

Regular Article

Juniperus chinensis Fruits Attenuate Oxazolone- and 2,4-Dinitrochlorobenzene-Induced Atopic Dermatitis Symptoms in Mice

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Juniperus chinensis, commonly Chinese juniper, has been used for treating inflammatory diseases. This study aimed to investigate anti-atopic dermatitis (AD) effects of standardized *J. chinensis* fruits extract on murine oxazolone- and 2,4-dinitrochlorobenzene (DNCB)-induced models of AD. Ear swelling, epidermis thickening, and eosinophils infiltration in the oxazolone-mediated dermatitis of BALB/c mice were significantly reduced upon topical application of *J. chinensis* fruits 95% EtOH extract (JCE). Besides, transdermal administration of JCE to SKH-1 hairless mice inhibited the development of DNCB-induced AD-like skin lesions by suppressing transepidermal water loss and improving skin hydration. Decreased total serum immunoglobulin E (IgE) and interleukin (IL)-4 levels could be observed in atopic dorsal skin samples of JCE-treated group. According to the phytochemical analysis, JCE was found to contain isoscutellarein-7-O- β -D-xyloside, cupressuflavone, and amentoflavone as main compounds. Therapeutic attempts with the *J. chinensis* fruits might be useful in the treatment of AD and related skin inflammatory diseases.

Key words *Juniperus chinensis* fruit; atopic dermatitis; transepidermal water loss; skin hydration; interleukin 4; immunoglobulin E

Atopic dermatitis (AD), a chronic inflammatory skin disorder, is increasingly becoming a clinical problem in industrialized countries.¹⁾ The prevalence of AD among children and adolescents has especially increased during the last decade.^{2,3)} Atopic disease is triggered by a variety of allergic factors including irritants, food, and stress factors.⁴⁾ Patients with AD have immediate immunoglobulin E (IgE)-mediated hypersensitivity reactions.⁵⁾ Especially, intense pruritus or itching is the most common and burdensome dermatologic feature of AD, thus negatively impact on the health-related QOL in patients suffering from AD.^{5,6)}

AD can be categorized into two types: extrinsic type or allergic AD and the intrinsic type or non-allergic AD.⁷⁾ Extrinsic AD with high IgE levels is the classical type of AD, while the incidence of intrinsic AD with normal IgE values is approximately 20% of patients.^{7,8)} The cause of AD is still unknown, though altered skin barrier as well as immune dysregulation appear to play important role in the pathogenesis of disease.⁹⁾ An impaired epidermal skin barrier is a characteristic feature and causative factor for the extrinsic AD.¹⁰⁾ Skin barrier damage contributes to the high serum IgE level, reduced skin surface hydration, and enhanced transepidermal water loss in patients with extrinsic AD.^{10,11)} Therefore, improvement in skin barrier function with moisturizing agents has great potential as a pharmacological target in atopic diseases.

Juniperus chinensis L. (Cupressaceae), common name Chinese juniper, is an evergreen coniferous plant that mainly distributed in Northeast Asian area including Korea, China, and Japan.^{12,13)} The heartwood of *J. chinensis* has been traditionally used for common cold and inflammatory diseases such as urinary infection and rheumatic arthritis, while the fruits are

known to treat convulsion, hyperhidrosis, and hepatitis.^{14,15)} Analysis of the chemical composition of *Juniperus* fruits represents the main class of non-volatile metabolites is phenolics including phenolic acids and flavonoids.^{16–18)} Recent reports have shown that the aerial parts of *J. chinensis* exert various biological properties including anti-inflammatory, antitumor, and antimicrobial activities.^{13,19)} However, studies on the fruits of *J. chinensis* are comparatively few in number, and furthermore, there have not been any attempts to reveal its anti-atopic effect. In this study, we investigated the potential therapeutic effects of *J. chinensis* fruits EtOH extract (JCE) on both oxazolone- and 2,4-dinitrochlorobenzene (DNCB)-induced AD animal models. Histological and blood analysis were also performed to observe the cutaneous changes in JCE-treated AD mice.

