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# Topical Application of 7,3′,4′-Trihydroxyisoflavone Alleviates Atopic Dermatitis-Like Symptoms in NC/Nga Mice

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## ABSTRACT

Atopic dermatitis is a skin disease characterized by chronic inflammatory lesions, and new therapies are needed to address its rising prevalence. Soy isoflavone has been highlighted as a potential new cosmeceutical material that may have applications in atopic dermatitis care. We have developed a technique to attach an additional -OH group to the ortho position of -OH in the phenol ring using a special enzyme. By adding the -OH group to daidzein, 7,3′,4′-trihydroxyisoflavone can be generated for possible use as a cosmeceutical and functional food material. In this study, we sought to examine the anti-atopic effects of 7,3′,4′-trihydroxyisoflavone, an analog of daidzein. Topical application of 7,3′,4′-trihydroxyisoflavone reduced *Dermatophagoides farina* extract-induced atopic dermatitis symptoms in NC/Nga mice. Histological analysis demonstrated that 7,3′,4′-trihydroxyisoflavone suppressed *D. farina* extract-induced infiltration of eosinophils and mast cells into skin lesions. We also found that 7,3′,4′-trihydroxyisoflavone significantly reduces the *D. farina* extract-induced increases in serum IgE and macrophage-derived chemokine (CCL22) levels. We observed that 7,3′,4′-trihydroxyisoflavone suppresses atopic markers including macrophage-derived chemokine (CCL22) and thymus and activation-regulated chemokine (CCL17) in HaCaT cells. 7,3′,4′-Trihydroxyisoflavone also reduced TNF-α/IFN-γ-induced phosphorylation of ERK1/2 and JNK1/2. These results highlight several desirable properties of 7,3′,4′-trihydroxyisoflavone, which support its use as a cosmeceutical ingredient for the treatment of atopic dermatitis.

* Sang Hun Park and Chang Hyung Lee contributed equally to this work.



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**ABBREVIATIONS**

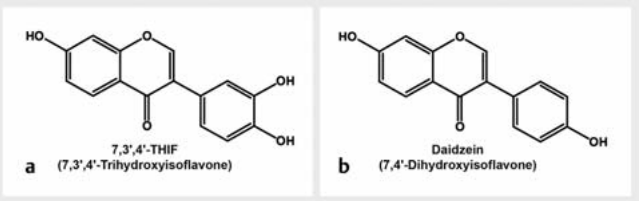
| | |
|---|---|
| **7,3′,4′-THIF** | 7,3′,4′-trihydroxyisoflavone |
| **AD** | atopic dermatitis |
| **CR** | Congo red |
| **DFE** | *Dermatophagoides farinae* extract |
| **H&E** | hematoxylin and eosin |
| **MDC** | macrophage-derived chemokine |
| **TARC** | thymus and activation-regulated chemokine |
| **TB** | toluidine blue |

## Introduction

AD is a chronic inflammatory skin disease. With rising global prevalence, AD often occurs in infancy and subsides, but can continue to develop in some adults. The condition is characterized by itching due to inflammation and subsequent scratching can cause wounds [1, 2]. With continued scratching, skin barriers collapse and moisture is lost, leading to dry skin and more inflammation in what can become a vicious cycle [3]. This can cause psychological and social stress for patients and their families and increases the risk of food allergies, asthma, allergic rhinitis, and other immune-mediated inflammatory diseases, as well as mental health disorders [2]. Local corticosteroids or calcineurin inhibitors are currently recommended for the prevention and treatment of AD, and are widely used for its management [4]. However, due to potentially serious side effects, including liver and kidney damage, diabetes, skin thinning, immunodeficiencies, and drug resistance, additional therapeutic options are needed [3].

Acute skin lesions common to AD patients often exhibit infiltration of immune cells such as mast cells and eosinophils, which contributes to the inflammatory response [5]. In skin lesions of AD, inflammatory cytokines and chemokines are locally overexpressed. Cytokines such as TNF-α (in keratinocytes, mast cells, and dendritic cells) bind to receptors in vascular endothelial cells to activate cell signaling cascades. These processes cause inflammatory cells to leak out of the blood vessels and infiltrate into surrounding tissues, causing reactions with chemoattractant cytokines and chemokines. In particular, chemokine receptor 4 ligands such as TARC/CCL17 and MDC/CCL22 promote inflammatory infiltration of Th2 lymphocytes and ultimately increase IgE expression in lesioned tissues. Severe levels of immune cell infiltration can be detected in mouse models of AD [6].

