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Inhibitory effects of *Juglans mandshurica* leaf on allergic dermatitis-like skin lesions-induced by 2,4-dinitrochlorobenzene in mice

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ABSTRACT

Allergic dermatitis among common skin diseases is a chronic and recurrent inflammatory skin disorder caused by genetic, environmental, allergens as well as microbial factors. Allergic dermatitis patients clinically present skin erythematous plaques, eruption, elevated serum immunoglobulin E (IgE) and T helper cell type 2 (Th2) cytokine levels. The leaf of walnut tree *Juglans mandshurica* Maxim (JM) is consumed food and traditional phytomedicine in Asia, China, Siberia and Korea. JM has been reported to have various pharmacological activities, such as anti-tumor, anti-oxidative, and anti-bacterial effects. However, no study of the inhibitory effects of JM on allergic dermatitis has been reported. Here, we demonstrated the effect of JM against 2,4-dinitrochlorobenzene-induced allergic dermatitis-like skin lesions. 0.5% JM or 1% dexamethasone (positive control) applied to the dorsal skin inhibited development of allergic dermatitis-like skin lesions and scratching behavior. Moreover, the Th2-mediated inflammatory cytokines IgE, tumor necrosis factor- α , interleukin (IL)-1, and IL-13, were significantly reduced by JM treatment. Thus JM can inhibit development of allergic dermatitis-like skin lesions in mice by regulating immune mediators, and may be an effective alternative therapy for allergic dermatitis.

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1. Introduction

Allergic dermatitis is a chronic inflammatory skin disease accompanied by several common symptoms including itching, erythema, eczema skin lesions, chronic relapse and pruritus (Leung, 2000). In allergic dermatitis, skin becomes extremely itchy followed by severe scratching behavior that induces the production of proinflammatory cytokines (Leung, 2000; Thestrup-Pedersen, 2000). This in turn activates immune cells and initiates inflammatory cycle of allergic dermatitis that accompanied by erythema, keratosis and its scaling (Mar et al., 2011; Thestrup-Pedersen, 2000). All these symptoms are the consequence of an imbalanced immune response to various allergens. Another defining characteristic of the allergic immune system is the capacity to generate elevated immunoglobulin E (IgE) antibodies and type 2 helper T cells (Th2) are critical for IgE synthesis (Pugliarello et al., 2011; Saeki et al., 2009). Th2 cells predominantly produce interleukin (IL) families, and these cytokines are associated with specific function of immune cells in allergic dermatitis (Pugliarello et al., 2011).

The Manchurian walnut, *Juglans mandshurica* Maxim (JM), is a fast-growing deciduous tree that is widely distributed in China and Korea (Park et al., 2012). It has been used as a folk medicine plant for the treatment of esophageal, gastric, cardiac, and lung cancer (Xu et al., 2010). JM is known to contain juglone, β -sitosterol, naphthoquinones, naphthalenyl glycosides, α -teralonyl glucopyranosides, flavonoids, and diarylheptanoyl glucopyranosides (Liu et al., 2010; Park et al., 2012; Xu et al., 2010, 2013). Additionally, JM has been shown to have anti-tumor, anti-oxidative, anti-bacterial, and anti-inflammatory effects (Xu et al., 2012, 2013). Furthermore, we previously demonstrated that JM protects human skin dermis fibroblasts against H_2O_2 -induced oxidative damage, possibly by regulating antioxidative defense enzymes via nuclear factor erythroid-derived 2-related factor 2 (Nrf2) (Park et al., 2012). El Ali et al. (2013) reported that transcription factor Nrf2 regulate the allergic dermatitis or skin inflammatory disease in chemical sensitizers. However, no study of the inhibitory effects of JM on allergic dermatitis has been reported.

Therefore, this study examined the protective effects of JM against 2,4-dinitrochlorobenzene (DNCB)-induced allergic dermatitis-like skin lesions using clinical skin severity scores and scratching behavior in mice. We additionally investigated possible JM mechanisms by assessing the levels of IgE, TNF- α , IL-1, and IL-13 and by examining 2,2-diphenyl-2-picrylhydrazyl

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(DPPH) free radical scavenging activity and 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation scavenging activity.

2. Materials and methods

2.1. Chemical

DNCB, acetone, and olive oil were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IgE, IL-1 and -13 were obtained from R&D Systems (Minneapolis, MN, USA) and BD Biosciences (San Diego, CA, USA). DPPH, ABTS, phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals and solvents were of the highest grade commercially available.

