

Mast Cell Stabilizing Effect of (–)-Elema-1,3,11(13)-trien-12-ol and Thujopsene from *Thujopsis dolabrata* Is Mediated by Down-Regulation of Interleukin-4 Secretion in Antigen-Induced RBL-2H3 Cells

Chul-Hee Kim,^{a,#} Taehun Lee,^{a,#} Ikhoon Oh,^a Kung-Woo Nam,^b Kyeong Ho Kim,^c Ki-Bong Oh,^d Jongheon Shin,^{*,a} and Woongchon Mar^{*,a}

^aNatural Products Research Institute, College of Pharmacy, Seoul National University; Seoul 151–742, Korea; ^bOffice of Industrial Cooperation, Soon Chun Hyang University; Asan-si, Chungcheongnam-do 336–745, Korea; ^cCollege of Pharmacy, Kangwon National University; Chuncheon-si, Gangwon 200–701, Korea; and ^dSchool of Agriculture Biotechnology, Seoul National University; Seoul 151–921, Korea.

Received April 25, 2012; accepted December 26, 2012

Several isolated compounds from the wood part of *Thujopsis dolabrata* were evaluated for their inhibitory effects against antigen-induced mast cell degranulation and interleukin-4 (IL-4) secretion, as well as IL-4 mRNA and protein expression in immunoglobulin E (IgE)-sensitized RBL-2H3 cells. Among the five isolated compounds, (–)-elema-1,3,11(13)-trien-12-ol (1) and thujopsene (2) exhibited the potent inhibitory activity against mast cell degranulation measured by β -hexosaminidase release with IC₅₀ values of 27.4 μ M and 25.1 μ M, respectively. These compounds also inhibited the release of IL-4 (IC₅₀ values of 7.0, 6.7 μ M, respectively), IL-4 mRNA expression (IC₅₀ values of 16.5, 7.2 μ M, respectively) and IL-4 protein expression (IC₅₀ values of 17.0, 9.6 μ M, respectively) in antigen-induced IgE-sensitized RBL-2H3 cells. These results suggested that (–)-elema-1,3,11(13)-trien-12-ol (1) and thujopsene (2) effectively inhibits mast cell degranulation as well as IL-4 production, suggesting that these compounds from *Thujopsis dolabrata* can be used as candidates for IgE-mediated allergic disorders.

Key words *Thujopsis dolabrata*; RBL-2H3 cell; mast cell degranulation; interleukin-4; β -hexosaminidase

The past two decades have witnessed a dramatic increase in the prevalence of allergic diseases.^{1–4)} Allergic diseases are a chronic disorder that is characterized by asthma, rhinitis, and atopic dermatitis. New therapeutic agents are urgently needed since it is still difficult to control and cure with currently used drugs for allergic diseases. Although environmental factors are important in the development of allergic diseases, and the etiology is not fully understood, many pathological molecules involved in this disease have been identified.^{5,6)}

It was previously reported that rat basophilic leukemia RBL-2H3 cells are a mucosal mast cell type that is a major model for the study of immunoglobulin E (IgE)-mediated degranulation^{7,8)} and the stimulation of RBL-2H3 cells with IgE-specific antigen can mimic cell activation by allergens under physiological conditions. Mast cells and basophils play a central role in allergic reactions mediated by IgE.⁹⁾ The interaction of multivalent allergens to specific IgE bound to the IgE receptor (FC ϵ RI) on the mast cells leads to the release of inflammatory mediators such as histamine, serotonin, and arachidonic acid metabolites.^{10,11)}

During immediate-type allergy (type I hypersensitivity), histamine is released from the activated mast cells via a degranulation process by IgE-allergenic stimulation.¹⁰⁾ Mast cell secretory granules contain β -hexosaminidase,¹²⁾ which is released in a quantitative relation to histamine release after the immunologic stimulation of the cells. It was suggested that special intra-cellular and extra-cellular functions for the mast cell granule could be detected by the contents of β -hexosaminidase and by the stimulus for mast cell activation.¹³⁾ Thus, the measurement of β -hexosaminidase release

could be used as a biomarker of the allergic response of the mast cells degranulation.^{13–15)}