The soybean plant is native to Manchuria, the area north of the Korean Peninsula, and is now widely cultivated around the world. Soy-based foods have been popular in Asian countries for both their nutritional and medical benefits. Soybean is a high-quality protein source that contains several key bioactive compounds [7, 8], including isoflavones, which are associated with beneficial effects on human health. These include anticarcinogenic properties and estrogen-like effects arising from their diphenolic structure [9, 10]. Daidzein and genistein are isoflavones and are well known as bioactive components of soybean. 7,3′,4′-THIF features an additional -OH group added to daidzein in the ortho position on the B ring (▶ **Fig. 1 a, b**). In previous studies, we observed that anticancer efficacy increases when there are two or more OH groups




▶ **Fig. 1** Chemical structure of 7,3′,4′-THIF (**a**) and daidzein (**b**).

in the B ring of flavonoids [11, 12] and subsequently developed a technique to attach an additional OH to the ortho position of OH in the beta phenol ring (regioselective o-hydroxylation) with the use of a modified tyrosinase [13]. The resultant 7,3′,4′-THIF can be generated very efficiently by attaching an OH group to daidzein and may have applications as a cosmeceutical and functional food material.

In our previous study, oral administration of 7,3′,4′-THIF in mice resulted in the alleviation of AD symptoms. However, oral administration of 7,3′,4′-THIF is thought to likely be significantly impacted by digestion, absorption, metabolism, and other processes. For this reason, topical formulations of 7,3′,4′-THIF treated directly to the skin of AD patients may be more effective. We sought to investigate whether 7,3′,4′-THIF has therapeutic effects when applied topically.

## Results

To investigate the topical effect of 7,3′,4′-THIF on AD symptoms, a DFE-induced NC/Nga mouse model was used. In comparison to the untreated control group, the DFE-induced group exhibited AD-like symptoms, including erythema, excursion, keratinization, and dryness. Topical application of 800 nmol or 0.1 % dexamethasone significantly lowered AD symptoms in comparison to the control group (▶ **Fig. 2 a**). The 200 and 800 nmol 7,3′,4′-THIF treatment groups showed improvements on their dermatitis score (▶ **Fig. 2 b**). Also, the 200 and 800 nmol 7,3′,4′-THIF groups showed a reduction in the duration of scratching of the skin (▶ **Fig. 2 c**), and 800 nmol treatment with 7,3′,4′-THIF improved skin moisture reduced by DFE (▶ **Fig. 2 d**).

Dorsal skin samples were prepared and stained with H&E (▶ **Fig. 3 a, b**). The external application of 7,3′,4′-THIF reduced epidermal thicknesses in the NC/Nga atopic mouse model. Epidermal thickness of NC/Nga mice was also increased in the DFE-induced groups, while it was reduced in the 7,3′,4′-THIF and dexamethasone groups. In contrast, there were no significant changes in the daidzein group (▶ **Fig. 3 a, b**). To investigate whether topical application of 7,3′,4′-THIF suppresses the infiltration of eosinophils and mast cells in DFE-induced skin lesions, dorsal skin tissue samples were stained with CR and TB. The number of eosinophils in the skin lesions significantly increased in the DFE-induced group compared to the control group. The number of eosinophils in the 7,3′,4′-THIF and 0.1 % dexamethasone groups was dramatically reduced (▶ **Fig. 3 c, d**). In addition, the number of mast cells in the skin lesions also increased in the DFE-induced group compared to control group. The greatest reduction in mast cell

