



Topical application of an ethanol extract prepared from *Illicium verum* suppresses atopic dermatitis in NC/Nga mice

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ABSTRACT

Ethnopharmacological relevance: *Illicium verum* is a traditional herbal medicine with anti-inflammatory properties used in Asia. However, its usefulness in the treatment of allergic diseases remains unclear. This study evaluated the anti-inflammatory and antiallergic effects of *I. verum* extract (IVE) in a mouse model of atopic dermatitis.

Materials and methods: We investigated the effects of IVE on compound 48/80-induced histamine release, and phorbol 12-myristate 13-acetate and calcium ionophore A23187-stimulated cytokines secretion in MC/9 mast cells. Atopic dermatitis was induced in NC/Nga mice by exposure to extract of house dust mite (*Dermatophagoides farinae*). After a topical application of IVE on ear and skin lesions, we evaluated the severity of skin symptoms, ear thickness, inflammatory cell infiltration, and serum levels of immunoglobulin E (IgE), histamine, interleukin (IL)-6, and intercellular adhesion molecule (ICAM)-1. In addition, we determined the expression of IL-4, IL-6, tumor necrosis factor (TNF)- α , interferon (IFN)- γ thymus- and activation-regulated chemokine (TARC), regulated on activation, normal T cell expressed and secreted (RANTES), ICAM-1, and vascular cell adhesion molecule (VCAM)-1 in ear tissues.

Results: IVE inhibited secretion of histamine, IL-4, IL-6, and TNF- α from mast cells in a dose-dependent manner. Topical application of IVE significantly reduced dermatitis scores, ear thickness, and serum levels of IgE, histamine, IL-6, and ICAM-1. Histopathological analysis demonstrated decreased epidermal thickening and dermal infiltration by inflammatory cells. In the ear lesions, IVE treatment reduced expression of IL-4, IL-6, TNF- α , TARC, RANTES, ICAM-1, and VCAM-1, but not IFN- γ .

Conclusions: These results indicate that IVE inhibits atopic dermatitis-like skin lesions by suppressing the expression of cytokines, chemokines, and adhesion molecules. These results suggest that IVE may be a potential therapeutic candidate for atopic dermatitis.

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1. Introduction

Illicium verum Hook. f. (Illiciaceae), an aromatic evergreen tree commonly called star anise, is distributed primarily in eastern Asia. In addition to its culinary use, the fruit of *I. verum* is used in traditional Chinese medicine to treat stomachache, colic, vomiting, insomnia, skin inflammation, and rheumatic pain (Chouksey et al., 2010; Editorial Committee of Chinese Pharmacopoeia, 2010). It is also useful in dyspepsia, facial paralysis, asthma, and bronchitis (Warrier et al., 1996; Nadkarni, 2002). The fruits

contain tannins and essential oil that consists of anethole, α -pinene, limonene, β -phellandrene, α -terpineol, anisaldehyde, farnesol, shikimic acid, and safrole (Chouksey et al., 2010; Huang et al., 2010). The essential oil of *I. verum* is traditionally used as a topical antiseptic and rheumatism treatment (Verghese, 1988), and previous studies have suggested that the essential oil also exhibits insecticidal, antioxidant, and antimicrobial properties (De et al., 2002; Padmashree et al., 2007). The major chemical constituent of *I. verum*, *trans*-anethole, exerts anti-inflammatory and anticarcinogenic actions by inhibiting tumor necrosis factor (TNF)-induced cellular responses (Chainy et al., 2000). Another essential oil component, shikimic acid, is a primary ingredient of the antiviral drug Tamiflu, a remedy for the H5N1 avian influenza virus (Wang et al., 2011). In addition, anethole and limonene have been reported to exert anti-inflammatory effects by suppressing

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nitric oxide, prostaglandin E₂, and cytokines in lipopolysaccharide-stimulated RAW 264.7 macrophages (Conforti et al., 2010; Yoon et al., 2010). However, it has not yet been determined whether *I. verum* can suppress the development of allergic inflammation.

Atopic dermatitis (AD) is a chronic inflammatory skin disease that occurs most often in infants and children and is increasing in prevalence (Spergel and Paller, 2003). AD is characterized by elevated serum immunoglobulin E (IgE) levels, peripheral eosinophilia, and pruritic and relapsing eczematous skin lesions infiltrated by inflammatory cells such as T lymphocytes, monocytes/macrophages, eosinophils, and mast cells (Soter, 1989; Leung and Bieber, 2003). In response to antigen cross-linking of the IgE receptor or direct activation, mast cells release inflammatory mediators including histamine, proteases, chemokines, and cytokines (Galli et al., 1991). In the acute phase of AD, T helper 2 (Th2) cells secrete interleukin (IL)-4, IL-5, and IL-13, which are important in the onset and development of AD. Th1 cells secrete interferon (IFN)- γ , which contributes to pathogenesis during the chronic phase (Leung et al., 2004).

The inbred strain NC/Nga mouse was established in 1957 from Japanese fancy mice (Nishiki-Nezumi). When placed in conventional surroundings, these mice spontaneously develop AD, which is accompanied by frequent scratching, elevated IgE levels, and inflammatory cell infiltration into the skin lesions (Matsuda et al., 1997; Vestergaard et al., 1999). However, this mouse model does not show AD-like symptoms under specific pathogen-free conditions and thus requires exposure to stimulus for developing a cutaneous hypersensitivity reaction. Patients with AD are highly

sensitized to house dust mite allergens. Topical application of an extract of *Dermatophagoides farinae*, a major species of house dust mite, induces AD-like skin lesions in NC/Nga mice (Matsuoka et al., 2003; Gao et al., 2004; Yamamoto et al., 2007). Thus, we investigated the therapeutic effects of *I. verum* extract (IVE) on AD in NC/Nga mice exposed to the dust mite allergen.

