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Essential Oil of *Thujopsis dolobrata* Suppresses Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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Abstract

We examined the effects of essential oil from *Thujopsis dolobrata* Sieb. et Zucc. var. *hondai* Makino (EOTD) (Cupressaceae) on atopic dermatitis (AD)-like skin lesions in NC/Nga mice. Treatment with EOTD twice daily for two weeks ameliorate AD-like skin lesions induced by DNCB (2,4 dinitrochlorobenzene), and clinical scores were reduced to 7.29, 7.07, and 4.5 points in the groups treated with 1.5%, 3.0%, and 6.0% extract (p<0.01) respectively, from the 15.0 score obtained using vehicle. EOTD inhibited the infiltration of mast cells into the AD-like skin lesion in NC/Nga mice (p<0.01) and also reduced serum histamine and IgE levels (p<0.05). Furthermore, it dose-dependently inhibited the release of beta-hexosaminidase from rat basophilic leukemia RBL 2H3 cells. These results indicate that EOTD reduces AD-like skin lesions by inhibiting the production of IgE and histamine, and, thus, IgE-mediated degranulation.

Key Words: *Thujopsis dolobrata*, Atopic dermatitis, Beta-hexosaminidase, NC/Nga mouse, IgE

INTRODUCTION

Atopic dermatitis (AD) is a multifactorial allergic inflammatory skin disorder characterized by pruritic and eczematoid skin lesions. AD is commonly present in early infancy and childhood, although it can occur later in life and persist into adulthood (Barker *et al.*, 2007; Brenninkmeijer *et al.*, 2009). It results from interactions between susceptibility genes, the host's environment, skin barrier defects, bacterial and viral skin infections, and immunological factors (Leung and Bieber, 2003). Recent immunological analyses of the pathogenesis of AD have revealed that activated mast cells and an excess in the number of differentiated T-helper 2 (Th2) cells (caused by chemical mediators and cytokines) may play major roles in the development of dermatitis by elevating serum immunoglobulin E (IgE) levels (Leung, 1995). They may trigger the massive infiltration of T cells, eosinophils, mast cells, and macrophages that characterizes AD (Alenius *et al.*, 2002; Milgrom, 2002; Chen *et al.*, 2005).

In the treatment of AD, topical glucocorticoids are frequently used to control acute exacerbation (Ference and Last, 2009; Saeki *et al.*, 2009). However, it is well known that prolonged use of high-dose glucocorticoids often cause a variety of adverse effects (Pariser, 2009). In recent years, therefore, alternative approaches have been used to target specific defects in AD (Levin and Maibach, 2002). Results from several studies indicate that AD patients may benefit from herbal Oriental medicine therapy (Qi *et al.*, 2009; Choi *et al.*, 2008). *Thujopsis dolobrata* Sieb. et Zucc. var. *hondai* Makino (Cupressaceae) is a plant that grows in Korea and Japan. *Thujopsis dolobrata* contains a number of neutral terpenoids (Takahashi *et al.*, 2001; Yamaji *et al.*, 2007) and several acidic phenols, tropolones, and lignans (Morita *et al.*, 2003; Morita *et al.*, 2004; Noshita *et al.*, 2009). It has been reported that hinokitiol, the major component of *Thujopsis dolobrata*, has anti-bactericidal and anti-fungal activity (Morita *et al.*, 2002; Morita *et al.*, 2004). However, whether or how EOTD suppresses AD has

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not been studied.

In this study, we examined the anti-AD effects of EOTD. We used NC/Nga mice for *in vivo* studies and rat leukemia RBL 2H3 cells (frequently used as an *in vitro* model of mast cells) to study the roles of EOTD in IgE-mediated de-granulation.

MATERIALS AND METHODS

Extraction and analysis of EOTD

EOTD was extracted from the stem of *Thujopsis dolobrata* Sieb. et Zucc. var. *hondai* Makino by boiling according to conventional methods. The EOTD was then weighed to calculate the yield and analyzed by HPLC (RP-18, 5 μm, 250×4.6 mm). A methanol (MeOH) gradient (100% MeOH (0 min), 30% MeOH (30 min)) was applied at a flow rate of 1 ml/min. The EOTD HPLC profile was quantified using its integrated area (Fig. 1).