Cytokines play a pivotal role in the development of allergy by regulating the expansion of T helper 2 (Th2) cells and by mediating many of the functions of Th2 effectors that underlie the pathogenic events of allergic responses. Interleukin-4 (IL-4) is a Th2 cytokine that participates in the immune responses against parasitic infections and in the development of allergic diseases playing an essential role in IgE regulation in the induction of Th2 phenotype differentiation on T cells.¹⁶⁾

In hematopoietic cells, IL-4 exerts its activities by interacting with a specific cell surface receptor comprised of a binding component, IL-4R α , and the common cytokine receptor γ -chain (γ C), which is shared by multiple cytokine receptors.^{17,18)} The binding of IL-4 to the IL-4 receptor (IL-4R) induces the initial response for Th2 lymphocyte polarization. The IL-4 produced by Th2 cells is capable of inducing the isotype class-switching of B-cells to produce IgE after allergen exposure.^{19,20)}

Thujopsis dolabrata, which belongs to the cypress family (Cupressaceae), is an endemic tree in Japan. The seeds, leaves, trunks, and branches contain characteristic secondary metabolites such as sesquiterpenes and hinokitiol-related compounds,²¹⁾ and their reported bioactivities were antifungal,²¹⁾ antimicrobial,^{22–24)} antiplatelet,²⁵⁾ insecticidal, and acaricidal activity.²⁶⁾ (–)-Elema-1,3,11(13)-trien-12-ol (1) has been reported that (–)-elema-1,3,11(13)-trien-12-ol (1) has inhibitory effects on Na⁺/K⁺-ATPase activity²⁷⁾ and inhibits lipopolysaccharide (LPS)-induced nitric oxide (NO) production,²⁸⁾ and thujopsene (2) has antibacterial activity.²⁷⁾ In our previous study, it was reported that essential oil from *Thujopsis dolabrata* reduced both serum levels of histamine and IgE in atopic dermatitis (AD)-like skin lesions induced

The authors declare no conflict of interest.

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed. e-mail: shinj@snu.ac.kr; mars@snu.ac.kr

© 2013 The Pharmaceutical Society of Japan

by 2,4-dinitrochlorobenzene (DNCB) in NC/Nga mice, and furthermore it presented a mast cell stabilizing effect by inhibiting the release of β -hexosaminidase from antigen-induced RBL-2H3 mast cells.²⁹⁾

In present study, two compounds, (-)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) isolated from essential oil of *Thujopsis dolabrata* were investigated for the inhibition of mast cell degranulation, IL-4 secretion, and IL-4 mRNA and protein expression in antigen-induced RBL-2H3 mast cells.

MATERIALS AND METHODS

Plant Material Wood part of *Thujopsis dolabrata* (Cupressaceae) were collected from Chunlanam-do, Korea in 2008 and identified by Dr. Hak Ju Lee (Korea Forest Research Institute). A voucher specimen (NPRI-CN02) is on deposit at the Natural Products Research Institute, College of Pharmacy, Seoul National University.

Extraction and Isolation The wood part of *Thujopsis dolabrata* (10 kg) was steam distilled for 3 h using a Clevenger-type apparatus, yielding 141 g of essential oil. An aliquot of the essential oil (54.9 g) was partitioned between *n*-hexane (43.6 g) and 15% aq. MeOH (7.8 g). An aliquot of the *n*-hexane (10.5 g) was subjected to silica normal-phase vacuum flash chromatography using sequential mixtures of *n*-hexane and EtOAc as eluents (elution order: 100% *n*-hexane, 10%, 20%, 30%, 40%, 50%, 60%, 70% EtOAc in *n*-hexane, and 100% EtOAc), 100% acetone, and 100% MeOH. Inhibitory effects of β -hexosaminidase released in antigen-induced mast cells were measured from each fraction and a bioactivity-guided fractionation procedure followed.

A portion (537 mg) of the fraction eluted with 20% EtOAc in *n*-hexane from flash chromatography was separated by reversed-phase HPLC (15% aq. MeOH), then further purified by reversed-phase HPLC (55% aq. MeCN) to afford 11.6 mg and 14.3 of (-)-elema-1,3,11(13)-trien-12-ol (**1**)³⁰⁾ and thujopsene-12-ol (**3**)²⁷⁾ respectively.