MATERIALS AND METHODS

Plant Material and Extraction Fresh fruits of *J. chinensis* were collected from Muju (Jeollanam-Do) area in South Korea and identified by Prof. Eun Ju Jeong of Department of Agronomy and Medicinal Plant Resources, Gyeongnam National University of Science and Technology. A voucher specimen (PNU-0022) has been deposited in the Medicinal Herb Garden, Pusan National University. Dried fruits of *J. chinensis* (3.9 kg) were extracted with 95% EtOH and evaporated under reduced pressure to yield JCE (824.2 g).

Animals Six-week-old female BALB/c and SKH-1 hairless mice were purchased from the animal facility of Orient Bio Inc. (Seongnam, Republic of Korea) and housed in an air-conditioned animal room at a temperature of 25 $\pm$ 5°C and 55 $\pm$ 5% humidity. Mice were given access to a standard laboratory diet and water *ad libitum*. All animal experiments were conducted in accordance with the Guide for the Care and Use

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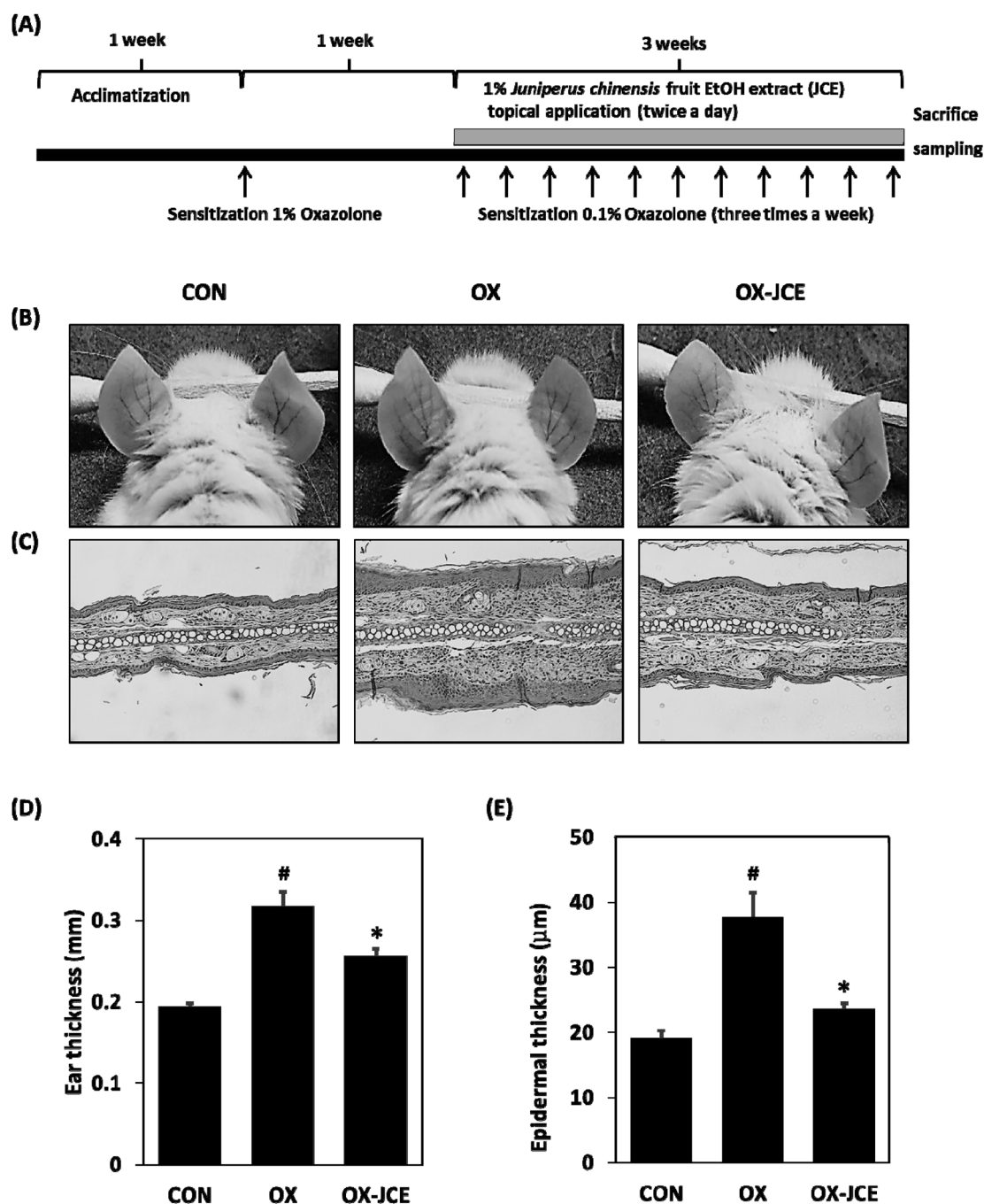