2.2. Preparation of the JM extract

The JM extract (JME) was prepared according to methods published previously (Park et al., 2012). Briefly, fresh JM was obtained from a producing district (Gangwon, Korea) and a voucher specimen (KHUOPS-GCH003) was deposited in the herbarium at the College of Pharmacy, Kyung Hee University (Seoul, Korea). 100 g of JM leaf were ground with 1 L of 70% ethanol for 24 h at room temperature. Then, the extract was filtered, evaporated on a rotary vacuum evaporator, and lyophilized (yield; 5.15%). The powder (JME) was kept at 4 °C before use.

2.3. Animals and treatments

Animal maintenance and treatment were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and the Use Guidelines of Kyung Hee University, Seoul, Korea. Male ICR mice (8 weeks, 23–24 g) were purchased from Daehan Biolink Co., Ltd. (Eumseong, Korea). The animals were housed at an ambient temperature of 23 ± 1 °C and relative humidity of $60 \pm 10\%$ under a 12 h light/dark cycle, and were allowed free access to water and food. The mice were assigned to four groups ($n = 5$ per group). Allergic dermatitis-like skin lesions were induced by applying DNCB to the dorsal skin. After complete removal of the dorsal hairs within an area of approximately 8 cm^2 , 200 μL of 1% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) was applied for 7 consecutive days for sensitization. Seven days after sensitization, the dorsal skin was challenged with 200 μL of 0.5% DNCB solution three times per week for 4 weeks. After inducing allergic dermatitis, the JME solution (dissolved in a 3:1 mixture of acetone and olive oil) was applied to the dorsal skin of the mice seven times per week for 4 weeks. In the control and DNCB-alone treated mice no JME solution was applied to the dorsal skin. The animals were sacrificed 9 weeks after sensitization with DNCB (Fig. 1).

2.4. Evaluation of clinical skin severity scores

Five signs of skin lesions: (1) pruritus/itching, (2) erythema/hemorrhage, (3) edema, (4) excoriation/erosion, and (5) scaling/dryness were graded as follows: 0 (no symptoms), 1 (mild), 2 (moderate), and 3 (severe).

2.5. Evaluation of scratching behavior

Mice were placed individually in a clear plastic cage and their behavior was monitored for 30 min 5 weeks after sensitization. Scratching of the rostral back and biting of the caudal back were observed; scratching movements by the hind paw were defined as a scratching bout that ended when the mice either licked its hind

paw or placed its hind paw back on the floor, and a series of one or more biting movements were counted as one episode that ended when the rat returned to the straight-forward position.

2.6. Evaluation of total serum IgE, TNF- α , IL-1 and IL-13 Levels

Blood was collected from the mice on the day of sacrifice and centrifuged at $1700 \times g$ for 10 min to obtain serum samples, which were stored at -70 °C until use. Total serum IgE, TNF- α , IL-1, and IL-13 were measured using an ELISA kit according to the manufacturer's instructions.

2.7. DPPH radical scavenging activity

JME at various concentrations of 1–1000 $\mu\text{g/mL}$ was mixed with 0.20 mM DPPH ethanol solution (1:1). After incubation at room temperature in the dark for 30 min, the mixture determined at the absorbance of 517 nm using spectrophotometer. Also, the antioxidant activity of JME was expressed as half maximal inhibiting concentration (IC_{50}) which is defined as the concentration of JME required to scavenge 50% of DPPH radicals. IC_{50} values were estimated by a non-linear regression using the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA). DPPH radical scavenging activity (%) = $\{\text{control} \times (\text{sample-blank})\} / \text{control} \times 100$.

2.8. ABTS cation scavenging activity

ABTS solution of 7.40 mM was added to 2.60 mM potassium phosphate for 1 day before starting the experiment in the dark. JME at various concentrations of 1–1000 $\mu\text{g/mL}$ was mixed with 7.40 mM ABTS solution. After incubation at room temperature for 5 min, the mixture was determined at the absorbance of 732 nm using spectrophotometer. Also, the antioxidant activity of JME was expressed as IC_{50} which is defined as the concentration of JME required to scavenge 50% of ABTS cations. IC_{50} values were estimated by a non-linear regression using the GraphPad Prism. ABTS cation scavenging activity (%) = $(\text{control} \times \text{sample}) / \text{control} \times 100$.