The aliquot (551 mg) of the fraction eluted with 100% *n*-hexane from flash chromatography was separated by silica HPLC (YMC-silica, 100% *n*-hexane), then further purified by reversed-phase HPLC (YMC ODS-A column, 1 cm $\times$ 25 cm, 5% aq. MeOH) to afford 451.4 and 14.3 mg of thujopsene (**2**)²⁷⁾ and cuparene (**4**)²⁷⁾ respectively.

Finally an aliquot (289 mg) of the fraction eluted with 30% EtOAc in *n*-hexane from flash chromatography was separated by reversed-phase HPLC (25% aq. MeOH) to afford 5.3, 6.1, 7.4 mg of (-)-elema-1,3,11(13)-trien-12-ol (**1**), thujopsene-12-ol (**3**), and γ -cuparene (**5**)²⁷⁾ respectively.

Based upon the results of combined spectroscopic analysis and comparison of the ¹H- and ¹³C-NMR spectral data, the purified compounds exhibited a comparable spectroscopy to previously published values.

Materials Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich (St. Louis, U.S.A.). Fetal bovine serum (FBS) was purchased from Gibco (Carlsbad, U.S.A.). Monoclonal anti-dinitrophenyl (DNP)-IgE and 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). DNP-conjugated bovine serum albumin (DNP-BSA) were obtained from Invitrogen (Carlsbad, U.S.A.). ONE-STEP reverse transcription-polymerase chain reaction (RT-PCR) PreMix kit and

easy-BLUE[®], total RNA extraction kit, PRO-PREP protein extraction solution and WEST-ZOL[®] plus were purchased from iNtRON (Kyunggi, Korea). IL-4 mouse monoclonal IgG was purchased from Santa Cruz Biotechnology, Inc. (CA, U.S.A.). Rat IL-4 Platinum enzyme-linked immunosorbent assay (ELISA) kit was purchased from eBioscience (San Diego, U.S.A.).

Cell Culture The Rat basophilic leukemia (RBL-2H3) cells (ATCC No. CRL-2256) were grown in 37°C in 5% CO₂ in DMEM supplemented with 8% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Cell Viability Assay Cell viability assay was conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.³¹⁾ RBL-2H3 cells (5 $\times$ 10⁴ cells/well) were cultured into a 96-well plate for 24 h and incubated with various concentrations of samples (0.32–200 μ M) for another 24 h. Cell viability was determined by treatment with MTT dissolved in phenol-red free medium (250 μ g/mL) at 37°C for 4 h. The medium was carefully removed and resuspended with 250 μ L of dimethyl sulfoxide (DMSO) and the production of the colored MTT metabolite formazan was quantified by measuring at 595 nm using a microplate reader. Values obtained from untreated cells were considered to represent 100% viability.

β -Hexosaminidase Release Assay RBL-2H3 cells were plated in 96-well plates (5 $\times$ 10⁵ cells/well) using DMEM with 10% FBS, incubated for 24 h with anti-DNP IgE (1 μ g/mL) for sensitization, and the cells were washed with *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.2) to eliminate free IgE. Samples were then treated for 20 min with different concentrations (1.6, 8, 20, 40 μ M) in releasing buffer (116.9 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄·7H₂O, 5.6 mM glucose, 25 mM HEPES, 2.0 mM CaCl₂ and 1.0 mg/mL BSA at pH 7.7). After that, DNP-BSA (400 ng/mL) was incubated for 1 h for mast cell activation and degranulation and the reaction was stopped by cooling in an ice bath for 20 min. After centrifugation, the supernatant was transferred into 96-well plates and 5 mM of substrate (*p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) solution was mixed and incubated for 90 min at 37°C. The reaction was stopped by the addition of 0.1 M Na₂CO₃/NaHCO₃ (200 μ L/well) and the absorbance was measured at 405 nm.³²⁾

Cytokine Secretion Assay RBL-2H3 cells (1 $\times$ 10⁶ cells/well) were plated in 6-well, incubated with anti-DNP-IgE for 24 h, and washed with PBS buffer to eliminate free IgE. The cells were then pre-treated with different concentrations of samples in fresh media for 3 h, and induced by antigen (400 ng/mL of DNP-BSA) in fresh media for additional 2 h, and supernatants were used for the measurement of cytokines using Rat IL-4 Platinum ELISA kit according to manufacturer's instruction (eBioscience, San Diego, U.S.A.).