Fig. 1. Effects of JCE on the Development of Oxazolone-Induced AD-Like Symptoms in BALB/c Mouse Ears

CON: control group, OX: oxazolone-treated group, OX-JCE: oxazolone and 1% *J. chinensis* fruits EtOH extract-treated group. Schematic representation of the experiment (A); Clinical features of AD-like skin lesions (B); Histopathological features of skin lesions. Tissues were excised, fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E) (magnification, 100×) (C); Ear thickness and epidermal thickness (D). Results are expressed as the mean±standard error of the mean (S.E.M.) ($n=7$). The means±S.E.M. of two independent experiments are shown. [#] $p<0.05$ vs. control; ^{*} $p<0.05$ vs. DNCB-treated group.

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 KIST (Certification No. KIST-2016-011).

Induction of Topical AD-Like Skin Dermatitis in Mice by Oxazolone and Evaluation of Ear Swelling and Erythema after Treatment of JCE Ears of BALB/c mice were sensitized topically with 20 μ L of 1% 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) in a propylene glycol, EtOH solution (7:3) once on day 1 according to previous method.²⁰⁾ The experimental scheme is shown in Fig. 1A.

After the first challenge, 20 μ L of 0.1% oxazolone was repeatedly applied to both right and left ears for an additional 3 weeks at 2-d intervals. At the same time, the ears of the BALB/c mice were exposed to 20 μ L of JCE daily in the OX-JCE group for 3 weeks, and the application of 1% JCE was separated by 4 h from that of oxazolone. No substances were applied to the skin surface on the last day of the experiment. On the last day, measurements of skin inflammation signs, including ear swelling and erythema, were carried out.

Induction of AD-Like Skin Lesions in Mice by DNCB and Treatment with JCE AD-like skin dermatitis in