Animals

Five-week-old male NC/Nga mice were purchased from Central Lab. Animal, Inc. (Seoul, Republic of Korea) and allowed to acclimatize for 1 week before use. Food and water were provided *ad libitum*. The EOTD was kindly provided by GNG corporation (Hwaseong-City, Gyeonggi-Do, Republic of Korea). All animal protocols used in this study were approved by the Committee for Animal Experiments of Seoul National University.

Cell culture

Rat basophilic leukemia (RBL 2H3) cells obtained from Korean Cell Line Bank (Seoul, Korea) were grown in Dulbecco's modified Eagle's medium (Hyclone, Thermo Fisher Scientific Inc., IL, USA) with 10% heat inactivated fetal bovine serum (GIBCO, Invitrogen, Seoul, Korea) at 37°C in 5% CO_2 and complete humidity.

Cell viability assay

Cell viability was analyzed using an MTT assay. RBL-2H3 cells (5×10^4 cells (0.2 ml)/well) were plated in 96-well plates. Samples were treated with various concentrations of EOTD for 24 h. The culture medium was replaced with phenol red-free DMEM containing MTT (5 mg/ml). Cells were incubated for 4 h at 37°C in 5% CO_2 and complete humidity. After 4 h, the MTT solution was removed and replaced with 250 μl of DMSO. Cells were further incubated for 5 min at room temperature, and the optical densities of the wells at 595 nm were determined using a plate reader.

Beta-hexosaminidase secretion assay

RBL-2H3 cells (5×10^4/well) were sensitized with 300 ng/ml 2,4-dinitrophenyl (DNP)-specific IgE for 12 h. After three washes in PIPES buffer, cells were exposed to compounds for 24 h and then challenged with 20 ng/ml 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) for 30 min. Next, 40 μl of supernatant was mixed with an equal volume of substrate solution (*p*-nitrophenyl-*N*-acetyl-β-d-glucosaminide (2 mM) in citrate 0.1 M citrate buffer, pH 4.5) for 1 h at 37°C. Reactions were terminated by adding 50 μl of stop solution (0.5 M Na_2CO_3/$NaHCO_3$, pH 10.0). Absorbances were measured at 405 nm using a microplate reader (Marciniak *et al.*, 2006). Inhibition (%) of β-hexosaminidase release by the test samples were calculated by according to the following equation: Inhibition (%) = [1−(T−B/ C−B)]×100

Control (C): cells (+), DNP-BSA (+), test sample (−)
Test sample (T): cells (+), DNP-BSA (+), test sample (+)
Blank (B): cells (−), DNP-BSA (+), test sample (+)

AD induction with DNCB (2, 4-dinitrochlorobenzene)

Five-week-old NC/Nga mice (n=35) were acclimatized for one week, and each 200 μl aliquot of 1% DNCB in acetone:olive oil (5:1) was applied to each side of ears and dorsal skin without EOTD. 1% DNCB was applied every two days for two weeks to induce atopic dermatitis. After two weeks of induction, EOTD (1.5%, 3%, and 6%) was applied for a further two weeks. The mixture of 1% betamethasone (BM) and 1% hydrocortisone (HC) in acetone:olive oil (5:1) was used as a positive control. Every volume of test sample (100 μl) was applied to each mouse.

Histological analysis of DNCB-induced AD

After two weeks, mice were sacrificed with CO_2 gas. Skin tissue was fixed with 10% neutral formalin solution and then embedded in paraffin by conventional methods. Tissue was cut to a thickness of 5 μm. Tissue sections were then stained with hematoxylin and eosin (HE) using Mayer's Hematoxylin solution, or with 0.05% toluidine blue (TB) solution.

