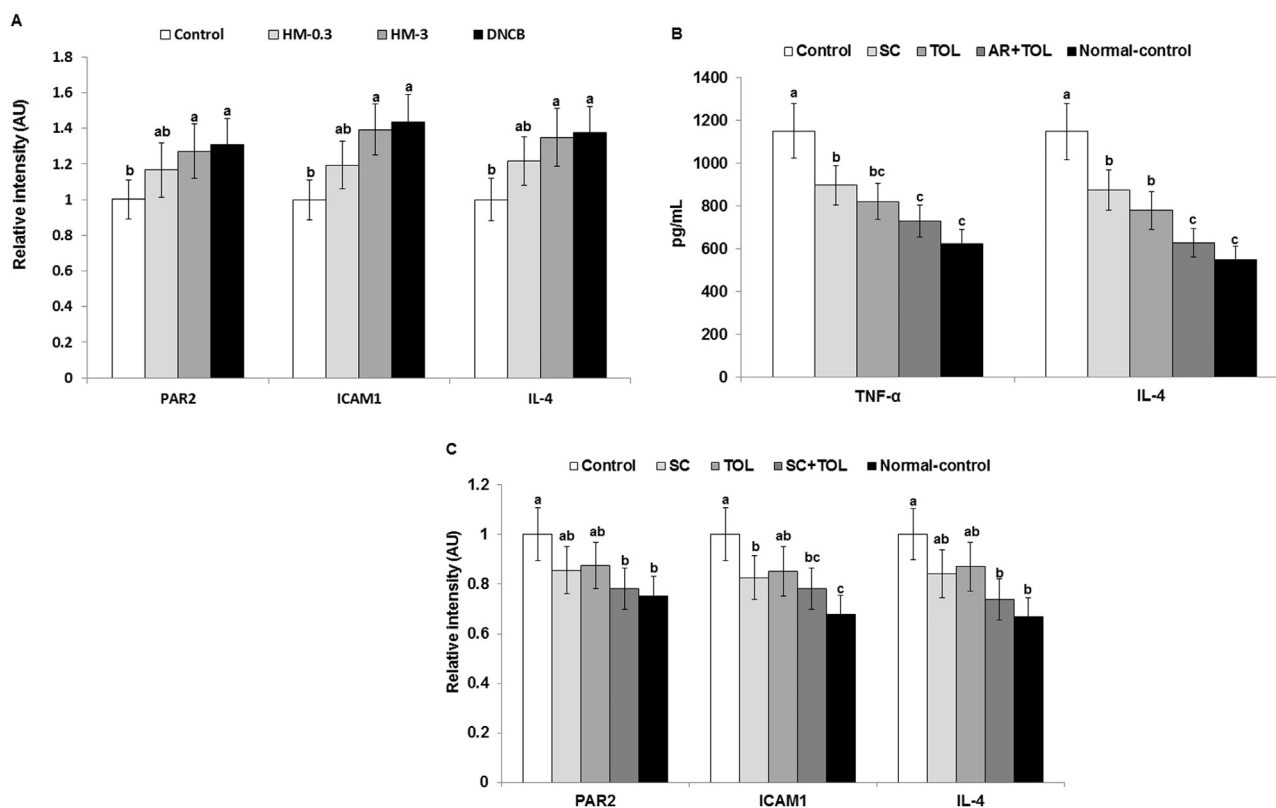


Fig. 2. Pro-inflammatory cytokines in the media and their expression in the HaCat cells induced with house dust mite extracts after treatment with of *Saussurea lappa* root (SC) and leaves of *Thuja orientalis* L. (TOL). A. mRNA expression of PAR-2, ICAM-1 and IL-4 after the treatment of house dust mite extracts (0.3 µg/mL, house mite extract (HM)–0.3; 3 µg/mL, HM-3 and 10 nM DNCB) for 24 h. B. TNF-α and IL-4 levels in the media after the treatment of 1,3-butylene glycol (control), SC (20 µg/mL), TOL (20 µg/mL) or SC (10 µg/mL)+TOL (10 µg/mL) at 30 min before inducing inflammation with house dust mite extracts (3 µg/mL). Normal-control was treated with of 1,3-butylene glycol without dust mite extracts. C. mRNAs expression of TNF-α and IL-4 in the cells in the same treatments of Fig. 2B. Each value represents the mean ± SD (n=3). * Significantly different among the different treatments in HaCat cells at $P < 0.05$. a, b, c Means of the bars with different superscripts were significantly different by Tukey test at $P < 0.05$.