2. Materials and methods

2.1. Preparation of IVE

Dried fruits of *I. verum* were purchased from Omniherb Co. (Yeoungcheon, Korea) and authenticated based on its macroscopic characteristics by the Classification and Identification Committee of the Korea Institute of Oriental Medicine (KIOM). A voucher specimen (HRA-60) was deposited in the herbarium of the Department of Herbal Resources Research at KIOM. The dried herb (300 g) was extracted twice with 70% ethanol (with a 2-h reflux), and the extract was concentrated under reduced pressure. The decoction was filtered, lyophilized, and stored at 4 °C. The yield of dried extract from starting crude materials was approximately 15.73% (wt/wt).

2.2. Reagents and cell culture

Compound 48/80, phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, L-glutamine, and 2-mercaptoethanol

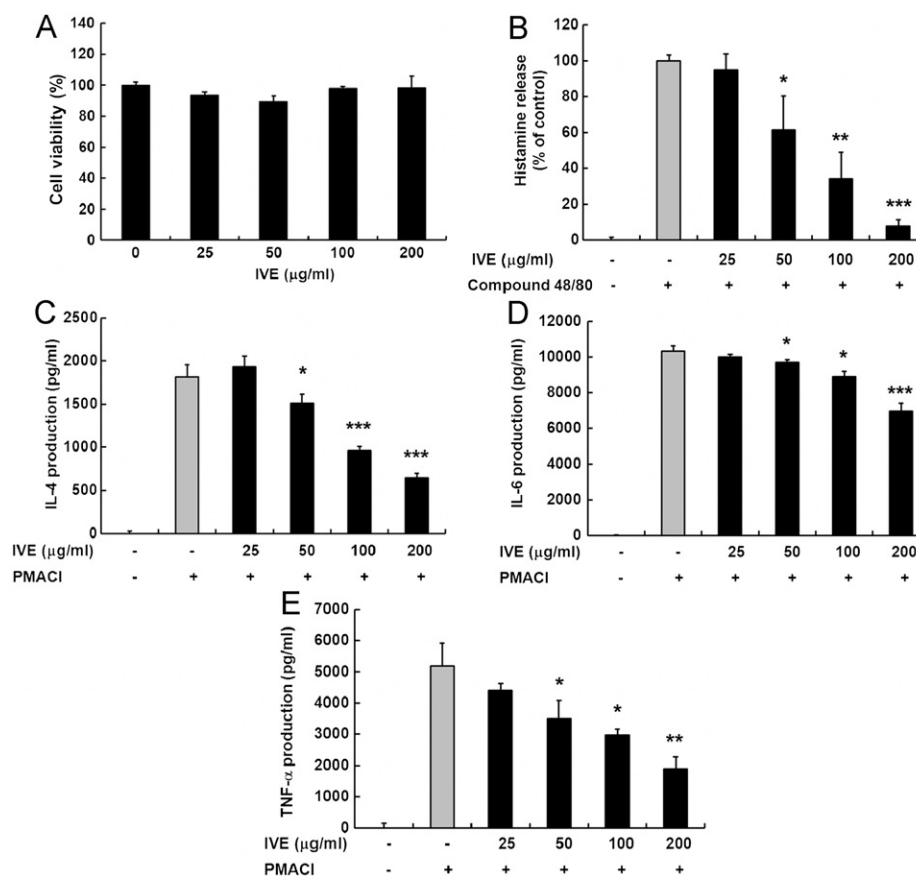


Fig. 1. Effects of *Illicium verum* extract (IVE) on compound 48/80-induced histamine release or PMACI-induced inflammatory cytokines production in mast cells: (A) cell viability. Cells were cultured in 96-well plates and stimulated with or without the extracts (0–200 µg/ml). After 24 h, cell viability was measured using the MTT assay. Production of (B) histamine, (C) IL-4, (D) IL-6, and (E) TNF- α in MC/9 cells was measured using an ELISA kit. MC/9 cells (2×10^5 cells/ml) were pretreated with IVE at 37 °C for 30 min prior to compound 48/80 (25 µg/ml) or PMA (20 µM) and A23187 (1 µM) stimulation. Values represent the means $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. compound 48/80- or PMACI-treated. PMA, phorbol 12-myristate 13-acetate; CI, calcium ionophore A23187.

were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). MC/9 mouse mast cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 10% Rat T-STIM (Becton Dickinson, Franklin Lakes, NJ, USA), and 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂.

2.3. Histamine and cytokines (IL-4, IL-6 and TNF- α) assay in mast cells

Histamine levels were measured using a histamine ELISA kit (Oxford Biomedical Research Inc.) according to the manufacturer's instructions. MC/9 mast cells were incubated with IVE (0–200 μ g/ml) for 30 min at 37 °C before the addition of compound 48/80 (25 μ g/ml) or PMA (20 nM) and A23187 (1 μ M), and incubated for additional times. The levels of IL-4, IL-6 and TNF- α in the culture supernatant of MC/9 cells were determined using a mouse ELISA kit (R&D Systems). The optical density of each well was measured using a Benchmark plus microplate spectrophotometer (BioRad, Hercules, CA, USA) (Fig. 1).