RT-PCR RBL-2H3 cells (5 $\times$ 10⁵ cells/well) were plated onto 12-well plates. The cells were sensitized with anti-DNP-IgE (1 μ g/mL) for 24 h at 37°C. The cells were washed with PBS buffer to eliminate free IgE. After washing, the cells were pre-treated at 37°C for 3 h with different concentrations of samples in fresh media, and incubated at 37°C for additional 1 h with antigen (400 ng/mL of DNP-BSA) in fresh media. After antigen-stimulation, total RNA was isolated by using easy-BLUE[®] (iNtRON, Kyunggi, Korea). cDNA synthesis was performed from 1 μ g of total RNA by using ONE-STEP

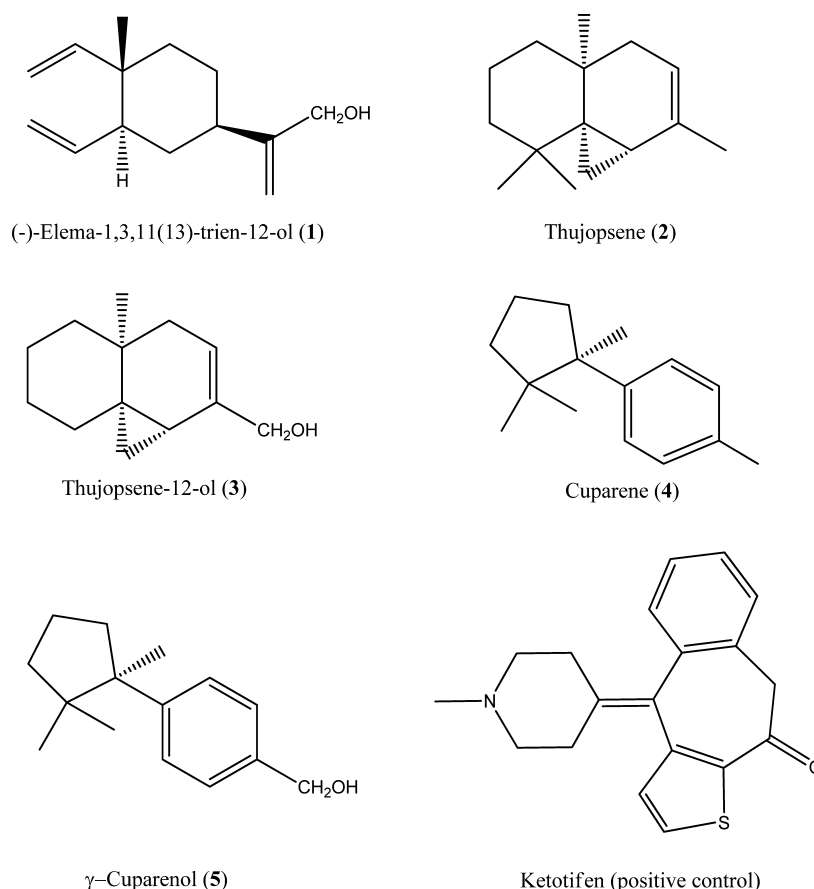


Fig. 1. Chemical Structures of Isolated Compounds from *Thujopsis dolabrata*

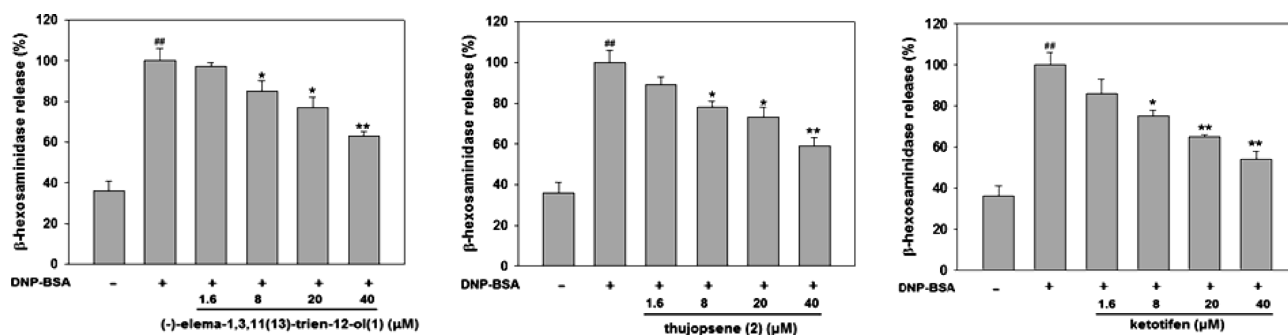


Fig. 2. Inhibitory Effect of (-)-Elema-1,3,11(13)-trien-12-ol (1) and Thujopsene (2) on β -Hexosaminidase Release from Antigen-Induced RBL-2H3 Cells