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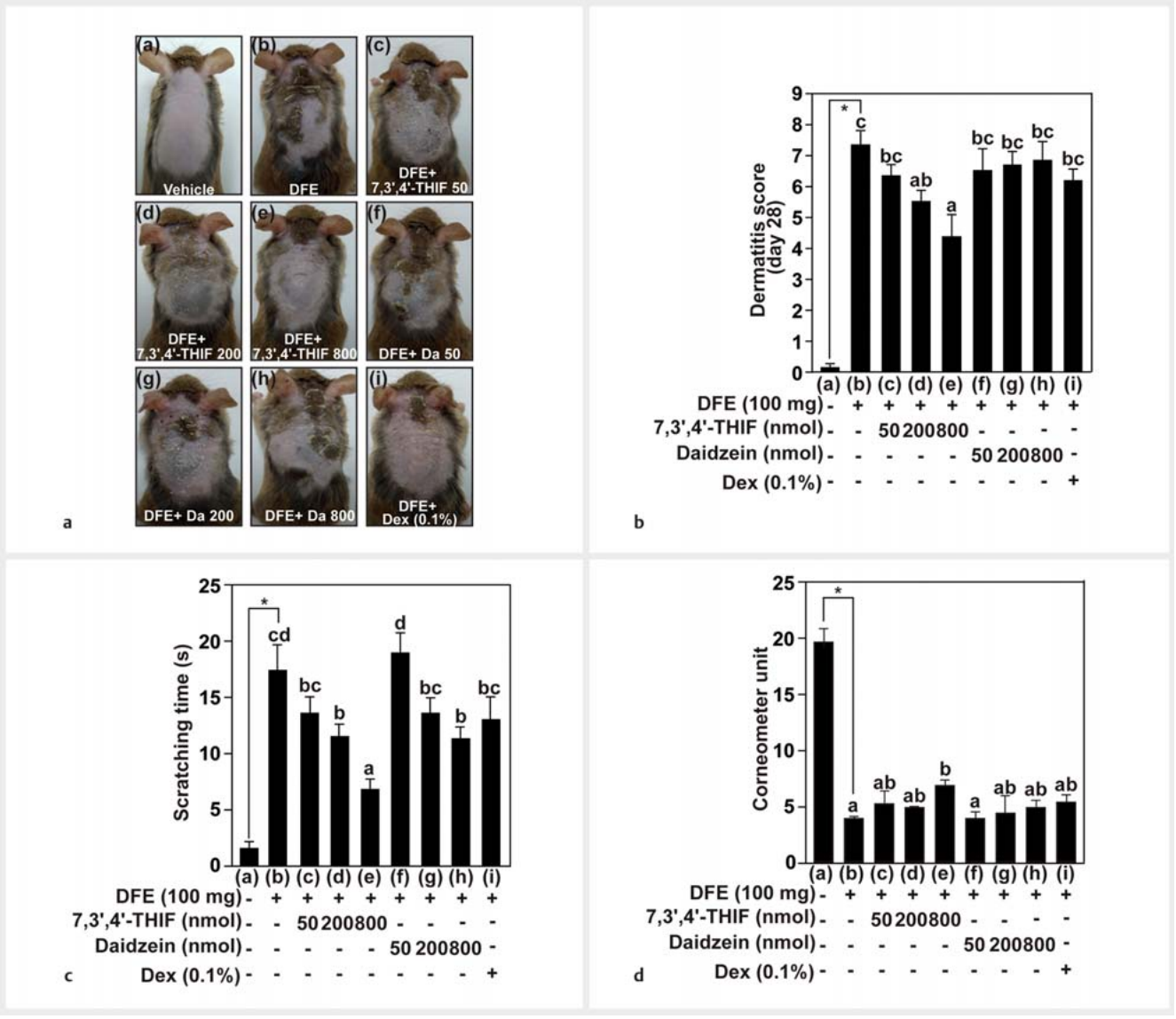


▶ **Fig. 2** Effect of 7,3′,4′-THIF on DFE-induced AD symptoms in NC/Nga mice. **a** Photo of experiment on the final day. **b** Dermatitis score on the final day. Data represent means ± SEM (n = 6–8). **c** Scratching time was assessed using recorded video. **d** Corneometer unit represents skin hydration. Data represent the mean values ± SEM (n = 8). Mean values within a graph are significantly different from each other at $p < 0.05$. Bars with the same letter are not different from each other. *Means are significantly different between the untreated control and DFE-treated groups at $p < 0.05$.

infiltration was observed in the 7,3′,4′-THIF and 0.1% dexamethasone groups, which occurred in a dose-dependent manner (▶ **Fig. 3e, f**).

To investigate the effect of 7,3′,4′-THIF in MDC as well as IgE levels in DFE-induced NC/Nga mice, serum samples were collected on the final day of the experiment (day 21). Serum MDC/CCL22 levels in the DFE-induced group also increased compared to the control group. Serum MDC levels of 7,3′,4′-THIF in the dexamethasone groups were lower than in the DFE-induced group (▶ **Fig. 4a**). Serum IgE levels in the DFE-induced group dramatically increased compared to that of the control group. In addition, topical application of 7,3′,4′-THIF elicited a significant reduction in IgE levels (▶ **Fig. 4b**).

To elucidate the mechanism of action of 7,3′4′-THIF, HaCaT cells (human skin keratinocytes) were used. TNF-α/IFN-γ was treated with TARC/CCL17 and MDC/CCL22, cytokines that play an important role in AD. 7,3′4′-THIF was treated to observe the expression level of these cytokines. HaCaT cells were treated with 7,3′4′-THIF and daidzein, followed by a TNF-α/IFN-γ cocktail. Production levels of TARC/CCL17 and MDC/CCL22 were measured by an ELISA assay. The results showed that 7,3′4′-THIF decreased TARC/CCL17 and MDC/CCL22 more than daidzein in the HaCaT cells (▶ **Fig. 5b, c**) at 20 µM of 7,3′4′-THIF, a concentration that does not cause cell toxicity (▶ **Fig. 5a**).