Calculation of tissue thicknesses from stained tissue samples

Epidermal thickness was measured from six serial sections using Image J freeware obtained from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Toluidine blue-stained mast cell counts

Tissue sections were stained with toluidine blue. After staining with toluidine blue working solution for 2-3 min, tissue sections were dehydrated using a series of ethanol solutions (30, 50, 70, 95, and 100%) and rinsed twice in xylene. Numbers of mast cells in three independent areas (40,000 μm^2) of six serial sections were counted and averaged.

Measurement of serum IgE levels by ELISA

Whole blood was obtained from mouse tails on day 0 and after 14 days of sample treatment. Sera were stored at −80°C prior to analysis. Total IgE levels were measured using an ELISA kit (Alpha Diagnostic International; #6370) according to the manufacturer's instructions. 100 μl of IgE standard, samples, and controls was added to the wells of a 96-well plate, which was tapped gently to mix the reagents and incubated for 60 min. 100 μl of working anti-mouse IgE-HRP conjugate antibody was added to each well, and the plate was incubated for 30 min before washing. 100 μl of enzyme substrate was added to each well. After incubation for 15 min in the dark, 100 μl of stop solutions was added to each well. The plate was then read at 450 nm using a microplate reader.

Quantification of serum histamine levels by ELISA

Serum samples were obtained by centrifugation (1,700×g, 10 min) and stored at −80°C prior to analysis. Total histamine levels were measured using an ELISA kit according to the manufacturer's instructions. 50 μl of histamine standard, samples, and controls was added to the wells of 96-well plates. 50 μl of ready-to-use enzyme conjugate was added to the wells.