activated by HM-3 whereas SC+TOL synergistically decreased them to levels similar to the normal-control (Fig. 2B). In addition, SC and TOL did not reduce the mRNA expression of PAR-2, ICAM-1 and IL-6 in HaCaT cells activated by HM but SC+TOL decreased their expressions to similar levels as the normal-control group (Fig. 2C).

3.3. Clinical severity of skin lesions

After repeatedly applying DNCB in the dorsal skin of the Nc/Nga mice the control mice developed AD lesions with symptoms of hypertrophy, hyperkeratosis, intercellular edema, and the liquefaction degeneration of the basal layer (Fig. 3). The clinical severity was evaluated by the sums of the scores for each symptom, such as erythema and hemorrhage, edema, erosion (excoriation), and scaling (dryness). The clinical severity of dorsal skin lesions in the control group increased remarkably until 4 weeks after topical application of DNCB, but the severity was reduced from week 5 (Fig. 3A, B). SC exacerbated the symptoms until 1 week but it attenuated the symptoms after 2 weeks. TOL exhibited similar patterns of the symptoms as SC but the severity in TOL was less than that in SC until week 4 (Fig. 3A, B). SC+TOL synergistically improved the clinical skin severity from the 1st week but the severity score was worse than the normal-control.

3.4. Serum levels of proinflammatory cytokines, TNF-α and IL-4, and IgE

Serum TNF-α levels of the control group were higher by 2.2 fold than those of the normal-control group (Fig. 4). TOL suppressed the increase of serum TNF-α levels in comparison to the control, and SC+TOL synergistically decreased the levels although SC itself did not

decrease circulating TNF-α (Fig. 4). Serum IL-4 and IgE levels exhibited similar results as serum TNF-α levels. Thus, SC and TOL decreased AD symptoms (serum IgE levels) and proinflammatory markers, whereas SC+TOL synergistically increased their effects on serum IgE and proinflammatory cytokines (Fig. 4).

3.5. Histological findings and mast cell counts in the inflamed skin

In H-E stained skin tissues, keratinocytes exhibited hypertrophy to make abnormal cell shapes and larger nuclei in the control group in comparison to the normal-control group (Fig. 5). SC and TOL prevented the cell damage and SC+TOL synergistically prevented the abnormalities. In addition, the thicknesses of dermal and epidermal tissues were greater in the control group than the normal-control group due to edema, hyperkeratosis, and hypertrophy (Fig. 5A, B). SC and TOL reduced the thickness of dermal and epidermal tissues and SC+TOL synergistically reduced their thickness (Fig. 5A, B). The surface of the skin was rough and hypertrophic in the control group in comparison to the normal-control. SC+TOL greatly improved the skin tissue surface, but it was still not like the normal-control group (Fig. 5A, B). Eosinophil infiltration, an indicator of inflammation, was greatly increased in the control group in comparison to the normal-control whereas SC and TOL reduced the number of eosinophils. SC+TOL additively decreased their numbers (Fig. 5C, D). In addition, the number of mast cells increased in the control group, compared to the normal-control group and SC+TOL suppressed the increase of mast cells as observed in the toluidine stained sections (Fig. 5B, D).