2.4. Animals

In this study, only male mice were used because female is able to show a heavy variation of population by hormonal control such as ovulation cycle, and female has few skin lesions as compared to male with mild skin lesions for AD experiments (Jiang et al., 2009; Funakushi et al., 2011). Male 8-week-old NC/Nga mice were purchased from SLC, Inc. (Hamamatsu, Japan) and housed in an air-conditioned animal room with a 12-h light/dark cycle at 22 $\pm$ 1 °C and 50 $\pm$ 10% humidity. Mice were provided with a standard laboratory diet and water ad libitum. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication no. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the KIOM.

2.5. Induction of atopic dermatitis in NC/Nga mice

Induction of AD-like skin lesions using mite antigen was performed as described previously (Sung et al., 2011). The schematic of the experimental procedure is shown in Fig. 2. Mice were anesthetized with ether, and the hair on the upper back of each mouse was removed with a clipper and a shaver 1 day before the experiments. Barrier disruption was achieved using 4% sodium dodecyl sulfate (150 μ l) on the shaved dorsal skin and both surfaces of each ear. After 3 h, the skin was treated with 100 mg ointment prepared from a crude extract of *D. farinae* (DfE)

(Biostir-AD, Biostir, Kobe, Japan); control mice were treated with the ointment base (hydrophilic petrolatum). Twice a week for 3 weeks, sodium dodecyl sulfate was applied to the skin, and then 100 mg DfE ointment or hydrophilic petrolatum was reapplied.

Mice were randomly divided into four groups: (1) normal controls that did not receive DfE application, (2) DfE-treated control group (100 mg/mouse), (3) DfE-treated mice that received 1000 μ g IVE, and (4) DfE-treated mice that received 1000 μ g Protopic ointment containing 0.1% tacrolimus (Astellas Pharma Inc., Deerfield, IL, USA) a positive control. The dose of IVE used in this study was determined from the prior experimental results of anti-allergic activity of herbal extracts (Lee et al., 2010; Sung et al., 2011). The IVE powder was dissolved in 70% ethanol solution. The same volume of vehicle was applied to the normal and DfE-treated control groups instead of IVE solution. IVE was applied daily for 23 days, beginning after the second DfE application (day 5). On the day of DfE application, IVE was applied 1 h before the antigen application. Ear thickness was measured twice a week using a micrometer (Mitutoyo Corporation, Kanagawa, Japan). Mice received the last IVE treatment on day 27 and were killed on day 28 to evaluate immunological and histological changes.

2.6. Evaluation of dermatitis severity

The severity of dermatitis on the ear and dorsal regions was evaluated twice a week. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores comprised the dermatitis score (Matsuda et al., 1997).

2.7. Measurement of serum IgE, histamine, IL-6, and intercellular adhesion molecule-1 levels

Serum total IgE levels were measured with a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi, Gunma, Japan) according to the manufacturer's instructions. Histamine levels were measured using a histamine ELISA kit (Oxford Biomedical Research Inc., Oxford, MI, USA). IL-6 and intercellular adhesion molecule (ICAM)-1 levels were measured with ELISA kits purchased from R&D Systems (Minneapolis, MN, USA).

2.8. Histopathological examination

Tissue specimens from the ear and dorsal skin of NC/Nga mice were removed, fixed in 10% formalin, embedded in paraffin, and serially sectioned at 2–3 μ m. Tissue sections were then stained with hematoxylin and eosin or toluidine blue. Histopathological changes were examined by light microscopy (Olympus CX31/BX51, Olympus Optical Co., Tokyo, Japan) and photographed (Olympus DP70, Olympus Optical Co.). Histopathology was scored as follows: no lesion, 0; minimal, 1; mild, 2; moderate, 3; and severe, 4. To determine mast cell infiltration, mast cells were counted in five randomly selected sites of toluidine blue-stained sections.

2.9. Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from mouse ear tissues using an easy-BLUE total RNA extraction kit (Intron, Seoul, Korea). Reverse transcription was carried out in a 20- μ l reaction with 2 μ g total RNA, Maxime RT premix (Intron), oligo-dT primers, and DEPC-treated water and incubated at 45 °C for 60 min. The reaction was terminated by heat inactivation at 95 °C for 5 min. Gene

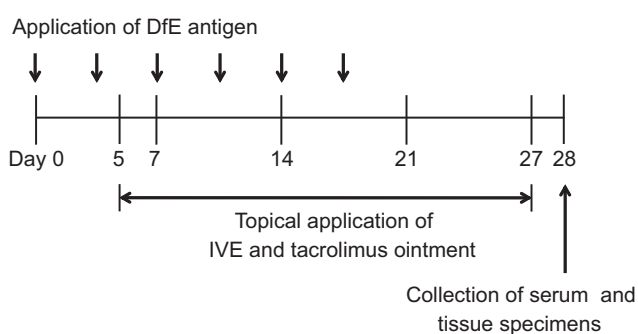


Fig. 2. Experimental induction of atopic dermatitis lesions. *Dermatophagoides farinae* extract (DfE) was applied to both ears twice per week for 3 weeks. After the second DfE application, *Illicium verum* extract (IVE) was applied to the ears every day for 23 days.

Table 1
Real-time RT-PCR primer sequences.