To identify the mechanism of action of 7,3′4′-THIF in the AD model, we examined changes in TNF-α/IFN-γ-induced signal





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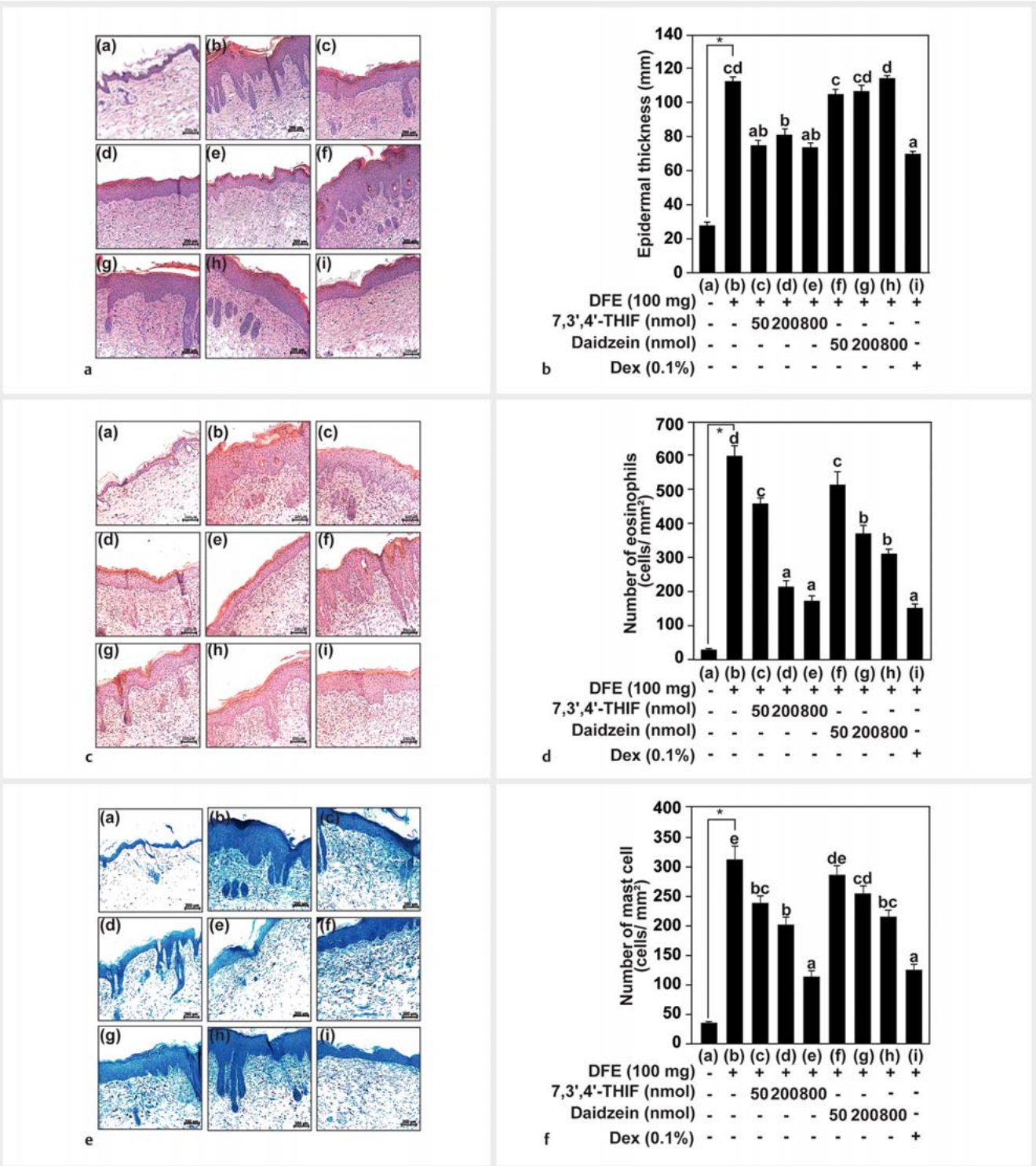


▶ **Fig. 3** Effect of 7,3′,4′-THIF on DFE-induced epidermal thickness and infiltration of eosinophils and mast cells into skin lesions in NC/Nga mice. **a, b** Images of epidermal and dermal thickness stained with H&E. Data represent the mean values ± SEM (n = 8). **c, d** Skin image stained with CR. The number of eosinophils within 1 $mm^2$ skin lesion sections were measured. Data represent the mean values ± SEM (n = 8). **e, f** Skin image stained with TB. The number of mast cells within 1 $mm^2$ skin lesion sections were measured. Data represent the mean values ± SEM (n = 8). Skin lesions were evaluated under a microscope at 400 × magnification. Scale bar: 200 µm. (a) Untreated control group; (b) DFE-treated group; (c) DFE plus 800 nmols of 7,3′,4′-THIF; (d) DFE plus 200 nmols of 7,3′,4′-THIF; (e) DFE plus 50 nmols of 7,3′,4′-THIF; (f) DFE plus 800 nmols of daidzein; (g) DFE plus 200 nmols of daidzein; (h) DFE plus 50 nmols of daidzein; (i) DFE plus 0.1 % dexamethasone. Mean values within a graph are significantly different from each other at $p < 0.05$. Bars with the same letter are not different from each other. *Means are significantly different between the untreated control and DFE-treated groups at $p < 0.05$.