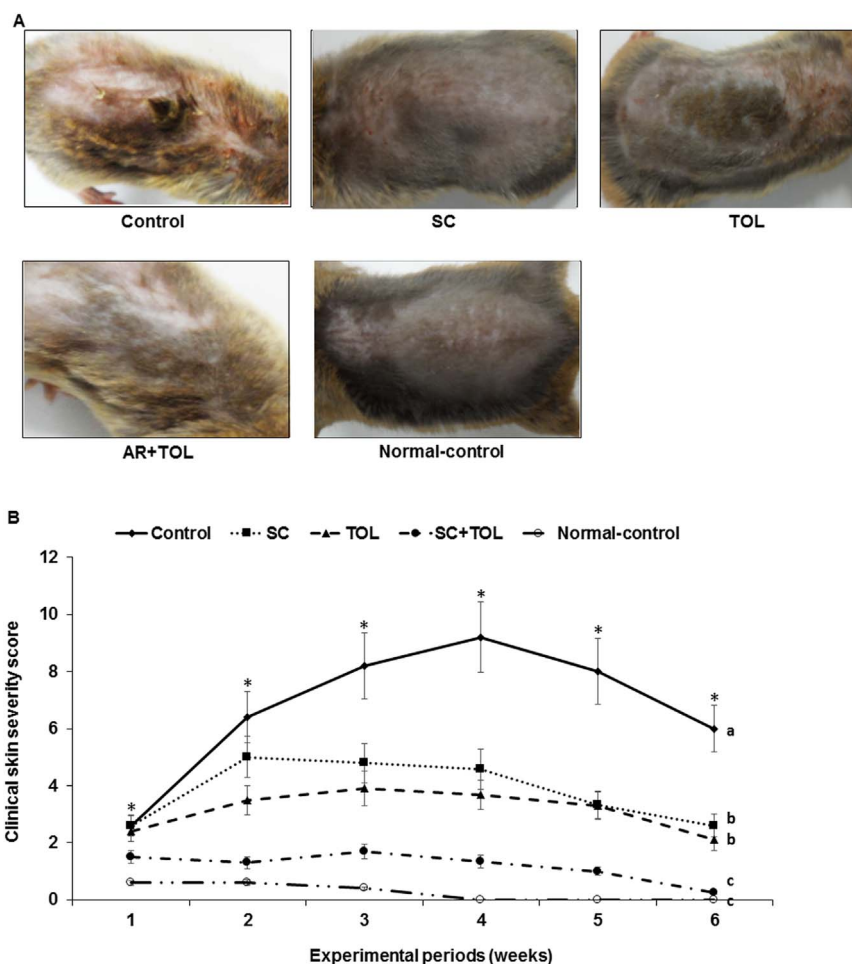


Fig. 3. Changes in severity scores of atopic dermatitis (AD) in the dorsal skin of Nc/Nga mice induced atopic dermatitis AD induced in Nc/Nga mice by topical application of 2,4-dinitrochlorobenzene (DNCB). Each value represents the mean $\pm$ SD ($n=10$). * Significantly different among the different treatments in Nc/Nga mice at $P < 0.05$. †Significantly different between control (BG) and normal-control at $P < 0.05$.

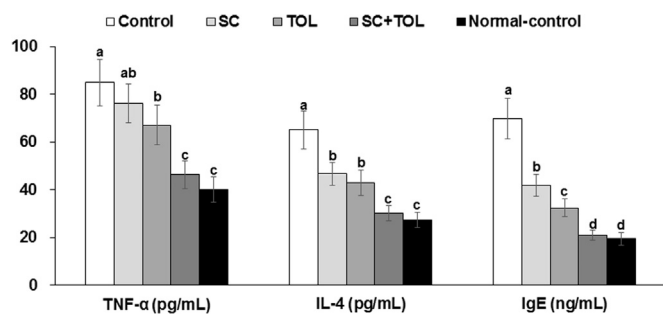


Fig. 4. Serum levels of IL-4, IFN- γ and IgE levels in Nc/Nga mice induced atopic dermatitis at the end of experimental periods. Each value represents the mean $\pm$ SD ($n=10$). * Significantly different among the different treatments in Nc/Nga mice at $P < 0.05$. ^{a, b, c}Means on the bars with different superscripts were significantly different by Tukey test at $P < 0.05$. †Significantly different between control (BG) and normal control at $P < 0.05$.