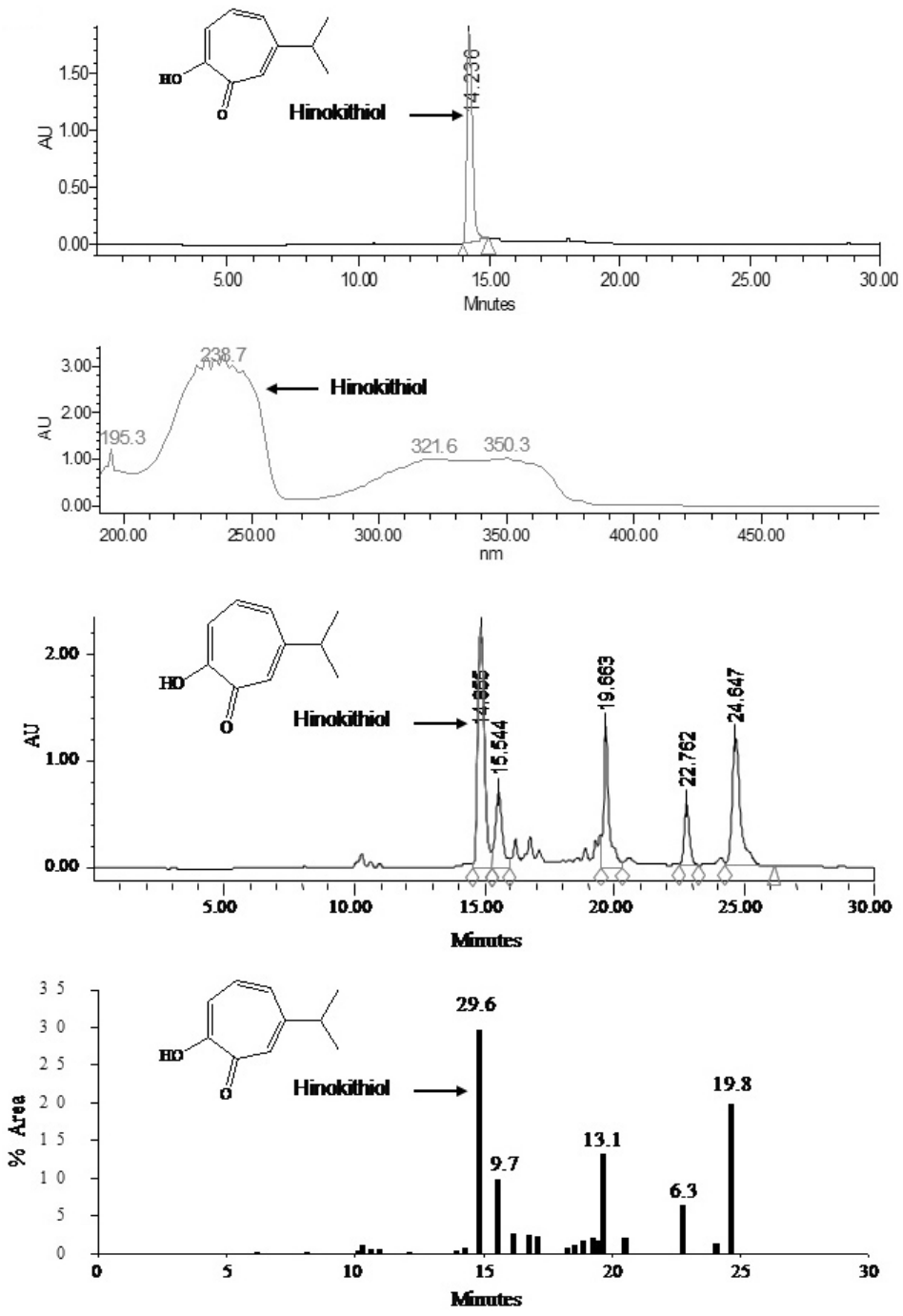

Fig. 1. HPLC profile of EOTD.

After washing, the plates were incubated for 45 min. Following washing, 150 μl of substrate was added to each well. After incubation for 30 min, the plates were read at 650 nm using a microplate reader. Percentage inhibition was calculated as follows: Inhibition % = [1−(Day14/Day0)]×100.

Clinical scoring of DNCB induced atopic dermatitis

Pruritus, edema, erosion, lichenification, and erythema were scored from 0 to 3 (0, no symptom; 1, mild; 2, moderate; and 3, severe). For each treatment group, mean values (± standard deviation) were calculated. Clinical scoring was performed to further investigate the efficacy of EOTD.

Statistical analysis

Numerical data are expressed as mean ± standard deviation. Statistically significant differences were identified using the Student's *t*-test. $p<0.05$ was considered to be statistically significant.

RESULTS

Clinical scores from EOTD-treated NC/Nga mice

AD lesions were scored from 0 to 3 on each of five parameters (pruritus, edema, erosion, lichenification, and erythema). In mice treated with 1.5%, 3%, and 6% EOTD, average scores were 7.27 ± 1.1, 7.07 ± 1.8 and 4.5 ± 1.4, respectively. Compared with animals treated with vehicle (averaged score 15.0 ± 0.0), the experimental group showed significant decreases in clinical AD scores (Table 1).

Effect of EOTD on AD-like skin thickness

Tissue sections were stained with hematoxylin and eosin, and epidermal thickness was measured (Fig. 2A). Mean epidermal thicknesses (in μm) in lesional tissue obtained from mice treated with 0%, 1.5%, 3%, and 6% EOTD were 186.4 ± 16.6, 74.7 ± 24.8, 72.8 ± 23.5 and 53.9 ± 19.0, respectively. Epidermal thickness in the positive control (BM+HC) group was 53.4 ± 16.0 μm. EOTD significantly decreased epidermal thickness (p<0.01).

Histological analysis of mast cell numbers

Following staining of AD tissue sections with toluidine blue, the number of mast cells was counted. Mast cell counts in mice treated with 0%, 1.5%, 3%, and 6% EOTD were 99.7 ± 13.7, 85.6 ± 7.9, 72.9 ± 6.2 and 54.8 ± 6.2, respectively (Fig. 2B). The mean count in the positive control (BM+HC) group was 44.3 ± 5.1.