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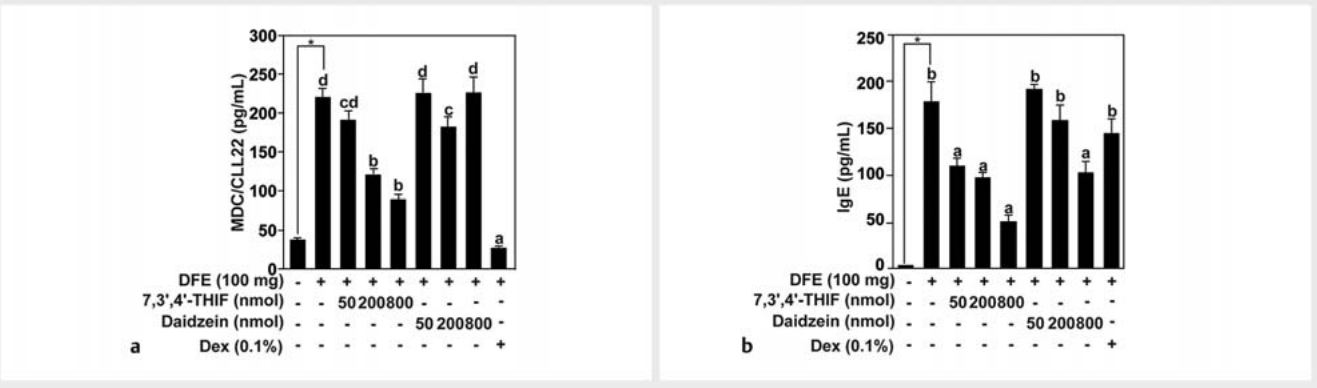
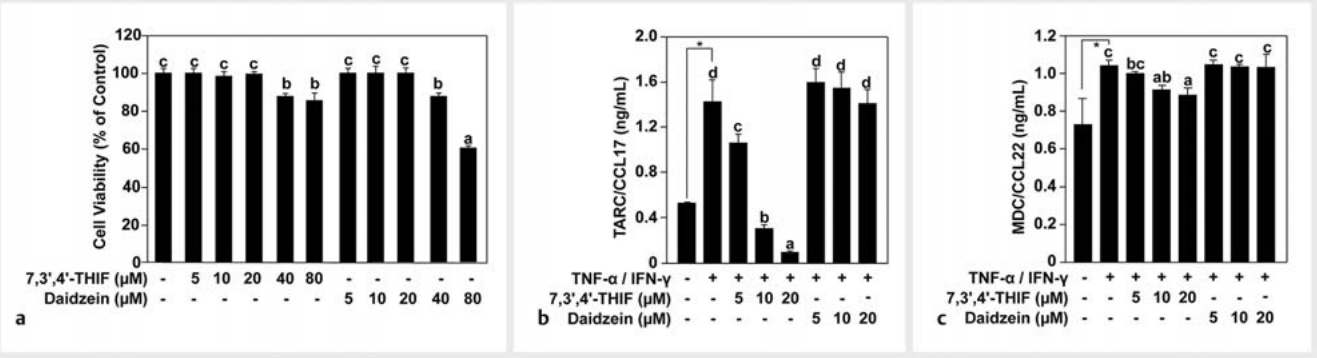



▶ **Fig. 4** Effects of 7,3′,4′-THIF on DFE-induced increases in MDC/CLL22 and IgE levels in serum. **a** Serum MDC levels in NC/Nga mice. Serum MDC levels were measured by ELISA. **b** Serum IgE levels in NC/Nga mice. Serum IgE levels were measured by ELISA. Data represent the mean values ± SEM (n = 6–8). Mean values within a graph are significantly different from each other at $p < 0.05$. Bars with the same letter are not different from each other. *Means are significantly different between the untreated control and DFE-treated groups at $p < 0.05$.