Ketotifen was used as a positive control compound. Data are expressed as mean $\pm$ S.D. ($^{##}p < 0.01$, compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, compared with DNP-BSA treatment group). All data were obtained from at least three independent experiments.

RT-PCR PreMix kit (iNtRON, Kyunggi, Korea). The following primer pairs were synthesized. IL-4, forward 5'-ACC TTG CTG TCA CCC TGT TC-3' and reverse 5'-TTG TGA GCG TGG ACT CAT TC-3'; β -actin, forward 5'-GAC CCA GAT CAT GTT TGA GA-3' and reverse 5'-GCT TGC TGA TCC ACA TCT GC-3'. The denaturation, annealing, extension, and cycle conditions were as follows: IL-4: 94°C for 45 s, 58°C for 45 s and 72°C for 45 s and 35 cycles; β -actin: 94°C for 45 s, 55°C for 45 s and 72°C for 45 s and 25 cycles. The PCR reaction was performed with a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, U.S.A.). The PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen, Calsbad, U.S.A.). The

detection and densitometric analysis of bands were performed with an image analyzer, LAS 1000plus (FUJIFILM, Tokyo, Japan) and Multi Gauge software (FUJIFILM, Tokyo, Japan). The sizes of bands were confirmed with reference to molecular size markers, 100bp DNA Ladder Marker (TaKaRa, Shiga, Japan). The value of each cytokine mRNA was normalized to the amount of β -actin signal.³³⁾

Immunoblotting RBL-2H3 cells (1×10^6 cells/well) were cultured for 24 h in 6-well, and then pre-treated with different concentrations of samples for 3 h and incubated at 37°C for additional 2 h with DNP-BSA ($4 \mu\text{g/mL}$) in fresh media. Then cells were lysed by PRO-PREP™ protein extraction solution (iNtRON, Kyunggi, Korea) for 20 min at -20°C . After

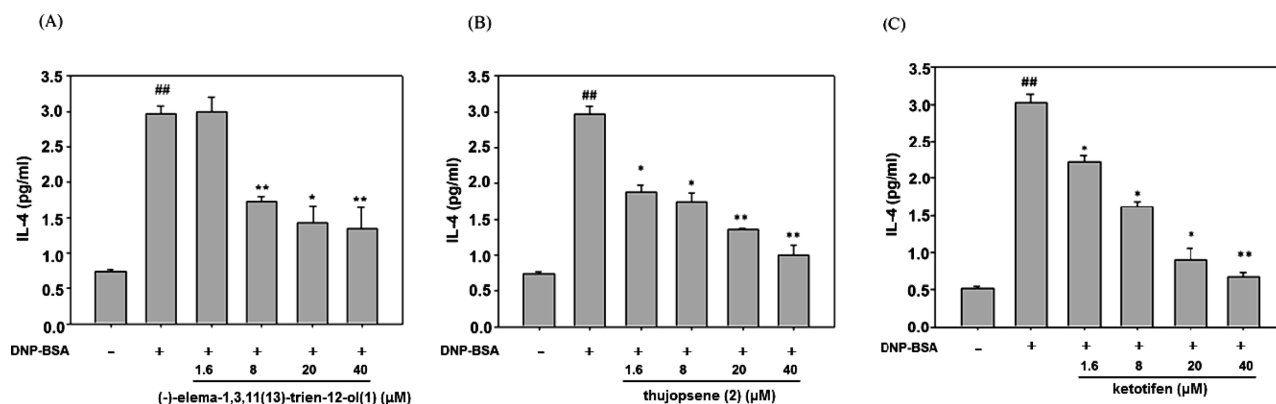


Fig. 3. Inhibitory Effect of (-)-Elema-1,3,11(13)-trien-12-ol (1) and Thujopsene (2) on the Secretion of IL-4 in Antigen-Induced RBL-2H3 Cells

Ketotifen was used as a positive control compound. Data are expressed as mean $\pm$ S.D. (## $p < 0.01$, compared with control group, * $p < 0.05$, ** $p < 0.01$, compared with DNP-BSA treatment group). All data were obtained from at least three independent experiments.