Table 1. Clinical DNCB-induced AD scores in NC/Nga mice 14 days after EOTD Treatment. Pruritus, edema, erosion, lichenification, and erythema were scored from 0 to 3 (0, no symptom; 1, mild; 2, moderate; and 3, severe)

EOTD (%)	Clinical score
Positive control (BM+HC)	2.89 ± 3.5[a]
0	15.0 ± 0.0
1.5	7.29 ± 1.1[a]
3.0	7.07 ± 1.8[a]
6.0	4.5 ± 1.4[a]

Data are presented as mean ± SE. Asterisks indicate statistically significant differences between groups. [a]p<0.01.

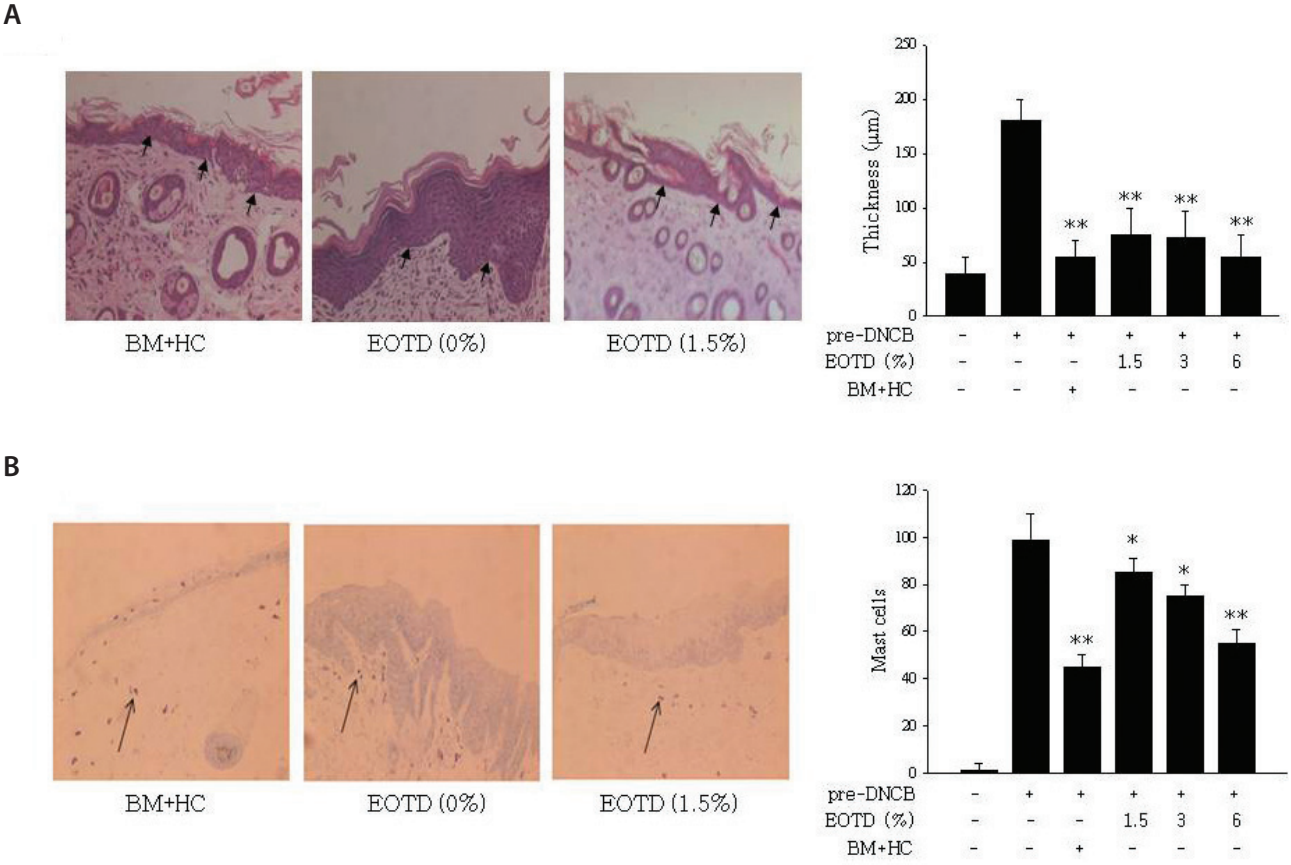

Fig. 2. Effect of EOTD on tissue thickness and mast cell recruitment in NC/Nga mice. (A) In each HE-stained slide, ten areas were randomly selected, and the tissue thickness measured. (B) Skin sections were stained with toluidine blue. Black arrows indicate histamine secretion by mast cells. All images were taken at 200X magnification. Data are presented as mean ± SD (n=6). Pre-DNCB, pretreated with DNCB for two weeks. *p<0.05, **p<0.01.

Change in serum levels of IgE and histamine in NC/Nga mice

After inducing AD through treatment for two weeks with DNCB, serum was isolated from whole blood obtained prior to sample application (day 0) 14 days after sample treatment. Compared to the control group, total IgE levels were reduced by 78.7 ± 5.3%, 75.3 ± 16.0% and 67.9 ± 10.1% in