▶ **Fig. 5** Effects of 7,3′,4′-THIF and daidzein on AD-associated chemokine production in HaCaT cells. **a** Cell viability after treatment with 7,3′,4′-THIF and daidzein. Viability was measured using an MTT assay as described in Materials and Methods. **b, c** Cells were pretreated with a TNF-α/IFN-γ cocktail for 1 h before treatment with each sample at the indicated concentrations for 24 h. Cell media was collected and the levels of TARC/CCL17 and MDC/CCL22 were assessed by ELISA. Data represent the means ± SEM (n = 3). Cells were pretreated with 7,3′,4′-THIF and daidzein at the indicated concentrations for 24 h at 37 °C. Data (n = 4) represent the mean values ± SD. Mean values within a graph are significantly different from each other at $p < 0.05$. Bars with the same letter are not different from each other. *Means are significantly different between the untreated control and TNF-α/IFN-γ-treated groups at $p < 0.05$.

transduction. 7,3′4′-THIF inhibited TNF-α/IFN-γ-induced phosphorylation of ERK1/2 and JNK1/2 in HaCaT cells, whereas daidzein did not show any effects (▶ **Fig. 6a**). However, p38 was not inhibited by 7,3′4′-THIF (▶ **Fig. 6b**). The STAT/JAK pathway plays critical roles in TNF-α/IFN-γ-induced TARC/CCL17 and MDC/CCL22 upregulation in HaCaT cells [14–16]. Therefore, we investigated the effects of 7,3′4′-THIF on the TNF-α/IFN-γ-induced STAT/JAK pathway. 7,3′4′-THIF could not inhibit TNF-α/IFN-γ-induced phosphorylation of STAT1 and JAK2 (▶ **Fig. 6c**).

## Discussion

Soybean is widely regarded as a healthy ingredient in a myriad of foods, and is a prime source of protein for vegetarians. Soy contains a variety of phytochemicals that are beneficial to human health, the most representative of which are the isoflavones, although antigens in soybean are known to cause allergies. Interestingly, isoflavones have been shown to inhibit allergic reactions and inflammation in certain scenarios. We observed that 7,3′,4′-THIF is more effective than daidzein in this regard. 7,3′,4′-THIF features only one additional OH group than its parent molecule daidzein, however, a significant difference in efficacy can be observed. In our previous study, topical application of 7,3′,4′-THIF clearly suppressed the incidence and multiplicity of UVB-induced tumors in hairless mouse skin. 7,3′,4′-THIF inhibits Cot and MKK4 kinase activity directly, resulting in suppression of UVB-induced COX-2 expression. We have also reported that luteolin directly inhibits Cot [17] as well as ERK1/2 and JNK1/2 phosphorylation in the MAP kinase signaling pathway, which induces TNF by direct inhibition of Cot. However, the phosphorylation of p38 appears unaffected. In this study, TNF-/IFN-induced phosphorylation of ERK1/2 and JNK1/2 were inhibited by 7,3′4′-THIF, while p38 was unaffected. It appears that 7,3′,4′-THIF can inhibit Cot to influence downstream signal transduction.

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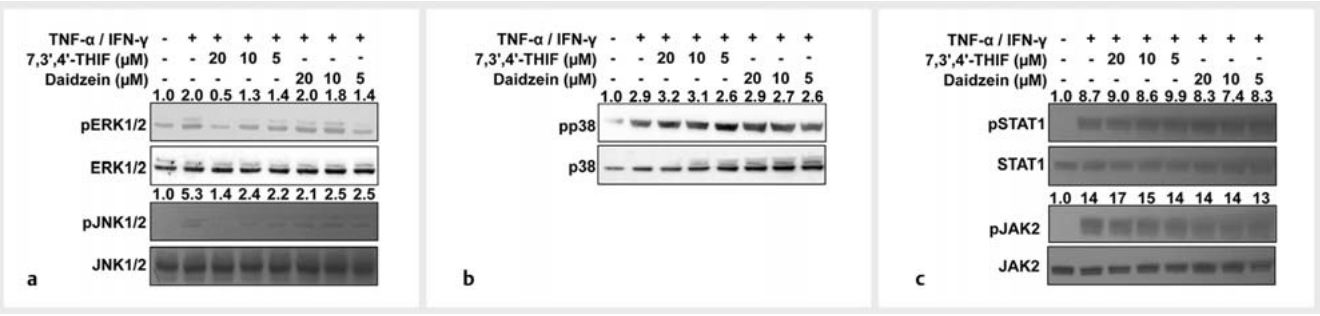


▶ **Fig. 6** Effects of 7,3′,4′-THIF on TNF-α/IFN-γ-induced signaling in HaCaT cells. Cells were pretreated with a TNF-α/IFN-γ cocktail for 1 h before treatment with 7,3′,4′-THIF or daidzein at the indicated concentrations for 30 min as described in Materials and Methods. **a** 7,3′,4′-THIF inhibits phosphorylation of ERK1/2 and JNK1/2. **b, c** 7,3′,4′-THIF could not inhibit phosphorylation of p38(b) and STAT1/JAK2 pathway (**c**). Western blot analysis was conducted using the specific antibodies as indicated. Protein band densities were measured using ImageJ and normalized to that of non-phospho form.