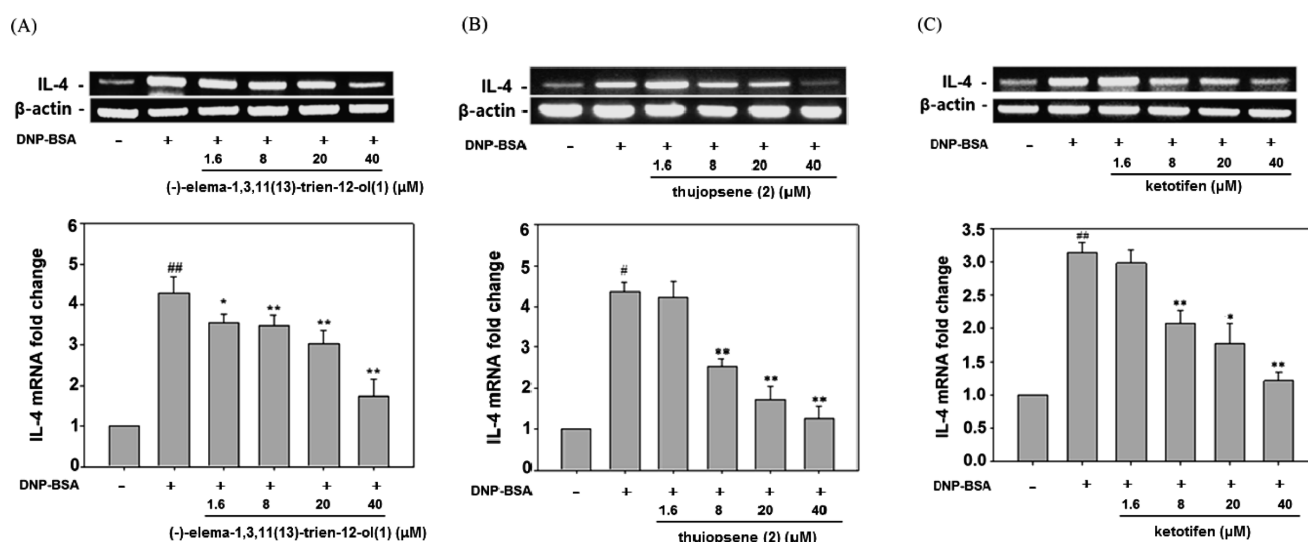


Fig. 4. Inhibitory Effect of (-)-Elema-1,3,11(13)-trien-12-ol (1) and Thujopsene (2) on IL-4 mRNA Expression in Antigen-Induced RBL-2H3 Cells

IL-4 mRNA expression was quantified using Image J software. Ketotifen was used as a positive control compound. Data are expressed as mean $\pm$ S.D. (# $p < 0.05$, ## $p < 0.01$, compared with control group, * $p < 0.05$, ** $p < 0.01$, compared with DNP-BSA treatment group). All data were obtained from at least three independent experiments.