3.6. mRNA expression levels of PAR-2, ICAM-1 and cytokines

PAR-2 and ICAM-1, which are involved in cell adhesions, were higher in the control group than the normal-control group whereas SC and SC+TOL lowered their expressions (Fig. 6A). The levels of PAR-2 and ICAM-1 expression in SC+TOL were similar to the normal-control group (Fig. 6A).

The mRNA expressions of IL-4, IL-13, and INF- γ cytokines in the dorsal skin tissues were analyzed. The dorsal skin tissues of the control mice exhibited higher expression levels of IL-4, IL-13, and INF- γ than

those of the normal-controls (Fig. 6B). TOL suppressed the mRNA expression of IL-4 and IL-13 produced by Th2 cells and SC+TOL synergistically decreased their expression (Fig. 6B). TOL and SC+TOL also suppressed the expression of IFN- γ produced by Th1 in comparison to the control group. However, the levels of cytokines in the TOL and SC+TOL groups remained higher than those in the normal-control group (Fig. 6B). The ratio of cytokines released from Th1 to Th2 cells greatly increased in the SC+TOL group in comparison to the control group.

4. Discussion

AD is one of many autoimmune diseases, and over-activation of immunity induces AD by promoting inflammation of the skin barriers. The prevalence of AD is markedly rising, especially in children. The possible causes that have been suggested include changes in lifestyle factors, including sanitation and dietary patterns such as consuming diets high in processed foods. However, the fundamental causes remain unknown. Current treatments such as topical corticosteroid ointments mainly suppress clinical symptoms such as itching and inflammation, but they also have some adverse effects. Therefore, alternative treatments are needed. Previous studies have reported that both SC and TOL can reduce inflammation and act immunomodulatory agents, but few studies have researched the anti-AD effects of SC and TOL. Lim et al. (2014) demonstrated that SC relieves AD symptoms induced by house mite exposure in cell-culture and animal studies. However, TOL has not been examined for potential effects on AD symptoms in animal