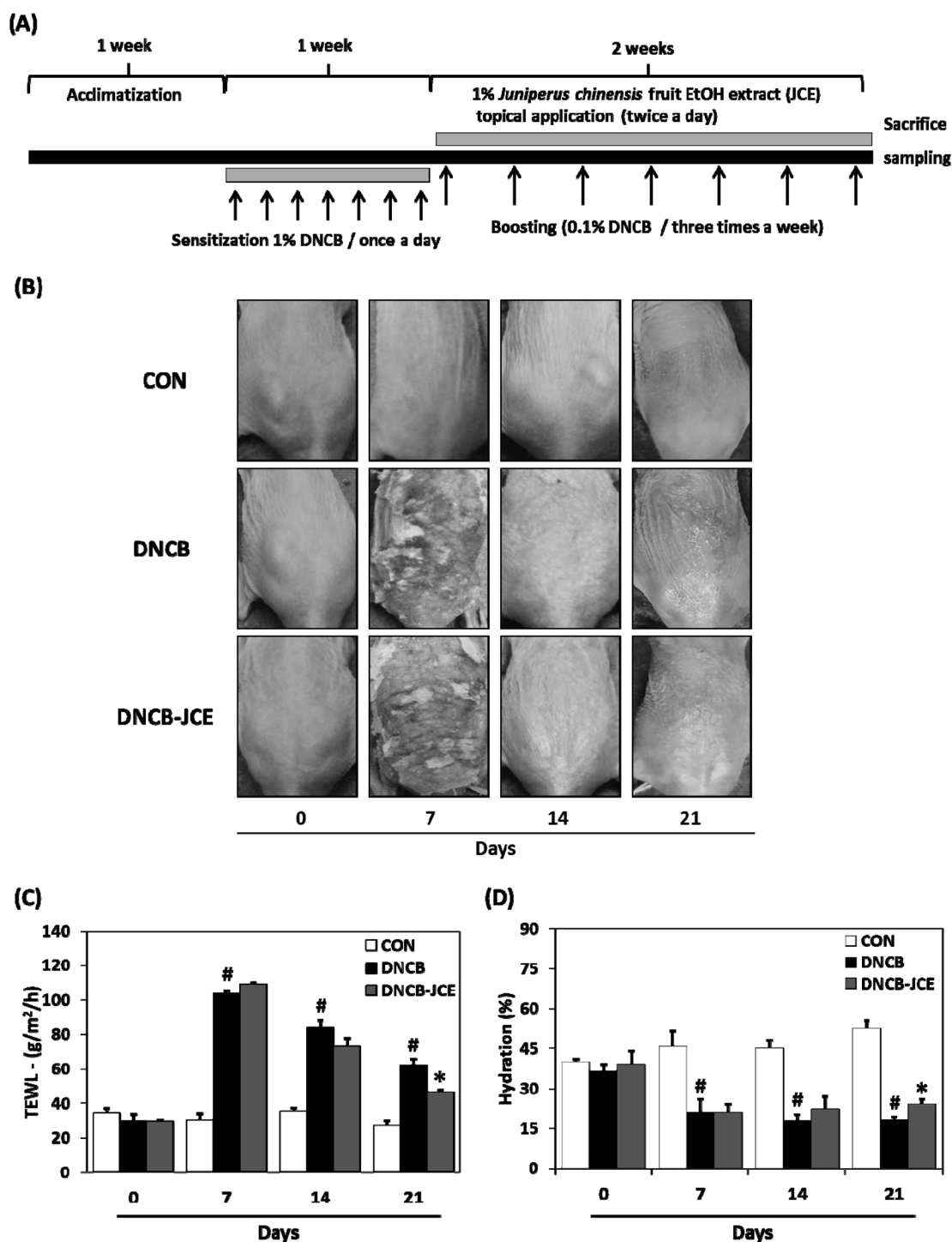


Fig. 2. Effects of JCE on the Development of DNCB-Induced AD-Like Symptoms in Hairless Mice

CON: control group, DNCB: DNCB-treated group, DNCB-JFE: DNCB and 1% *J. chinensis* fruits EtOH extract-treated group. Schematic representation of the experiment (A); Clinical features of AD-like skin lesions (B); The amount of transepidermal water loss (TEWL) (C); Estimation of skin hydration (D). The results are expressed as the mean $\pm$ S.E.M. ($n=7$). The means $\pm$ S.E.M. of two independent experiments are shown. [#] $p<0.05$ vs. control; ^{*} $p<0.05$ vs. DNCB-treated group.

SKH-1 hairless mice were induced by DNCB as previously described with small modification.^{21,22} The experimental scheme is shown in Fig. 2A. Briefly, the dorsal skin of hairless mice in both the DNCB-treated group and the DNCB and 1% JCE-treated (DNCB-JCE) group were sensitized once a day by painting 100 μ L of 1% DNCB daily. From day 7, the mice were challenged with 100 μ L of 0.1% DNCB for an additional 2 weeks at 3-d intervals. The DNCB-JCE group animals were painted with 100 μ L of 1% JCE for 2 weeks, and the applica-

tion of JCE was separated by 4 h from that of DNCB. The normal control animals (CON) were treated with distilled water alone. No substances were applied to the skin surface on the last day of the experiment. On the last day, mice were sacrificed, and dorsal skin and blood samples were collected for further analysis.

Measurement of Transepidermal Water Loss (TEWL) and Skin Hydration The severity of skin dermatitis in the SKH-1 hairless mice was evaluated by estimating erythema

(redness), TEWL, and skin hydration. Tewameter TM210 device (Courage and Khazaka, Cologne, Germany) and SKIN-O-MAT (Cosmomed, Ruhr, Germany) were used to evaluate the skin surface of the hairless mice according to the manufacturer's instructions. Skin hydration and TEWL were measured once per week after the twice daily application of JCE or vehicle.

Measurement of Serum IgE and Interleukin (IL)-4 Levels The blood samples were centrifuged at 10000rpm for 15 min at 4°C, and then serum was collected and stored at -80°C for further investigations. Total IgE and IL-4 concentration in mouse serum were measured *via* enzyme-linked immunosorbent assay (eBioscience, San Diego, U.S.A.) according to the manufacturer's instructions.