Prolonged topical steroid use is known to have serious adverse effects on other tissues and organs [4]. The HaCaT cell line consists of skin cells of the epidermis, which is the first skin tissue type to be damaged during AD-associated scratching. Th2 chemokines including TARC/CCL17 and MDC/CCL22 are involved in inflammatory cell infiltration and ultimately affect the expression of IgE. In this study, we demonstrated that 7,3′,4′-THIF reduces TNF-α/IFN-γ-induced TARC and MDC expression in HaCaT cells. The topical application of 7,3′,4′-THIF also reduces skin thickness and scratching in the NC/Nga atopic mouse model. Serum analysis showed that MDC/CCL22 and IgE levels were also reduced in the group treated with 7,3′,4′-THIF. In the case of dexamethasone, serum MDC/CCL22 was significantly reduced, while IgE levels showed a relatively modest reduction compared to 7,3′,4′-THIF. Skin histological analysis revealed that the inflow of mast cells and eosinophils was reduced in the 7,3′,4′-THIF group.

In our previous study, oral administration of 7,3′,4′-THIF reduced DFE-induced AD symptoms. The consumption of isoflavones renders them subject to metabolization in the body and they are rapidly converted into metabolites [18]. The topical approach may therefore be more suitable for the development of cosmeceuticals and therapeutic ointments to prevent AD. As we have developed a method to efficiently produce 7,3′,4′-THIF in large quantities, ease of manufacturing is another advantage.

In conclusion, we have shown that topical application of 7,3′,4′-THIF clearly suppresses DFE-induced AD symptoms in NC/Nga mice. Following verification in clinical trials, 7,3′,4′-THIF may be used as a cosmeceutical ingredient in topical formulations to prevent and treat AD.

## Materials and Methods

### Chemicals and reagents

7,3′,4′-THIF was provided by Dr. Byung Gee Kim (Seoul National University). 7,3′,4′-THIF was converted from daidzein via an enzyme conversion process [19], and was generated after purification, elution, and drying steps. The purity of 7,3′,4′-THIF was measured by HPLC at more than 98%. All samples (7,3′,4′-THIF, daidzein, dexamethasone) were dissolved in PEG300 and 1,3-Butyleneglycol (7 : 3) for animal treatment and in DMSO for cell treatment. Dexamethasone was obtained from Sigma-Aldrich. DMEM was purchased from Welgene. FBS was obtained from Sigma-Aldrich. MTT powder was purchased from USB Co. Penicillin-streptomycin solution was purchased from Mediatech, Inc. Protein assay reagent kits were obtained from Bio-Rad Laboratories. Cosmetic solvents for topical application were purchased from Shinjin Cosmetic Co. DFE-AD cream was purchased from Biostir Inc. Phospho-antibodies were purchased from Cell Signaling Technology, Inc., and unphosphorylated antibodies were purchased from Santa Cruz Biotechnology.

### Animal experiments

Three-week-old NC/Nga male mice were purchased from SLC Japan. The animals were housed in individual ventilated cages under specific pathogen-free conditions at 22 °C with a 12-h light-dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University (approved on June 9th, 2016, SNU-160408-7), Korea. After 1 week of acclimation, mice were randomly divided into the following nine groups (n = 8 per group): (1) control (non-induction) + vehicle (PEG300:1,3-BG 7 : 3); (2) DFE 100 mg + vehicle (PEG300:1,3-BG 7 : 3); (3) DFE + 7,3′,4′-THIF 800 nmols in vehicle; (4) DFE + 7,3′,4′-THIF 200 nmols in vehicle; (5) DFE + 7,3′,4′-THIF 50 nmols in vehicle; (6) DFE + daidzein 800 nmols in vehicle; (7) DFE + daidzein 200 nmols in vehicle; (8) DFE + daidzein 50 nmols in vehicle; and (9) DFE + 0.1% dexamethasone (510 nmols) in vehicle. To disrupt the skin barrier, one day after complete dorsal hair removal (approximately 4 $cm^2$), 200 μL of 4% (w/v) sodium dodecyl sulfate was topically applied to the shaved dorsal skin surface 1 h before DFE-AD cream application. To induce AD-like symptoms and skin lesions, DFE was applied to the dorsal skin and the back of both ears of the NC/Nga mice. DFE-AD cream was applied twice per week for 3 weeks (100 mg per mouse per application). All samples (7,3′,4′-THIF, daidzein, dexamethasone) were dissolved in vehicle (PEG300:1,3-BG 7 : 3) and were topically applied to the dorsal skin and back of both ears 4 h after DFE treatment, from 50 nmols to 800 nmols (500 μL