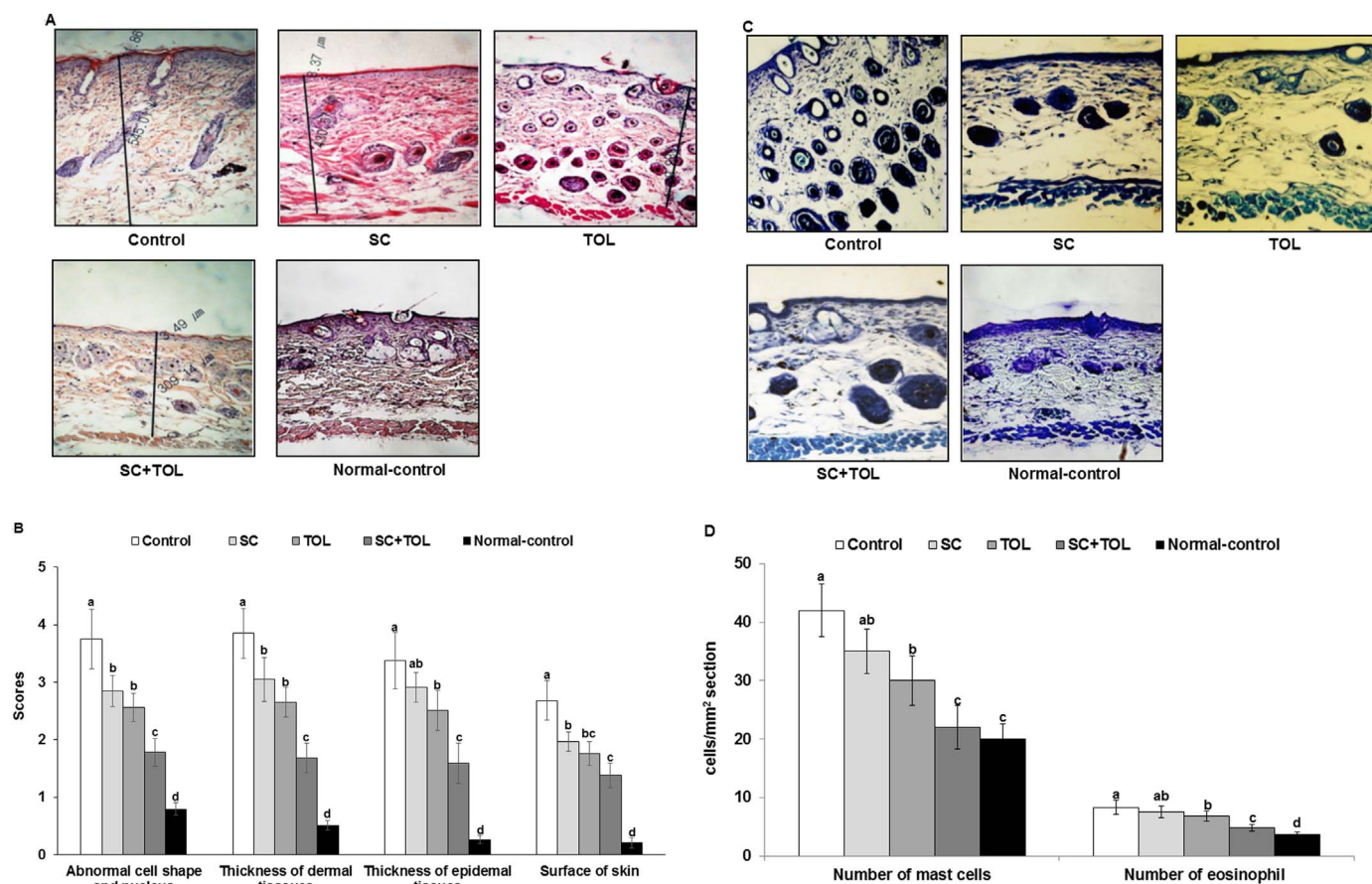


Fig. 5. The histological differences, number of mast cells and eosinophils of the dorsal skin with histopathological analysis. A. The histological pictures in hematoxylin and eosin (H-E) staining. B. Scores of histological changes in H-E staining. C. The histologic pictures in toluidine blue staining. D. The number of eosinophils and mast cells. Each value represents the mean $\pm$ SD (n=5). * Significantly different among the different treatments in Nc/Nga mice at $P < 0.05$. ^{a, b, c} Means on the bars with different superscripts were significantly different by Tukey test at $P < 0.05$. [†] Significantly different between control (BG) and normal control at $P < 0.05$.

studies. Furthermore, no studies have examined the efficacy of SC+TOL to reduce AD symptoms. Thus, the current study is the first one to demonstrate the synergistic efficacy of SC+TOL on AD symptoms and to explore its mechanism. We revealed that SC+TOL decreased the expressions of PAR-2 and ICAM-1 and the release of TNF- α and IL-6 in HaCaT cells activated by 3 μ g/mL house mite extracts in comparison to either SC or TOL alone. In Nc/Nga mice, the clinical symptoms of AD induced by DNCB were synergistically attenuated with SC+TOL. Histological analysis of the dorsal skin also showed that SC+TOL treatment significantly and additively decreased the inflammatory cell infiltrates, including mast cells and eosinophils in comparison to either

of SC or TOL alone. This was associated with the decrease of proinflammatory cytokines (TNF- α , IL-4, an IL-13), serum IgE levels and IFN- γ , IL-4, and IL-13 mRNA expressions in dorsal skin. Thus, SC +TOL might be a good alternative candidate for treating AD symptoms.