Histology For histologic examination, the ear or dorsal skin from mice were fixed in 10% formalin and processed for paraffin embedding. Tissue sections (2–3 mm) were then stained with hematoxylin and eosin. Histopathological changes were examined by light microscopy (Olympus CX31/BX51, Olympus Optical Co., Tokyo, Japan) and photographed (TE-2000U, Nikon Instruments Inc., Melville, U.S.A.).

Chromatographic Conditions The JCE sample was analyzed using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, U.S.A.) for phytochemical characterization. A Poroshell 120 EC-C18 column (3.0×100 mm, 2.7 μm, Agilent) was used for analysis at a flow rate of 0.3 mL/min. The mobile phase consisted of acetonitrile (solvent A) and water (solvent B), using a linear gradient elution: 5–95% A (0–10 min); 10% A (10–20 min). All acquisitions were performed under positive ionization mode. Mass spectra

were recorded across the range $m/z=100$ –1500 with accurate mass measurement of all mass peaks.

Statistical Analysis Data are expressed as the mean±standard deviation (S.D.) The values were expressed as percent changes from the mean value of the control experiment. Statistical analyses were performed by a one-way ANOVA using Statistical Package. *p* values less than 0.05 were considered statistically significant.

RESULTS

Effects of JCE on AD Symptoms Induced by Oxazolone in BALB/c Mouse Ears Symptoms of AD were elicited with the topical application of 1% oxazolone on both surfaces of each ear for 4 weeks. Oxazolone-treated mice exhibited an enhanced ear swelling compared with non-treated mice throughout the experimental period. Results revealed that 1% JCE was having remarkable effects in reducing swelling and erythematic intensity (Fig. 1B). In agreement with the phenotypic observation, histological findings demonstrated that JCE decreases skin thickening and the number of infiltrating lymphocytes as shown in Fig. 1C. Ear and epidermal thickening were also reduced by 50 and 79%, respectively, on day 28 compared with the oxazolone-treated group (Figs. 1D, E).

Effects of JCE on AD Symptoms Induced by DNCB in Hairless Mice The severity of dermatitis including erythema, dryness, and swelling increased rapidly in DNCB-treated mice. Severe skin barrier damage, such as increased TEWL and decreased hydration, were observed in the DNCB-treated control group. This AD-like skin disorder was reversed by