2.9. Statistical analysis

All statistical parameters were calculated using Graphpad Prism 4.0 software. Values were expressed as the mean $\pm$ standard error of the mean (S.E.M.). The results were analyzed by one-way analysis of variance (ANOVA) and post hoc multiple mean comparisons (Tukey's HSD test). Differences with a p -value less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of JME on clinical skin severity scores

To investigate the effect of JME against DNCB-induced allergic dermatitis-like skin damage, we measured clinical skin severity scores. Treatment with DNCB significantly increased clinical skin severity scores compared with control cells (by $3.83 \pm 0.44\%$), while treatment with 1% dexamethasone or 0.5% JME reduced DNCB-induced clinical skin severity scores (by $1.16 \pm 0.15\%$ and $1.50 \pm 0.32\%$, respectively) (Fig. 2A).

3.2. Effects of JME on scratching behavior

To investigate the effects of JME against DNCB-induced allergic dermatitis-like skin damage, we measured scratching behavior. Treatment with DNCB significantly increased scratching scores compared with control cells (by $325.01 \pm 25.79\%$), while treatment with 1% dexamethasone or 0.5% JME reduced DNCB-induced

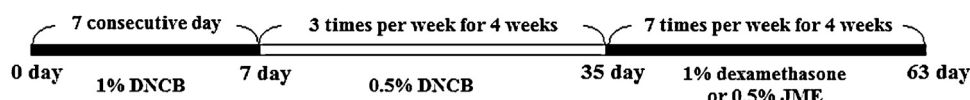


Fig. 1. Summary of the experimental design.

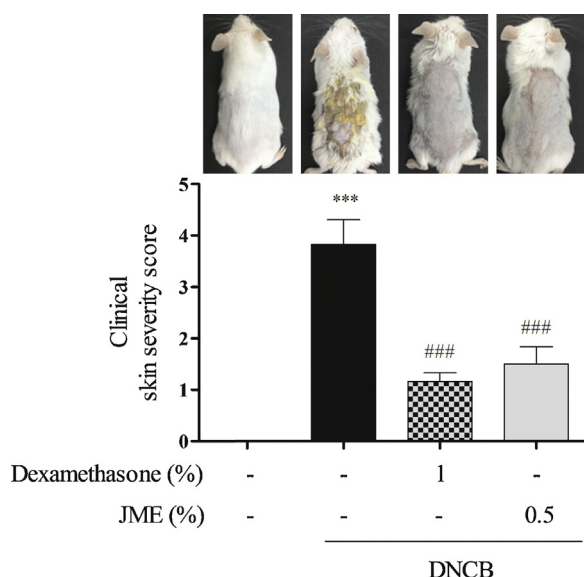


Fig. 2. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced clinical skin severity scores in mice. Nine weeks after the initial sensitization, the clinical skin severity scores of the DNCB-treated mice were assessed. Clinical skin severity scores were evaluated as the sum of the scores for five clinical symptoms. Values are means $\pm$ standard error of the mean. *** p < 0.001 compared to the control group, and ### p < 0.001 compared to the DNCB-alone group.

scratching scores (by $52.33 \pm 11.29\%$ and $96.33 \pm 14.15\%$, respectively) (Fig. 3B).

3.3. Effects of JME on cytokine expression

To determine whether JME affected allergic dermatitis-related cytokines, we determined the serum levels of TNF- α , IgE, IL-1, and -13. Treatment with DNCB significantly increased levels of TNF- α and IgE compared with control cells (by $158.15 \pm 5.92\%$ and

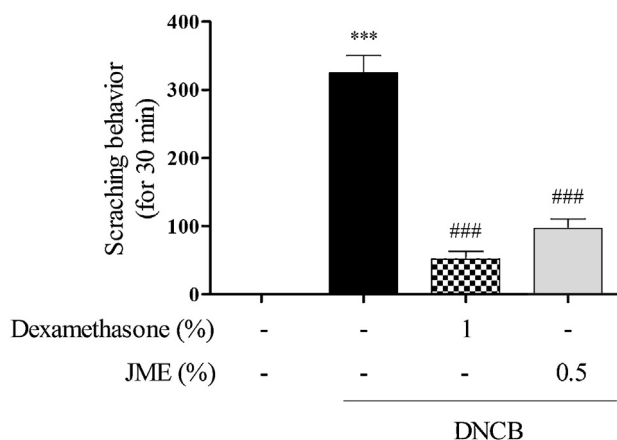


Fig. 3. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced scratching behavior in mice. Nine weeks after the initial sensitization, the clinical skin severity scores of the DNCB-treated mice were assessed. The number of scratching behaviors within 30 min was measured 1 h after each sample treatment. Values are means $\pm$ standard error of the mean. *** p < 0.001 compared to the control group, and ### p < 0.001 compared to the DNCB-alone group.