Genes	Forward	Reverse	Accession number	Length (bp)
IL-4	TCAACCCAGCTAGTTGTCA	CATCGAAAGCCGAAAGAG	NM_021283	313
IL-6	CCGGAGAGGAGACTTCACAG	TCCAGTTGGTAGCATCCATC	NM_031168	220
TNF- α	CCTGTAGCCACGTCGTAGC	TTGACCTCAGCGCTGAGTTG	NM_013693	373
IFN- γ	GCTACACACTGCATCTGGCTTG	CACCTCGGATGAGCTCATTGAATGC	NM_008337	404
TARC	CAGGAAGTTGGTGAGCTGGTATA	TTGTGTTCCGCTGTAGTGCATA	NM_011332	300
RANTES	GCTCCAATCTTGACGTCGTGT	ATTCTTGGGTTTCGTGGTCG	NM_013653	283
ICAM-1	CCTCTGCTCTGGCCCTGGT	CGGACTGCTGCTCCTCCCGA	NM_010492	237
VCAM-1	TCGCGGTCTTGGGAGCCTCA	TCGCGGTCTTGGGAGCCTCA	NM_011693	213
GAPDH	AAGCTGTGGCGTGATGGCCG	TGGGCCCTCAGATGCCTGCT	NM_008084	228

expression was quantified by real-time polymerase chain reaction (PCR) with SYBR green master mix (Qiagen, Tokyo, Japan). Amplification was carried out using the Rotor-gene 3000 (Corbett Research, Sydney, Australia) according to the following protocol: 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. Fold change in target gene expression relative to control mice was normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) according to the formula $2^{-\Delta\Delta C_t}$. Primers for target genes were designed using Primer 3 software (Table 1).

2.10. Quantitative high-performance liquid chromatography analysis

The sample was analyzed by reverse phase-high performance liquid chromatography using Waters Alliance 2695 system (Waters Co., Milford, MA, USA), coupled with a 2996 photodiode array detector. Data processing was carried out using Empower software (Waters Co.). Phenomenex Luna C18 column (250 mm $\times$ 4.6 mm; particle size 5 μ m, Phenomenex, Torrance, CA, USA) was used as stationary phase. The mobile phase consisted of (A) water and (B) methanol using a gradient elution of 60% A/40% B–20% A/80% B for 40 min. Ultraviolet (UV) detectors measured absorption over the range of 190–400 nm and recorded at 260 nm. The flow rate was 1.0 ml/min, and the injection volume was 10 μ l.

IVE was dissolved in 70% ethanol at concentration of 10 mg/ml, and standard stock solution containing the two components, *p*-anisaldehyde and *trans*-anethole, were prepared in 70% ethanol at concentration of 0.4 mg/ml for each of them. A serial dilution of standard stock solution resulting to 200 μ g/ml, 100 μ g/ml, 50 μ g/ml, 25 μ g/ml, and 12.5 μ g/ml were used for preparing calibration curve (Concentration vs. Area under curve). The amounts of *p*-anisaldehyde and *trans*-anethole present in IVE were quantified from the standard graph. The standard *p*-anisaldehyde and *trans*-anethole were purchased from ChromaDex (Irvine, CA, USA), and analytical solvents were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA).

2.11. Statistics

Results are expressed as mean $\pm$ standard deviation (SD). Mast cells counts were compared by one-way analysis of variance followed by Dunnett's *t*-test. All other data were compared by unpaired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of IVE on histamine release from mast cells

Cytotoxic effect of IVE was assessed at different concentrations (0–200 μ g/ml) by MTT assay in MC/9 cells. IVE did not affect cell

viability and was not toxic to MC/9 cells (Fig. 1A). We evaluated the ability of IVE to inhibit release of compound 48/80-induced histamine from MC/9 cells. Treatment of IVE inhibited dose-dependently compound 48/80-induced histamine release at concentrations of 50–200 μ g/ml (Fig. 1B).

3.2. Effects of IVE on secretion of inflammatory cytokines from mast cells

We evaluated the effect of IVE on secretion of inflammatory cytokines (IL-4, IL-6 and TNF- α) in PMACI-stimulated MC/9 cells. Stimulation of cells with PMACI during 7 h induced the secretion of cytokines. Treatment of IVE inhibited the secretion of IL-4, IL-6 and TNF- α in PMACI-stimulated MC/9 cells in a dose-dependent manner (Fig. 1C–E). These results show that IVE suppresses the production of inflammatory mediators in mast cells in vitro.

3.3. Effects of IVE on dust mite allergen-induced NC/Nga mice

To establish a dust mite allergen-induced AD model, DfE was applied twice per week to the dorsal skin of NC/Nga mice. Skin conditions were evaluated twice per week using the dermatitis severity score. Fig. 3A–C show dermatitis scores, ear thickness, and clinical features of the treatment groups. Dermatitis severity scores increased rapidly in the DfE-treated control mice and were significantly higher than those of normal controls after 4 days. However, dermatitis severity was significantly lower in IVE-treated mice after 7 days compared with DfE-treated controls (Fig. 3A). Ear swelling increased gradually in DfE-treated control mice (Fig. 3B), but the DfE-induced ear swelling was reduced by IVE. Repeated application of DfE induced skin dryness, followed by erythema, hemorrhage, edema, scarring, erosion, and excoriation (Fig. 3C). However, IVE inhibited these symptoms of AD. Taken together, these results demonstrate that IVE suppresses DfE-induced AD in NC/Nga mice.

3.4. Histopathological features

Fig. 4 shows histopathological features of the ear and dorsal skin lesions. Epidermal thickening by squamous cell hyperplasia (acanthosis), inflammatory cell infiltration of the dermis, dermal fibrosis, crust formation, and ulceration were observed in the DfE-treated control mice (Fig. 4A and B). However, IVE inhibited these pathological changes. To evaluate DfE-induced mast cell infiltration, ear and skin sections were stained with toluidine blue. The number of mast cell in the dermis increased markedly in DfE-treated control mice compared with normal mice. This increase was significantly reduced by IVE treatment (Fig. 5A and B).