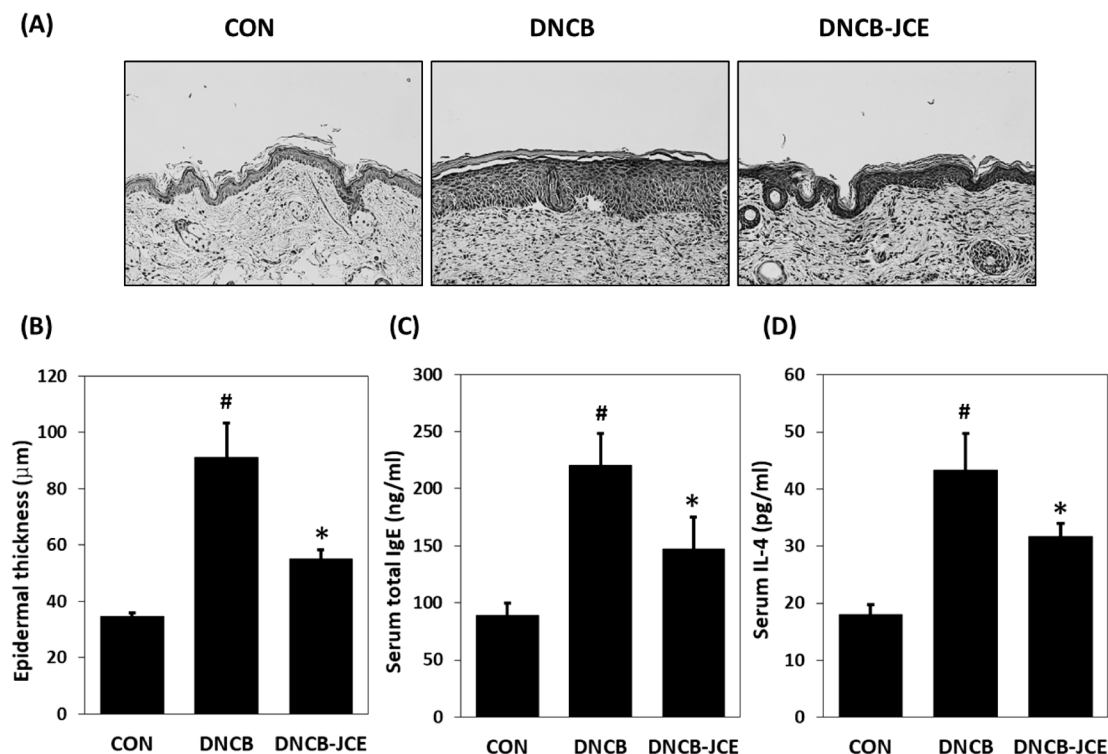


Fig. 3. Histopathological Analysis and Effects of JCE on Serum IgE and IL-4 Levels in DNCB-Induced AD Mice

CON: control group, DNCB: DNCB-treated group, DNCB-JFE: DNCB and 1% *J. chinensis* fruits EtOH extract-treated group. Histopathological features of skin lesions in JCE-treated DNCB-induced AD mice (A); Tissues were excised, fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E) (magnification, 100×). Dorsal skin epidermis thickness (B); Serum total IgE (C); IL-4 levels (D) were determined by enzyme-linked immunosorbent assay (ELISA). The results are expressed as the mean±S.E.M. (*n*=7). The means±S.E.M. of two independent experiments are shown. [#]*p*<0.05 vs. control; ^{*}*p*<0.05 vs. DNCB-treated group.

treatment with JCE (Fig. 2B). After 21 d of treatment, DNCB greatly increased TEWL to 44.7 J [g/(m²h)], whereas it was markedly reduced to 19.8 J [g/(m²h)] by the transdermal administration of 1% JCE (Fig. 2C). Consistent with this finding, the level of skin hydration in JCE-treated hairless mice was slightly higher than that in the DNCB-treated CON group (Fig. 2D).

Histopathological Features The histopathological features of the dorsal skin lesions from JCE-treated AD hairless mice are shown in Fig. 3A. Epidermal thickening by cell hyperplasia, slight spongiosis, and lymphocytes infiltration in the dermis were observed in DNCB-treated control mice. Dramatic increase in epidermis thickness of AD mouse model was significantly attenuated by treatment of 1% JCE. JCE inhibited the DNCB-induced epidermal hyperplasia by 72% in AD hairless mice. Inflammatory cell infiltration was also markedly reduced in DNCB-JCE group.

Effects of JCE on Serum IgE and IL-4 Levels After 21 d experiment, sensitization with DNCB elicited a 2.7-fold and 2.4-fold increase in total IgE and IL-4 concentrations, respectively. Treatment of JCE decreased the DNCB-induced serum IgE levels by 58% of DNCB-treated controls (CON: 82 ng/mL, DNCB: 223 ng/mL, and DNCB-JCE: 141 ng/mL) (Fig. 3C). Inhibitory effect of JCE on IL-4 levels in atopic disease mice induced by DNCB was also detected. The 1%

JFE application noticeably attenuated serum IL-4 concentrations (CON: 18 pg/mL, DNCB: 42 pg/mL, and DNCB-JCE: 30 pg/mL) in DNCB-induced AD mice (Fig. 3D).

The Standardization of JCE Using HPLC/MS For the simultaneous determination of major constituents of JCE, the optimized chromatographic condition has been investigated. The optimal mobile phase, which consisted of acetonitrile/water, was subsequently employed for the analysis of JCE and led to a good resolution and satisfactory peak shape. The presence of four compounds, **1**: isoscutellarein-7-*O*- β -D-xyloside (*m/z* 417.08 at *t_R* 8.1 min), **2**: kaempferol-3-*O*- β -D-rhamnoside (*m/z* 432.01 at *t_R* 10.8 min), **3**: cupressuflavone (*m/z* 537.08 at *t_R* 11.6 min), **4**: apigenin (*m/z* 269.04 at *t_R* 11.9 min), **5**: amentoflavone (*m/z* 537.08 at *t_R* 14.1 min), and **6**: hinokiflavone (*m/z* 551.10 at *t_R* 14.4 min), in JCE was verified by comparing each retention time and UV spectrum with those of each standard compound and spiking with authentic standards (Figs. 4A, B).