Increases in PAR-2 expression are involved in inflammation in various tissues, including keratinocytes, possibly inducing AD (Bar-Shavit et al., 2002; Kanke et al., 2001). The association between PAR-2 activation and development of AD has been shown by several studies (Kawagoe et al., 2002; Steinhoff et al., 2003). Contact dermatitis is remarkably decreased in PAR-2 knock-out mice (Kawagoe et al., 2002). PAR-2 is expressed by various inflammatory cells including mast cells

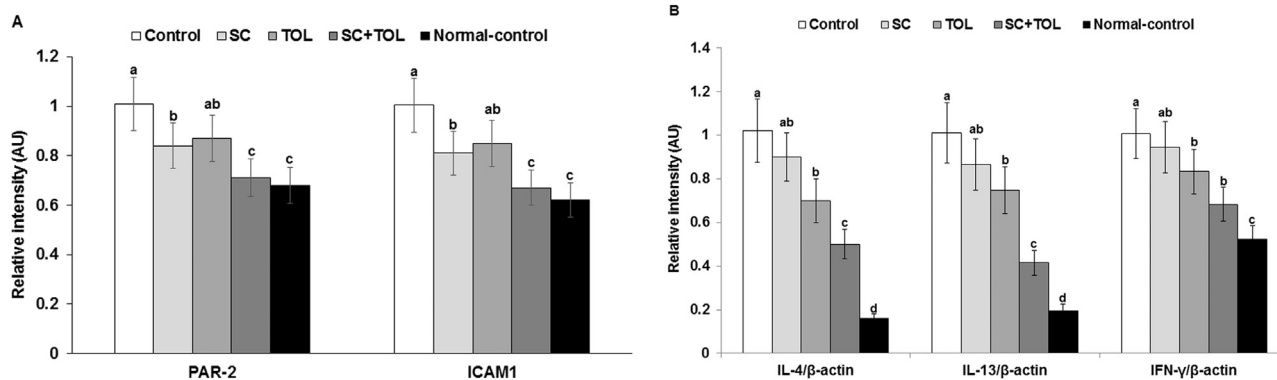


Fig. 6. PAR-2, ICAM-1, IL-4, IL-13 and IFN- γ expression in the dorsal tissues A. The mRNA expression of PAR-2 and ICAM-1 B. The mRNA expression of IL-4, IL-13 and IFN- γ . Each value represents the mean $\pm$ SD (n=5). * Significantly different among the different treatments in Nc/Nga mice at $P < 0.05$. ^{a, b, c} Means on the bars with different superscripts were significantly different by Tukey test at $P < 0.05$. [†] Significantly different between control (BG) and normal control at $P < 0.05$.

(D'Andrea et al., 2000) and T cells (Bar-Shavit et al., 2002). Activation of PAR-2 stimulates NF- κ B signaling and the release of IL-1 β , IL-4 and IL-6 by keratinocytes (Kanke et al., 2001). The present study demonstrated that allergens such as house dust mite extracts and DNCB increase PAR-2 expression with subsequent increases in the expressions of ICAM-1, IL-4, and TNF- α in HACAT cells. The PAR-2 expression was significantly lowered with SC+TOL and the decrease was similar to the normal-control. The expression of ICAM-1 and IL-4 was parallel with PAR-2 expression. However, TNF- α and IL-4 concentrations in the media were significantly lower with SC and TOL than the control and the decrease was much greater in SC+TOL than either SC or TOL alone. Thus, SC and TOL synergistically suppressed allergen-induced proinflammatory activities in keratinocytes.

Nc/Nga mice are a good animal model for AD induced by allergens such as house dust mite extracts and DNCB since the mice have similar clinical features as human AD (Inoue and Aramaki, 2007; Park et al., 2015). AD develops as a consequence of immune dysregulation that is associated with Ig E-mediated hypersensitivity reaction. This immune dysregulation is induced by the activation of Th2 cells which release IL-4, IL-5, IL-10, and IL-13 from immune-related cells such as inflammatory dendritic epidermal cells, lymphocytes, mast cells, etc (Spergel et al., 1999). Mast cells also stimulate the release of TNF- α and IL-4, and the proinflammatory cytokines promoted IgE synthesis. Allergen induced Nc/Nga mice have clinical characteristics of pruritus, redness, and swelling of the skin due to epidermal hypertrophy and hyperkeratosis, and also have increased expression of proinflammatory cytokines, histamine and Ig E (Park et al., 2015). In the present study Nc/Nga mice challenged with DNCB were used as animal model for moderate AD; and SC, TOL and SC+TOL improved the clinical pathologies with SC+TOL decreasing them similar levels as normal-control mice.