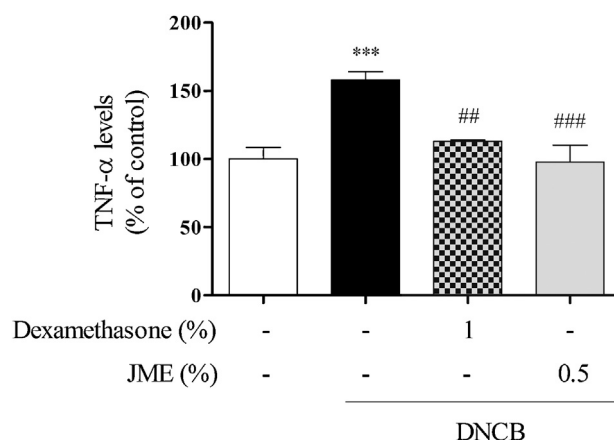


Fig. 4. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced serum tumor necrosis factor (TNF)- α levels in mice. Values are means $\pm$ standard error of the mean. *** p < 0.001 compared to the control group, and ## p < 0.01 and ### p < 0.001 compared to the DNCB-alone group.

$159.47 \pm 3.51\%$, respectively), while treatment with 1% dexamethasone or 0.5% JME reduced DNCB-induced TNF- α (by $113.06 \pm 0.84\%$ and $97.69 \pm 12.61\%$, respectively) and IgE (by $103.37 \pm 3.51\%$ and $132.60 \pm 9.88\%$, respectively) (Fig. 4). Moreover, treatment with DNCB significantly increased levels of IL-1 and -13 compared with control cells (by $193.51 \pm 9.36\%$ and $1684.95 \pm 74.83\%$, respectively), while treatment with 1% dexamethasone or 0.5% JME reduced DNCB-induced IL-1 (by $93.52 \pm 9.22\%$ and $98.25 \pm 13.51\%$, respectively) and IL-13 (by $121.09 \pm 46.54\%$ and $320.65 \pm 21.50\%$, respectively) (Figs. 5–7).

3.4. Radical scavenging activities of JME

To evaluate the anti-oxidant activities of JME, we carried out two radical scavenging assays: DPPH radical scavenging and ABTS radical cation decolorization activity. DPPH radicals are stable free radicals with absorption at wavelengths 512–528 nm that react with hydrogen donors. Also, ABTS radical cations are capable of

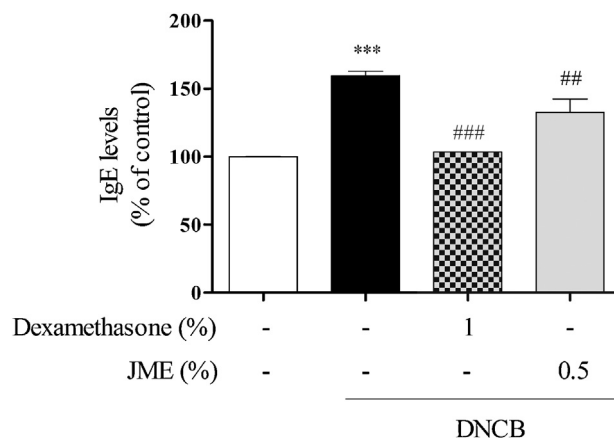


Fig. 5. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced serum immunoglobulin E (IgE) levels in mice. Values are means $\pm$ standard error of the mean. *** p < 0.001 compared to the control group, and ## p < 0.01 and ### p < 0.001 compared to the DNCB-alone group.