animals treated 6%, 3%, and 1.5% EOTD, respectively (Fig. 3A). Blood total histamine concentrations were also significantly decreased (by 40.9 ± 9.5%, 42.5 ± 8.8 % and 49.2 ± 9.2% in animals treated with 1.5%, 3%, and 6% EOTD, respectively (Fig. 3B)).

Measurement of β-hexosaminidase activity

Rat RBL 2H3 cells were treated with various concentrations (0.00128-4 μg/ml) of EOTD. Levels of degranulation were quantified by measuring β-hexosaminidase activity. Cells were sensitized with IgE and activated with DNP-BSA to elicit atopic responses. Treatment with 4, 0.8, 0.16, 0.032, 0.0064, and 0.00128 μg/ml EOTD oil inhibited β-hexosaminidase responses by 45.0 ± 5.2, 43.9 ± 3.9, 38.0 ± 4.6, 17.5 ± 3.9, 11.3 ± 2.7%, and 2.5 ± 3.2%, respectively (Fig. 4A). Treatment with ketotifen (4 μg/ml) (positive control) reduced degranulation 44.2 ± 5.1%, which represents a similar levels of inhibition to that achieved with EOTD (Fig. 4A).

Cytotoxic effects of EOTD on RBL 2H3 cells

RBL 2H3 cells were treated with various concentrations (0.00128-50 μg/ml) of the EOTD oil, and cell viability was determined by means of an MTT assay (Fig. 4B). EOTD reduced cell viability dose-dependently (p<0.05 (50 μg/ml EOTD)). Overall, however, EOTD displayed relatively low cytotoxicity in RBL 2H3 cells.