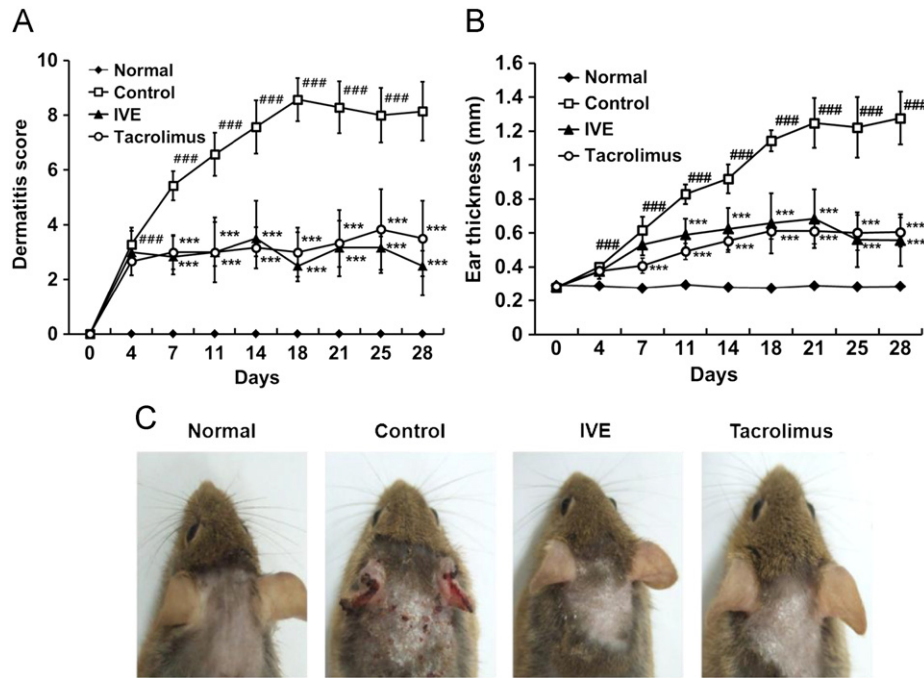


Fig. 3. Effects of *Illicium verum* extract (IVE) on *Dermatophagoides farinae* (DfE)-induced atopic dermatitis in NC/Nga mice. DfE was administered twice per week for 3 weeks: (A) dermatitis score and (B) ear thickness were determined twice per week. The total clinical skin severity score was the sum of the scores for erythema, hemorrhage, scarring, dryness, edema, excoriation, and erosion graded as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). (C) Representative photographs of clinical features on day 28. Results are expressed as mean $\pm$ SD ($n=6$). Normal, untreated; Control, DfE-treated; Tacrolimus, tacrolimus-treated; IVE, *Illicium verum* extract-treated group. ### $p < 0.001$ vs. Normal; *** $p < 0.001$ vs. Control.

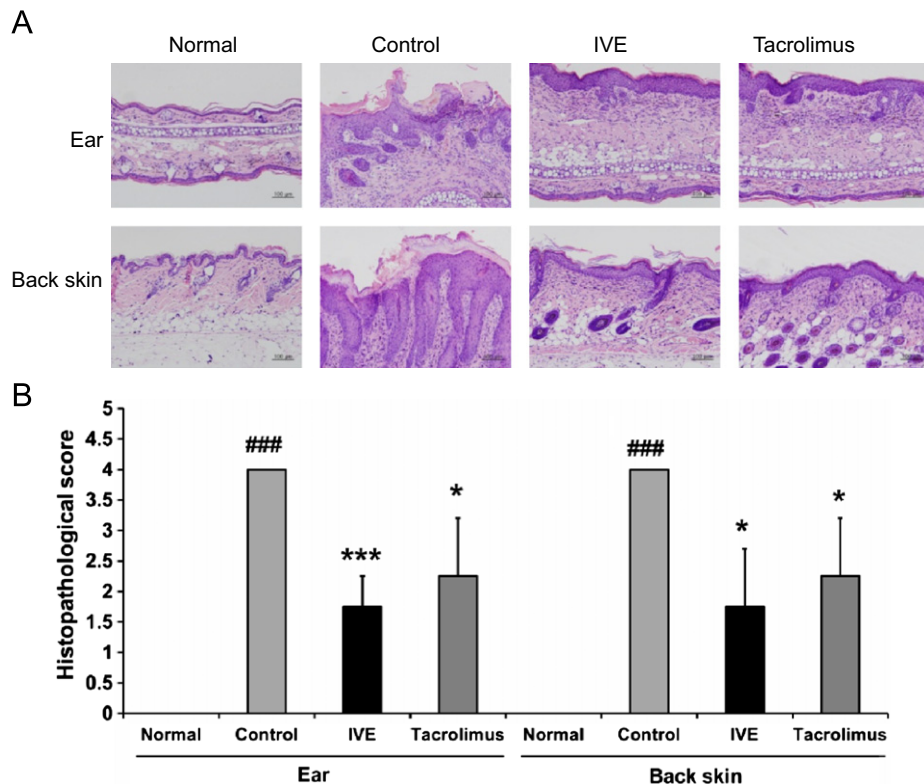


Fig. 4. Histopathological features of ear and skin lesions in NC/Nga mice. Tissues were excised, fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The sections were stained with (A) hematoxylin and eosin, and (B) histopathology was scored as described in Section 2 (magnification $\times 100$). Results are expressed as mean $\pm$ SD ($n=6$). Normal, untreated; Control, DfE-treated; Tacrolimus, tacrolimus-treated; IVE, *Illicium verum* extract-treated group. ### $p < 0.001$ vs. Normal; * $p < 0.05$, *** $p < 0.001$ vs. Control.