Saussurea lappa root and *Thuja orientalis* L. are well-known traditional herbal medicines that are used for the treatment of inflammatory disease such as asthma, rheumatism, coughs, tuberculosis and numerous other diseases (Seo et al., 2015; Shin et al., 2015). The 70% methanolic extracts of *Saussurea lappa* root contain three sesquiterpene lactones: costunolide, dehydrocostus lactone, and alantolactone at 17.32, 28.26, and 0.01 mg/g, respectively (Seo et al., 2015). However, not many studies have conducted with SC. SC and its components are reported to inhibit the proliferation of cancer cells, but not normal cells (Bocca et al., 2004). It is associated with increased apoptosis by inducing the expression of apoptosis related genes. In addition, recent studies have shown that 70% ethanol extract and aqueous extracts of SC have anti-inflammatory, antioxidant and immunomodulatory activities, and it improves the symptoms of adjuvant-induced arthritis and house dust mite-induced AD (Chang et al., 2012; Lim et al., 2014, 2015). These studies have shown that SC reduces proinflammatory activities of TNF- α and IFN- γ by blocking STAT1 phosphorylation (Lim et al., 2014). In the present study 1,3-butylene glycol extract of SC was also shown to reduce proinflammatory cytokines and to inhibit the over-activation of immune response in HACAT cells with house mite extract induced AD and Nc/Nga mice challenged with DNCB in comparison to the control. However, the efficacy was less than TOL and much less than SC+TOL. Thus, the present study can provide more information on how to improve the efficacy of SC for treating AD symptoms.

The main components of TOL are monoterpene ketones α - and β -thujone, fenchone, sabinene, diterpenes beyerene and rimuene (Kupeli Akkol et al., 2015). TOL and its components are also reported to reduce inflammatory response in ovalbumin-induced allergic asthma, transient focal cerebral ischemia and bone marrow-derived mast cells by reducing the expression of pro-inflammatory cytokines; including TNF- α , inducible nitric oxide synthase, cyclooxygenase-2 (COX-2) and IL-1 β and by suppressing phosphorylation of c-Jun N-terminal kinase and p38 MAPK and nuclear translocation of NF- κ B (Chae and Chin, 2012; Jung et al., 2013; Shin et al., 2015). It also has anti-viral activity by

activating the immune response to combat the common cold and respiratory infection (Naser et al., 2005a, 2005b). No adverse events from using TOL have reported in any studies, suggesting that it is safe to use as an herbal treatment (Naser et al., 2005a, 2005b). The present study also showed that TOL reduced the TNF- α and IL-1 β expressions in HACAT cells given house mite extract and Nc/Nga mice with AD induced by DNCB application, and TOL was more effective than SC.

No study has previously been conducted using SC+TOL. Interestingly, in the present study SC+TOL synergistically attenuated the symptoms of AD better than either SC or TOL alone. This may be due to the different action mechanisms of SC and TOL as previously described. SC reduced downstream proinflammatory activities of TNF- α and IFN- γ by blocking STAT1 phosphorylation, whereas TOL decreased the expression of pro-inflammatory cytokines in the previous studies. Thus, SC+TOL was shown to synergistically improve the clinical AD symptoms by reducing inflammation and attenuating over-activated immune response in the present study. Therefore, SC+TOL may be a good candidate for treating AD.

In conclusion, both SC and TOL itself improved the symptoms of AD better than the control but SC+TOL exhibited a synergistic efficacy for treating the symptoms. SC+TOL reduced pro-inflammatory cytokine release and their signaling pathways and attenuated over-activated immunity. These data suggest that SC+TOL may be an effective alternative substance for the management of AD.

Author contributions

SP and SK participated in the experimental design; DSK, NRL and SK conducted the animal studies; NRM and DSK performed biochemical studies. SP and SK wrote the manuscript. All authors participated in analysis of the data and review of the manuscript.

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