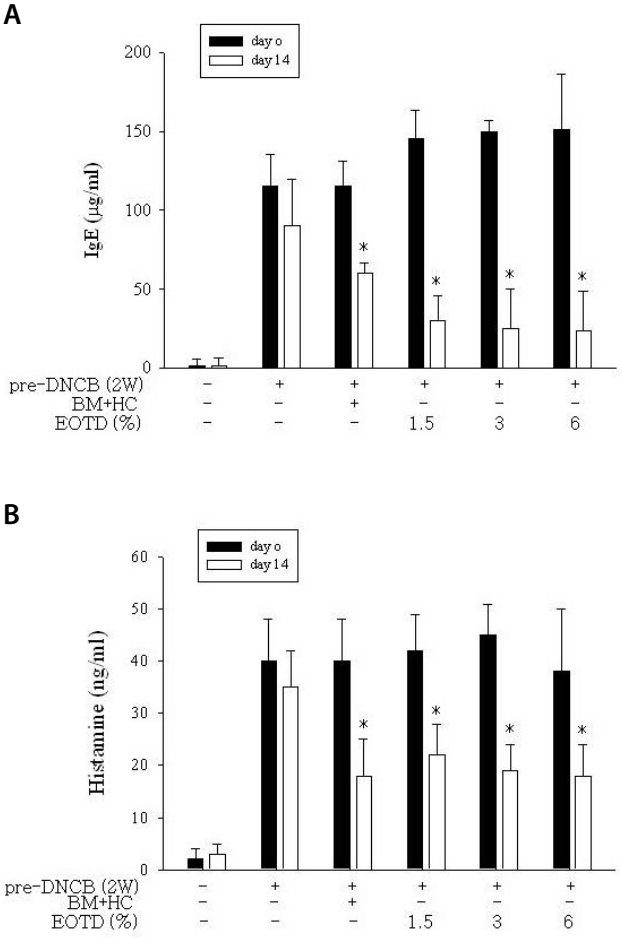

Fig. 3. Effect of EOTD on serum levels of histamine and IgE in NC/ Nga mice. Day 0 and 14 serum samples from mice treated with EOTD (1.5, 3, and 6%) (positive control: BM+HC). (A) Serum IgE levels measured by ELISA. (B) Serum histamine levels measured by ELISA. Data are presented as mean ± SD (n=6). Pre-DNCB (2W), pretreated with DNCB for two weeks. *p<0.05.

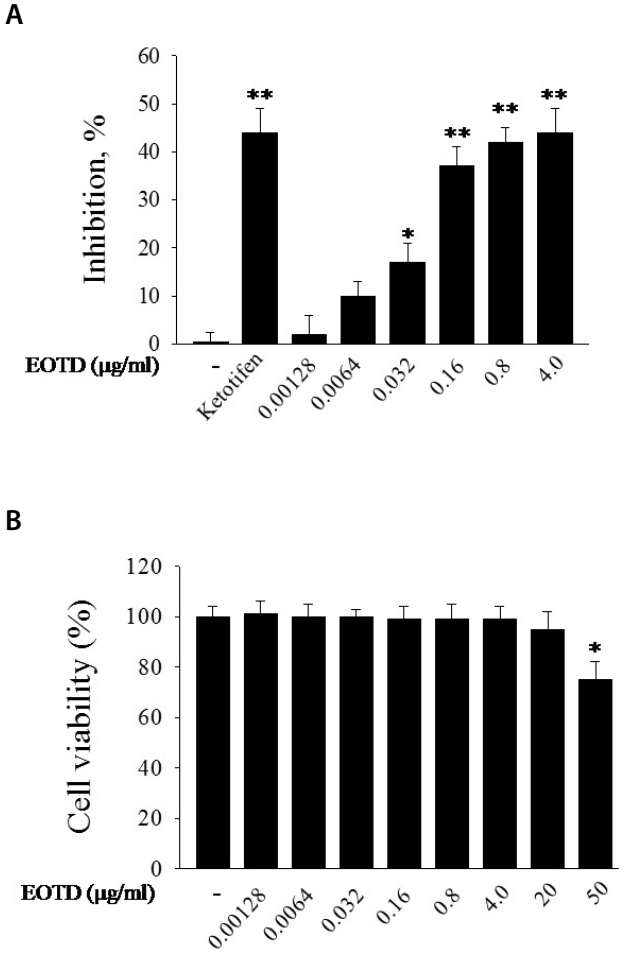

Fig. 4. Effect of EOTD on RBL-2H3 cell viability and β-hexosamidase release. (A) The effect of EOTD (0.00128, 0.0064, 0.032, 0.16, 0.8, and 4 μg/ml) and Ketotifen (4 μg/ml) on β-hexosaminidase release from RBL-2H3. Data are presented as the mean ± SD (n=3). *p<0.01, **p<0.001 (vs. control). (B) Effect of EOTD (0.00128, 0.0064, 0.032, 0.16, 0.8, 4, 20, and 50 μg/ml) on RBL-2H3 cell viability. Survival rate data are presented as mean ± SD (n=3). *p<0.01 (vs. control).