3.5. Effects of IVE on serum IgE, histamine, IL-6, and ICAM-1 levels

An elevated serum level of IgE is a hallmark of AD; therefore, we tested whether IVE could control serum levels of IgE in DfE-treated

NC/Nga mice. On day 28, the total IgE level in normal controls was 104.56 ± 73.99 ng/ml. DfE treatment increased this level by approximately 20-fold (2050.25 ± 459.69 ng/ml). Treatment with IVE decreased DfE-induced total IgE levels by approximately 4-fold

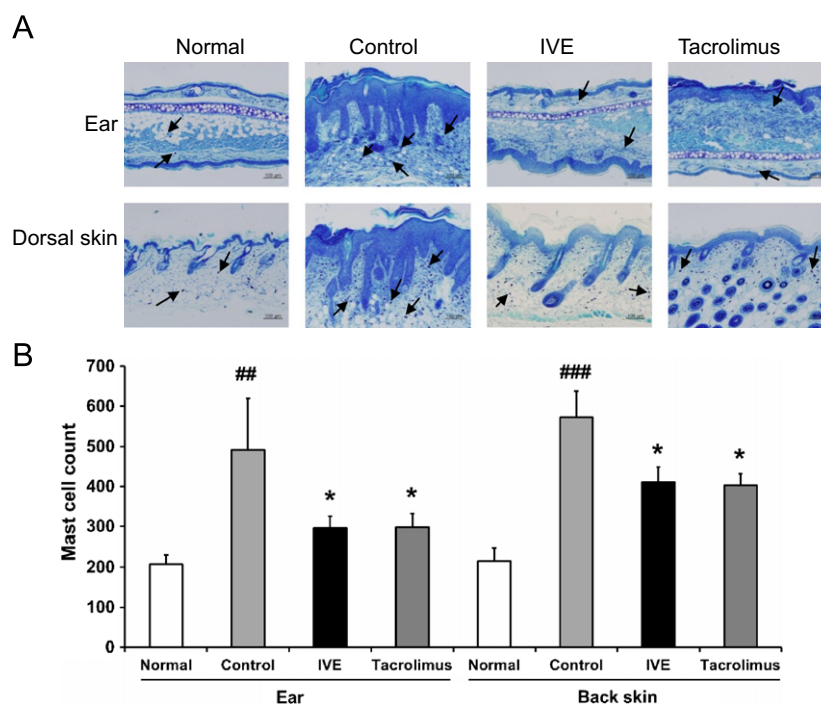


Fig. 5. Mast cells in ear and skin lesions of NC/Nga mice. Tissues were excised, fixed in 10% formaldehyde, embedded in paraffin, and sectioned: (A) sections were stained with toluidine blue, and (B) mast cells were counted under a microscope ($\times 100$). Results are expressed as mean $\pm$ SD ($n=6$). Normal, untreated; Control, DfE-treated; Tacrolimus, tacrolimus-treated; IVE, *Illicium verum* extract-treated group. ^{##} $p < 0.01$, ^{###} $p < 0.001$ vs. Normal; ^{*} $p < 0.05$ vs. Control.

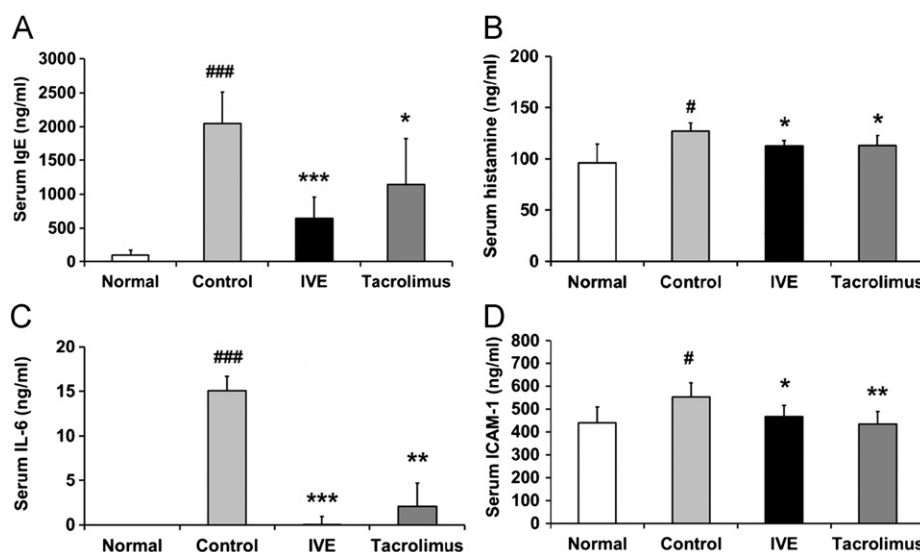


Fig. 6. Effect of *Illicium verum* extract (IVE) on serum levels of IgE, histamine, IL-6, and ICAM-1 in NC/Nga mice. Serum was collected 24 h after the last IVE treatment: (A) serum total IgE, (B) histamine, (C) IL-6, and (D) ICAM-1 levels were determined by ELISA. Results are expressed as mean $\pm$ SD ($n=6$). Normal, untreated; Control, DfE-treated; Tacrolimus, tacrolimus-treated; IVE, *Illicium verum* extract-treated group. [#] $p < 0.05$, ^{###} $p < 0.001$ vs. Normal; ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. Control.