DISCUSSION

Naturally occurring products are a rich source of medicinal agents. Natural product-derived drugs account for over 50% of the most-prescribed drugs in the U.S.A.²³⁾ Natural origin substances have been widely used in skin problems due to their therapeutic efficacy in dermatology including anti-

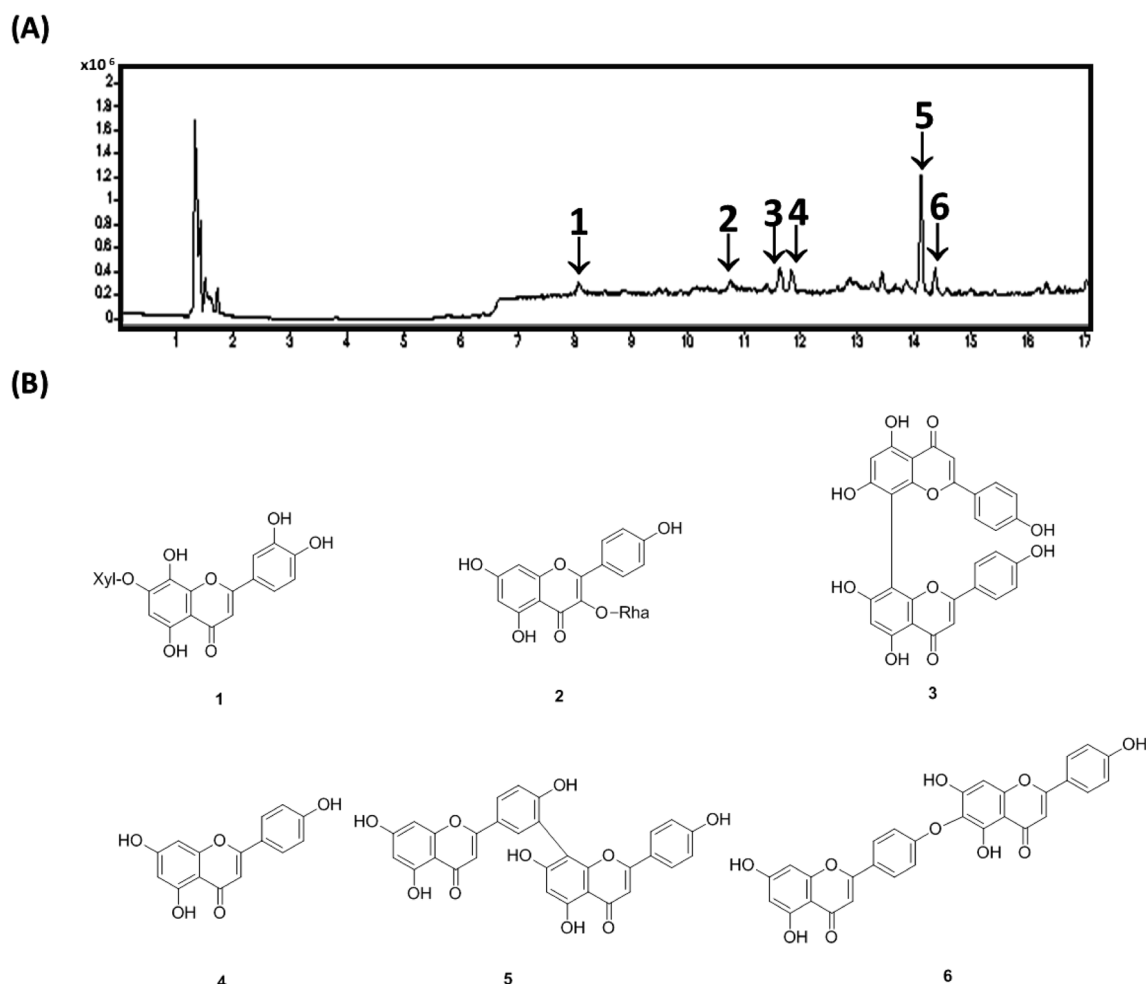


Fig. 4. HPLC Chromatogram of JCE (A) and Chemical Structures of Major Components (B)

Fingerprint analysis of *J. chinensis* fruits total extract was performed in positive ion mode using LC-TOF/MS. 1: isoscutellarein-7-*O*- β -D-xyloside, 2: kaempferol-3-*O*- β -D-rhamnoside, 3: cupressuflavone, 4: apigenin, 5: amentoflavone, 6: hinokiflavone.