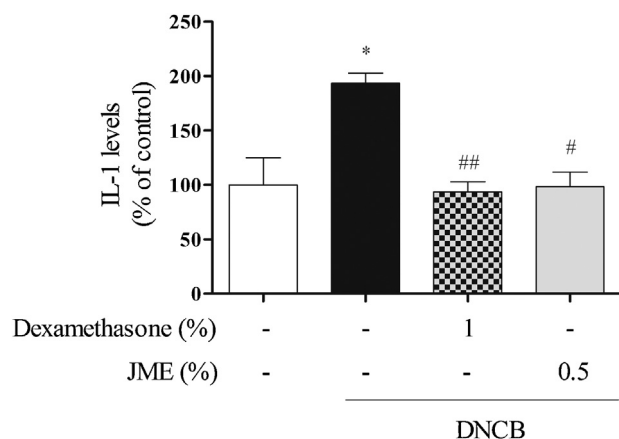


Fig. 6. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced serum levels of interleukin (IL)-1 levels in mice. Values are means $\pm$ standard error of the mean. * $p < 0.05$ and *** $p < 0.001$ compared to the control group, and # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to the DNCB-alone group.

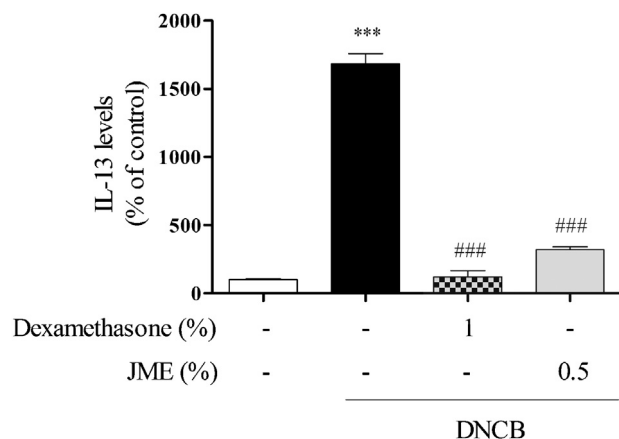


Fig. 7. The effect of *Juglans mandshurica* extract (JME) on 2,4-dinitrochlorobenzene (DNCB-) induced serum levels of interleukin (IL)-13 levels in mice. Values are means $\pm$ standard error of the mean. * $p < 0.05$ and *** $p < 0.001$ compared to the control group, and # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ compared to the DNCB-alone group.

measuring the anti-oxidant activity on hydrophilic and hydrophobic compounds. In both assays, JME was tested in parallel with positive controls, green tea extract, which are known to have strong anti-oxidant effects. At a concentration of 1000 $\mu\text{g/mL}$, JME showed 83.55% and 89.90% of scavenging activity in DPPH (Fig. 8A) and ABTS (Fig. 8B), respectively. DPPH IC_{50} s for JME and green tea extract were 104.40 and 5.03 $\mu\text{g/mL}$, respectively, whereas ABTS IC_{50} s were 44.89 and 11.42 $\mu\text{g/mL}$, respectively.

4. Discussion

We demonstrated the effect of JME on DNCB-induced allergic dermatitis-like skin lesions in mice using clinical skin severity scores and scratching behavior, via its regulation of inflammatory effects, here the serum IgE, TNF- α , IL-1, and IL-13 levels.

Scratching is an essential and a skin specific behavior induced by itching, which is a common symptom of allergic dermatitis and other types of dermatitis (Jin et al., 2009; Novak et al., 2003). Generally, scratching causes physical injury to epidermis, and induces the release of inflammatory cytokines (Jin et al., 2009). Treatment with DNCB significantly increased the clinical skin severity and scratching scores compared to the control group, while treatment