(Fig. 6A). In contrast, levels of IgG subtypes (IgG1 and IgG2a) did not differ significantly between IVE-treated mice treated DfE-treated controls (data not shown). Taken together, these results suggest that IVE suppresses the development of dermatitis by decreasing IgE levels.

Histamine release from mast cell granules was significantly higher in DfE-treated controls than in normal mice, but treatment with IVE significantly reduced DfE-induced histamine levels (Fig. 6B). Similarly, the 28-day DfE treatment increased serum IL-6 and ICAM-1 levels compared with normal mice, but IVE attenuated this increase (Fig. 6C and D).

3.6. Expression of cytokines, chemokines, and adhesion molecules in ear lesions

We investigated the effects of IVE on mRNA levels of cytokines, chemokines, and adhesion molecules, important markers of AD, in ear lesions. An increase in mRNA levels of Th2 cytokine (IL-4), and inflammatory cytokines (IL-6, TNF- α), and Th1 cytokine (IFN- γ) was observed after DfE application (Fig. 7). IVE treatment attenuated the DfE-induced upregulation of IL-4, IL-6, and TNF- α , but not IFN- γ . DfE-induced expression of adhesion molecules (ICAM-1 and VCAM-1) and chemokines (TARC and RANTES) was also attenuated by IVE. These

results suggest that IVE prevents skin inflammation caused by inflammatory cell infiltration by downregulating the expression of cytokines, chemokines, and adhesion molecules.

3.7. Quantification of IVE components by HPLC

The present study evaluated the antiallergic effect of IVE, standardized with respect to the two markers, *p*-anisaldehyde and *trans*-anethole appeared as major peaks in the HPLC/PDA analysis. To establish the fingerprint chromatogram and quantify the marker components, an optimized HPLC/PDA analysis technique was employed. According to the plot of peak-area ratio (*y*) vs. concentration (*x*, µg/ml), the regression equations (coefficient of correlation) for *p*-anisaldehyde and *trans*-anethole were $y = 38926x - 34.91$ ($R^2 = 0.9998$) and $y = 75143x - 145.43$ ($R^2 = 0.9998$), respectively. IVE contained 6.14 ± 0.05 mg/g anisaldehyde and 1.98 ± 0.03 mg/g anethole, identified at a retention time of approximately 12.4 min and 36.8 min, respectively (Fig. 8).

4. Discussion

The results of this study demonstrate that IVE significantly inhibited the release of histamine, IL-4, IL-6, and TNF- α from

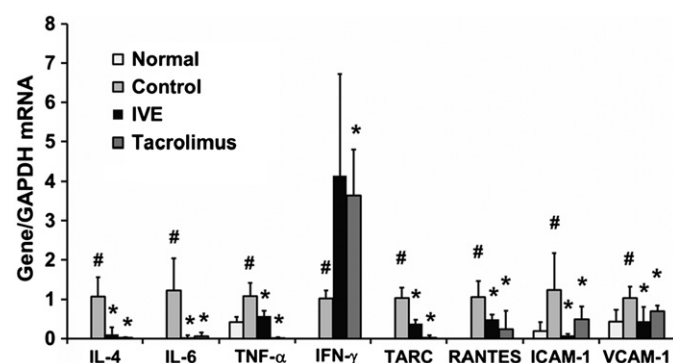


Fig. 7. Effects of *Illicium verum* extract (IVE) on *Dermatophagoides farinae* extract-induced inflammatory gene expression in NC/Nga mice. Total RNA was isolated from ear tissues and evaluated by real-time RT-PCR. Results are expressed as mean $\pm$ SD ($n=6$). Normal, untreated; Control, DfE-treated; Tacrolimus, tacrolimus-treated; IVE, *Illicium verum* extract-treated group. # $p < 0.05$ vs. Normal; * $p < 0.05$ vs. Control.

MC/9 cells in a dose-dependent manner. Furthermore, topical application of IVE to NC/Nga mice exposed to dust mite allergen markedly inhibited the development of AD-like skin lesions, as determined by dermatitis score, histopathologic analysis, serum levels of IgE and histamine, and levels of cytokines, chemokines, and adhesion molecules in serum and ear lesions. Taken together, these results demonstrate the therapeutic effects of IVE against AD.

Immediate-type allergic responses and chronic inflammation are associated with the release of histamines, lipid mediators, chemical mediators, and cytokines released by mast cells after activation by antigen-specific IgE antibodies bound to their Fc ϵ R1 receptors (Metcalf et al., 1997). Histamine release exerts a variety of effects related to the immediate phase of allergic inflammation, including vasodilation, increased vascular permeability, tissue erythema, and increased mucus production (Guo et al., 1997). The released cytokines promote the infiltration of inflammatory cells such as lymphocytes and eosinophils into the lesions. In allergic disease, Th2 cells mediate IgE-dependent mast cell activation and degranulation. IL-4 is a Th2 cell-secreted cytokine responsible for IgE class switching in B cells and differentiation of naive T-cells into Th2 cells (Bergstedt-Lindqvist et al., 1988, Kang et al., 2008). In contrast, the Th1 cell-secreted cytokine IFN- γ inhibits IgE synthesis and Th2 cell proliferation (Leung and Bieber, 2003). IL-6, which is produced by macrophages, dendritic cells, T and B lymphocytes, and keratinocytes in response to external stimuli such as IL-1 and TNF, promotes Th2 differentiation by upregulating IL-4 expression, and inhibits Th1 differentiation by upregulating suppressor of cytokine signaling (SOCS)-1 expression to interfere with IFN- γ signaling during CD4 $^{+}$ T cell activation (Diehl and Rincón, 2002). In this study, IVE inhibited compound 48/80-induced histamine release, and PMACI-stimulated cytokines (IL-4, IL-6 and TNF- α) production from MC/9 mast cells in a dose-dependent manner. In the mouse model of AD, IVE decreased serum levels of total IgE, histamine, and IL-6 and downregulated expression of IL-4, IL-6, and TNF- α , but not IFN- γ , in ear lesions. These results suggest that IVE attenuates the development of AD by inhibiting Th2 cell responses.