inflammatory, antimicrobial, and cell-stimulating properties.²⁴⁾ Therefore, research in natural products continues to be an important part of the drug development for new medicines to treat skin-related diseases.^{23,25)} A powerful example of this is in the case of pimecrolimus (Elidel®), one representative component available for the treatment of AD, derived from ascomycin which is a natural microorganism from soil actinomycetes.²⁶⁾ For this reason, plant resources including extracts, pure compounds, and phytochemical combinations have attracted continuous interest to find drug candidates for the prevention and/or treatment of atopic diseases.

In our effort to find new anti-inflammatory materials from Korean medicinal plants, the 95% EtOH extract of *J. chinensis* fruits exhibited a strong IL-4 inhibition in RBL-2H3 cells (data not shown). To evaluate whether the JCE affects atopic symptoms, we treated this sample to the oxazolone- and DNCB-sensitized AD mice for 2 and 3 weeks, respectively. The clinical severity of AD was evaluated by quantitative estimates of skin symptoms including pruritus, erythema, fissuring, and lichenification (skin thickening). Repeated topical and broad applications of the JCE resulted in a significant improvement in characteristic signs and symptoms of AD. Since elevated serum levels of IgE and IL-4 are considered as important biological markers of allergic diseases such as AD and asthma, we also evaluated whether the sample influences the blood IgE and IL-4 concentrations of AD mice.²⁷⁾ Treatment of JCE markedly diminished total IgE and IL-4 levels in the serum of DNCB-induced atopic mice. The results indicated that both topical and broad applications of JCE could improve atopic damage in murine AD models.

According to the previous literature, patients with AD are normally found to have higher basal TEWL and skin thickness than control group.²⁸⁾ Lichenification (skin thickening) and TEWL leads to severe skin barrier defect, pruritus, and atopy. And also, a defective skin barrier is thought to accelerate the development and aggravation of AD in human beings.²⁹⁾ Therefore, use of appropriate emollients or moisturizers improves clinical symptoms of AD via an immediate skin barrier-repairing effect by alleviation of dryness and TEWL.³⁰⁾ In our present study, the abnormal condition of skin barrier (thick, dry, and reddish) was obtained in murine AD models by treatment of sensitizers such as oxazolone and DNCB. This defective skin barrier was reversed by dermal application of the JCE according to the quantitative estimates of parameters (TEWL, stratum corneum hydration, and skin thickness) which is essential for the integral evaluation of the epidermal barrier status. As a result, *J. chinensis* fruits were considered to have a great potential as a pharmacological target in atopic or allergic diseases, especially because it accelerates skin barrier recovery.

Standardization of *J. chinensis* fruits extract is necessary to provide information on quality standards of natural product-derived drug development. In our recent study, thirteen flavonoids, namely hypolaetin-7-*O*- β -D-glucoside, luteolin-7-*O*- β -D-glucoside, isoscutellarein-7-*O*- β -D-xyloside, hypolaetin-7-*O*- β -D-xyloside, quercetin-7-*O*- β -D-rhamnoside, kaempferol-3-*O*- β -D-rhamnoside, amentoflavone, 7-*O*-methylamentoflavone, cupressuflavone, moghatin, cupressuflavone-4'-*O*- β -D-glucoside, hinokiflavone, and isocryptomerin, were isolated from JCE (unpublished data). Phytochemical screening, using thirteen isolates as bioactive marker compounds,

was performed to standardize the JCE sample. HPLC/MS analysis of the JCE revealed the presence of isoscutellarein-7-*O*- β -D-xyloside, cupressuflavone, and amentoflavone as major components.

In conclusion, both topical and broad treatments with JCE were protective against oxazolone- and DNCB-induced AD-like lesions in mice. The JCE improved skin barrier function and suppressed the overproduction of serum IgE and IL-4 in murine atopic animal models. Based on the fingerprint analysis, isoscutellarein-7-*O*- β -D-xyloside, cupressuflavone, and amentoflavone were confirmed to be the major constituents of JCE. Further study is warranted to identify the therapeutic effects of JCE against AD symptoms in human skin.

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Conflict of Interest The authors declare no conflict of interest.

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