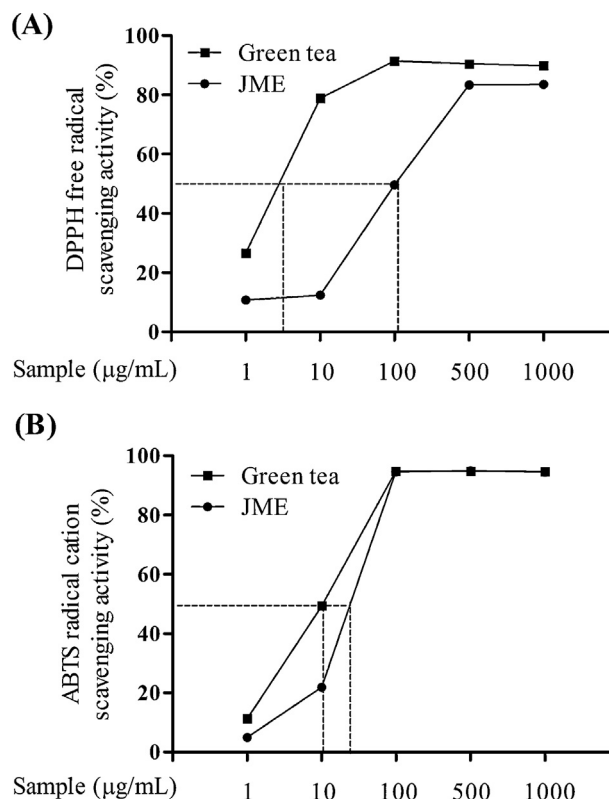


Fig. 8. Radical scavenging activities of *Juglans mandshurica* extract (JME). JME showed DPPH free radical scavenging activity (A) and ABTS radical cation decolorization activity (B) in a dose dependent manner at concentrations of 1–1000 $\mu\text{g/mL}$, respectively. Values are means $\pm$ standard error of the mean.

with 0.5% JME or 1% dexamethasone reduced the DNCB-induced clinical skin severity score (Fig. 2A) and scratching score (Fig. 3B). These results indicate that JME inhibits allergic dermatitis-like skin lesions and scratching behavior.

Th1 and Th2 cytokines released from activated T cells play important roles in the pathology of allergic dermatitis (Akdis et al., 2000). Cytokines representative of Th2 cells, including IL-4, IL-5, IL-10, and IL-13, are known to play important roles in allergic dermatitis (Akdis et al., 2000). Furthermore, it was reported that Th1-mediated cytokines, such as TNF- α and IFN- γ , produced by Th1 cells act as antagonists for these Th2 cytokines (Klunker et al., 2003). In the immediate allergic reaction phase, IgE is produced, which is bound to the mast cell via the $\text{Fc}\epsilon$ receptor (Klunker et al., 2003). Crosslinking of allergen-specific IgE leads to the release of various chemical mediators from mast cells (Buske-Kirschbaum et al., 2001; Klunker et al., 2003). The release of these chemical mediators results in various reactions, including itching in the tissue, after a few minutes (Buske-Kirschbaum et al., 2001). In the late allergic reaction phase, IL-4, IL-13, and other mediators including chemokines and enzymes are produced and released (Buske-Kirschbaum et al., 2001; Thepen et al., 1996). Chemokines attract eosinophils to the allergic dermatitis lesion (Thepen et al., 1996). We demonstrated that DNCB significantly increased the levels of IgE and TNF- α compared to the control group, while treatment with 0.5% JME or 1% dexamethasone reduced DNCB-induced IgE and TNF- α (Figs. 4 and 5). Moreover, DNCB significantly increased the serum levels of IL-1 and IL-13 compared to the control group, while treatment with 0.5% JME or 1% dexamethasone reduced DNCB-induced IL-1 and IL-13 (Figs. 6 and 7). These results suggest that JME inhibits Th1 and 2-mediated cytokines, including IgE, TNF- α , IL-1, and IL-13.

In the present study, we demonstrated for the first time that JME has a protective effect against DNCB-induced allergic dermatitis-like skin lesions via its regulation of IgE, TNF- α , IL-1, and IL-13. Skin inflammation in atopic dermatitis is histologically characterized by intense infiltration of lymphocytes, monocytes and eosinophils which have been reported to release proinflammatory cytokines and reactive oxygen species (ROS) such as O₂⁻, H₂O₂ and peroxynitrite, upon immunological and non-immunological stimulation (Briganti and Picardo, 2003; Okayama, 2005). Therefore, in DPPH radical scavenging and ABTS cation decolorization assays, usually used to evaluate anti-oxidant activity, JME has shown dose-dependent lower or similar activity in DPPH and ABTS compared with the green tea extract known to be a potential natural anti-oxidant (Fig. 8). Additionally, we reported previously that JME protects human skin dermal fibroblasts against H₂O₂-induced oxidative damage by regulating antioxidative defense enzymes via nuclear factor erythroid-derived 2-related factor 2 (Park et al., 2012). Thus, JME may contribute partially to the observed protection by JME of skin from DNCB-induced allergic dermatitis-like skin lesions.

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