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each), five times per week for 3 weeks. At the end of the experiments, the animals were anesthetized with 2% isoflurane. Dorsal thickness was measured with Vernier calipers. Blood and dorsal skin samples were collected on the last day of the experiment (day 21) and stored until use. Serum MDC and IgE levels were measured using an enzyme-linked immunosorbent assay ELISA kit (R&D Systems) according to the manufacturer's instructions. To investigate epidermal thickness, H&E staining was performed. Mouse skin samples were fixed with 10% neutral-buffered formalin and embedded in paraffin. Serial sections (4 µm) were mounted onto slides. After deparaffinization, skin sections were rehydrated and stained with hematoxylin solution for 5 min. Slides were then washed and counterstained in eosin Y solution for 30 seconds. Next, the slides were dehydrated in 95% alcohol and washed in absolute alcohol, for 5 min each. Lastly, they were incubated in xylene overnight to remove water and then were dried. Skin sections were examined at 400× magnification using an Olympus AX70 light microscope. To detect eosinophil and mast cell infiltration, the dorsal skin of each mouse was prepared on the last day of the experiment (day 21) as described above. Deparaffinized skin sections were stained with CR and TB, respectively. The number of eosinophils and mast cells per 0.025 $mm^2$ skin was counted at 400× magnification. Tissue sections were examined using an Olympus AX70 light microscope.

### Assessment of dermatitis score

Mice images of the skin lesions were taken using a digital camera once time each week. Skin dermatitis scores were assessed five times in 4 weeks with a slight modification of criteria. Scores of 0 (none), 1 (mild), 2 (moderate), or 3 (severe) were given for each of the four symptoms (erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness). A total dermatitis score indication of clinical severity was defined as the sum of all scores (maximum: 12).

### Scratching behavior

Video of the scratching behavior was recorded using a digital camera one day before the final day. Scratching behavior of the nose, ears, and dorsal skin was recorded for 10 min and the total scratching time (sec) was analyzed.

### Corneometer units

Skin hydration was assessed by using a Corneometer CM 825 (Courage-Khazaka). This device measures the degree of skin moisture by electrical capacitance. The measurements were conducted five times for the same area. The corneometer units are arbitrary units.

### Cell culture and treatments

Human skin keratinocytes (HaCaT) were purchased from CLS Cell Lines Service GmbH. HaCaT cells were cultured at 37 °C in an atmosphere of 5% $CO_2$ in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin. Cell cytotoxicity was measured by the MTT assay. The cells were cultured in 96-well plates at a density of $5 \times 10^4$ cells/well and incubated at 37 °C in a 5% $CO_2$ atmosphere prior to serum deprivation for 24 h. Various concentrations of chemicals (7,3′,4′-THIF and daidzein) were added to the wells for 24 h.

### Measurement of thymus and activation-regulated chemokine and macrophage-derived chemokine levels

HaCaT cells were seeded at a density of $1 \times 10^6$ cells/well in 6-well plates for sandwich ELISA. After incubation for 48 h, the cells were stimulated with 10 ng/mL TNF-α/IFN-γ each. After 1 h, 7,3′,4′-THIF or daidzein was added for a further 24 h. Cell culture supernatant TARC and MDC levels were measured using ELISA kits according to the manufacturer's instructions.

### Western blot analysis

The cells were washed with PBS and harvested by scraping in cell lysis buffer. Protein concentrations were measured using a protein assay reagent kit (Bio-Rad) as described by the manufacturer. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). The membranes were blocked in 5% skim milk for 1 h, and then incubated with a specific primary antibody (phospho-ERK1/2 [1 : 1000], phospho-JNK1/2 [1 : 1000], phospho-p38 [1 : 1000], phospho-STAT1 [1 : 1000], phosphor-JAK2 [1 : 1000], beta-actin [1 : 5000]) at 4 °C overnight. Protein bands were visualized by a chemiluminescence detection kit (GE Healthcare) after hybridization with an HRP-conjugated secondary antibody (Life Technologies). Band intensities were quantified using ImageJ (National Institutes of Health).

### Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan's multiple range test, and p values of less than 0.05 were considered statistically significant.

## Contributors' Statement

Investigation: S. H. P., C. H. L., and J. Y. L.; Formal analysis: Y. H., J. H. K., and J. H. P.; Original draft preparation: J. E. K.; Funding acquisition: K. W. L.


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## Conflict of Interest

The authors declare that they have no conflict of interest.

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