DISCUSSION

Thujopsis dolobrata is widely distributed in Korea and Japan. From this plant, many terpenoids, acidic phenols, tropolones, and lignans have been isolated. Hinokitiol, the major component of essential oil of *Thujopsis dolobrata* (Fig. 1), has been reported to possess antimicrobial and antifungal activities (Morita *et al.*, 2002; Morita *et al.*, 2004). However, the inhibitory effects of EOTD in AD-like skin lesion have not been explored. In this study, we investigated weather EOTD ameliorates AD-like skin lesions and alters the serum levels of allergic factors such as IgE and histamine in NC/Nga mice. AD-like skin lesions were induced in NC/Nga mice by the repeated application of DNCB for two weeks. Then, mice were treated with or without EOTD for a further two weeks. EOTD effectively lowered skin trauma, as measured by clinical scoring (Table 1). AD is often accompanied by severe itching. This causes scratching, which results in secondary infections by bacteria such as *Staphylococcus aureus*, and infiltration lesions by mast cells. Hence, it is noteworthy that EOTD suppress mast cell infiltration into the AD-like skin lesions (Fig. 2). It has previously been demonstrated that elevated serum levels of IgE play an important role in the development of AD-like skin lesions in NC/Nga mice (Matsubara *et al.*, 2009). Furthermore, elevated serum IgE levels were correlated with the degranulation of mast cells and eosinophils. Our results showed that administration of EOTD improved AD-like skin lesions and down-regulated serum histamine and IgE levels (Fig. 3). Based on these findings, we concluded that EOTD inhibits the development of DNCB-induced AD-like skin lesions in NC/Nga mice by reducing serum levels of IgE and histamine. To exclude the possibility that the development of some chronic AD-like skin lesions in NC/Nga mice occurs independently of plasma IgE (Amon *et al.*, 1995; Fischer *et al.*, 2006; Chen *et al.*, 2008), we further investigated the inhibitory effects of EOTD on mast cells degranulation in an *in vitro* assay system. The release of β-hexosaminidase from secretory granules has frequently been used as a measure of mast cell degranulation. As shown in Fig. 4A, EOTD significantly reduced the release of β-hexosaminidase from RBL-2H3 cells without affecting cell proliferation or viability. The decreased β-hexosaminidase activity suggests that EOTD inhibits the mast cell degranulation process, which would be expected to affect serum IgE and histamine levels. When activated mast cells release soluble mediators such as histamine and IgE, they typically induce strong Th2 immune responses and, as a result of acute inflammation, increase scratching behavior. A persistent Th2 immune response may lead to chronic inflammation (Elias, 2008). Serum IgE levels were previously shown to be elevated parallel to the development of AD-like skin lesions in NC/Nga mice (Matsumoto *et al.*, 1999; Kawakami *et al.*, 2009). In the present study, EOTD dose-dependently reduced *in vitro* mast cell degranulation (Fig. 4), lesional epidermal thickness (Fig. 2), and serum IgE and histamine levels (Fig. 3). Serum levels of total serum IgE and hiatamine were measured lower than 25 μg/ml and 30 ng/ml, respectively. While the DNCB (1%) treatment for two weeks reached high more than 120 μg/ml and 40 ng/ml, respectively. However, EOTD treated groups significantly decreased these total IgE and histamine levels in the serum.

Hence, it seems that EOTD effectively suppresses the development of atopic dermatitis-like skin lesions by inhibiting of histamine and IgE secretion and the IgE-mediated degranulation, which may in turn reduce infiltration of mast cells into skin lesions (Fig. 2).

In conclusion, we identified anti-allergic effects of EOTD, both *in vivo* and *in vitro*. EOTD reduced serum histamine and IgE levels and mast cell recruitment to skin lesions in NC/Nga mice with DNCB-induced AD and also inhibited the release of β-hexosaminidase from RBL 2H3 cells. Although further detailed studies are necessary to understand the anti-AD mechanisms of EOTD, our results suggest that EOTD may find application as a natural medicine for alleviating AD-like skin lesions.

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