On the other hand, IFN- γ mRNA expression increased in IVE treated ear lesions, which is attributable to the decrease of IL-6. Since IFN- γ signaling is inhibited by IL-6 production (Diehl and Rincón, 2002), this can be confirmed by looking for signal

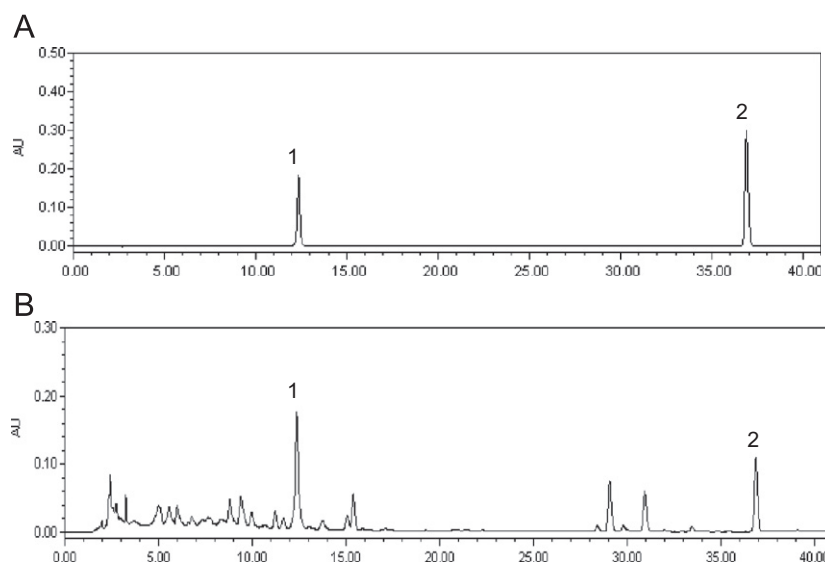


Fig. 8. HPLC chromatograms of (A) standard mixture and (B) 70% ethanol extract of *Illicium verum* at 260 nm: (1) *p*-Anisaldehyde; and (2) *trans*-anethole.

transducers and activators of transcription (STAT)1 and STAT3 phosphorylation in T-cells of mice. Further study is necessary to characterize the mechanism of action of IVE via *ex vivo* experimentation utilizing inflammatory cells extracted from mice of the four treatment groups.

Inflammatory cell infiltration is dependent on the local production of chemokines with leukocyte chemoattractant activity (Oshio et al., 2009). The chemokine receptor predominantly expressed on Th2 cells is CC chemokine receptor 4, and its ligands are CCL17/TARC and CCL22/macrophage-derived chemokine (Oshio et al., 2009), which are involved in Th2-mediated AD (Kakinuma et al., 2002; Shimada et al., 2004; Jahnz-Rozyk et al., 2005). In addition, increased levels of eotaxin and CCL5/RANTES in AD skin lesions promote the migration of eosinophils and mast cells via CC chemokine receptor 3 (Taha et al., 2000). In the present study, IVE significantly reduced TARC and RANTES expression in the ear lesions in our mouse model of AD.

ICAM-1 and VCAM-1 promote leukocyte infiltration in immune responses by mediating the adhesion and activation of these cells (Roebuck and Finnegan, 1999), and contribute to leukocyte accumulation in mice exposed to dust mite allergen (Kang et al., 2007). Expression of these adhesion molecules is induced by TNF- α at sites of inflammation (Ackermann and Harvima, 1998; de Vries et al., 1998). Because serum levels of soluble ICAM-1 are higher in patients with atopic bronchial asthma than normal control subjects (Lee et al., 1997), we examined serum levels of VCAM-1 and ICAM-1 and their expression in ear lesions. We found that IVE treatment decreased the DfE-induced upregulation of these molecules in our mouse model of AD. These results suggest that IVE inhibits leukocyte migration to sites of inflammation in AD by down-regulating the expression of chemokines and adhesion molecules.

Since human is the genetically heterogeneous population, these results from the NC/Nga mouse, homogeneous inbred strain, would be difficult to imply every human therapeutic usages. But the activity of IVE that modulates the Th1/Th2 imbalance by the inhibiting Th2 cell responses may play a role in their effectiveness for treating AD, thus IVE may be a potential therapeutic candidate for AD. In this study, we did not determine the components responsible for the antiallergic effect of IVE. Therefore, additional studies are needed to identify the primary active components.

5. Conclusion

The topical application of IVE inhibits the development of AD skin lesions and decreases serum IgE and histamine levels. The inhibition of cytokines, chemokines, and adhesion molecules and the subsequent blockade of inflammatory cell infiltration appears to be responsible for the inhibitory effects of IVE. These results indicate that IVE may be useful to prevent or treat AD.

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