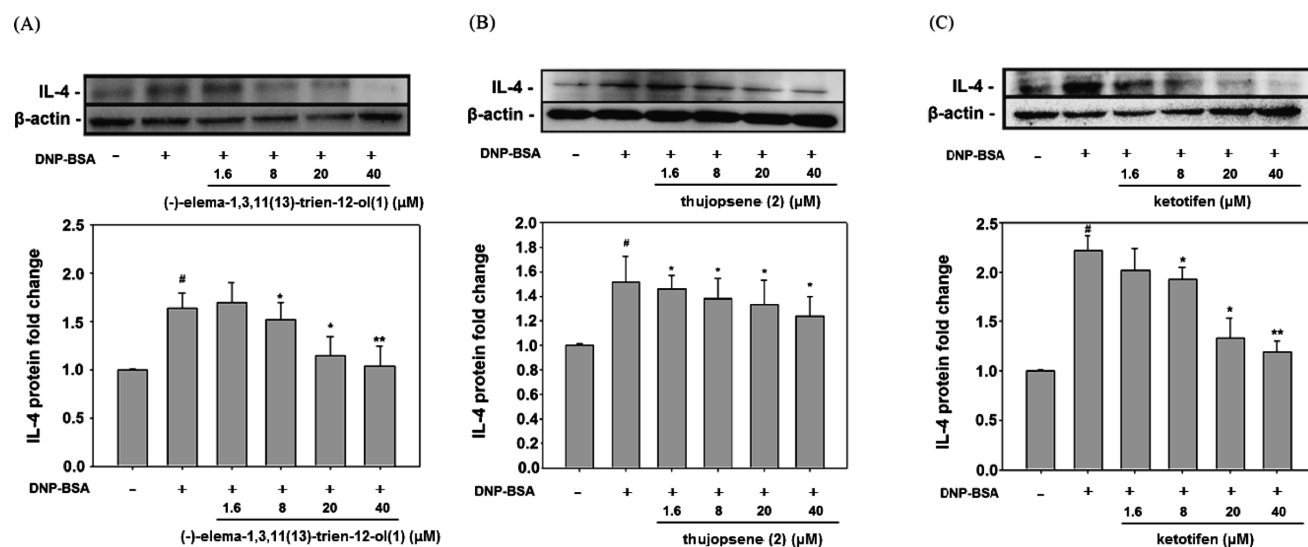


Fig. 5. Inhibitory Effect of (-)-Elema-1,3,11(13)-trien-12-ol (1) and Thujopsene (2) on IL-4 Protein Expression in Antigen-Induced RBL-2H3 Cells

IL-4 protein expression was quantified using Image J software. Ketotifen was used as a positive control compound. Data are expressed as mean $\pm$ S.D. (# $p < 0.05$, compared with control group, * $p < 0.05$, ** $p < 0.01$, compared with DNP-BSA treatment group). All data were obtained from at least three independent experiments.

incubation, lysates were centrifuged at 13000rpm for 10 min at 4°C for clarification. Protein (20 µg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) transfer membrane, and blocked with 5% skim milk at room temperature for 2h. IL-4 mouse monoclonal IgG (1:1000, Santa Cruz Biotechnology, CA, U.S.A.) were incubated with membranes at 4°C overnight. Membranes were then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibodies for 1h and developed with West-zol™ plus (iNtRON, Kyunggi, Korea). Immunoreactivity was detected by an image analyzer, LAS-1000plus (FUJIFILM, Tokyo, Japan).³⁴⁾

Statistical Analysis All data are expressed as mean S.D. of at least four independent experiments performed. Statistical significance was determined by using GraphPad Prism (GraphPad Software, CA, U.S.A.). The differences among groups were evaluated by one-way analysis of variance (ANOVA) test with Bonferroni's multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Allergic reactions are stated by uptake of allergens and are recognized by antigen-presenting cells.³⁵⁾ Recognized cells affect the precursor state of T helper cells to differentiate T_H type 1 cells and T_H type 2 cells. T helper cells affected by allergens simply prefer to differentiated into T_H type 2 cell and this leads to an inappropriate balance between T_H type 1 and T_H type 2 responses resulting allergic reactions.³⁶⁾

Five compounds, (–)-elema-1,3,11(13)-trien-12-ol (**1**), thujopsene (**2**), thujopsene-12-ol (**3**), cuparene (**4**), and γ -cuparene (**5**), were isolated from the wood part of *Thujopsis dolabrata* (Fig. 1). All five compounds were tested for their cytotoxicity in RBL-2H3 cells determined by using MTT assay at various concentrations and these showed no significant cytotoxicity at concentrations below 100 µM (Fig. 1S).

The interaction of allergens with IgE-sensitized mast cells stimulates cells to degranulate and release allergic mediators such as histamine, serotonin, β -hexosaminidase³⁷⁾ and cytokines, including IL-4 and IL-5.³⁸⁾ The inhibition of mast cell degranulation was determined by measuring the release of β -hexosaminidase in antigen-induced RBL-2H3 cells. (–)-Elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) and revealed an inhibitory effects with IC_{50} values of 27.4 µM and 25.1 µM, respectively, whereas thujopsene-12-ol (**3**), cuparene (**4**) and

γ -cuparene (**5**) revealed no significant effect at concentrations tested (IC_{50} values >100 µM). A positive control compound, ketotifen, well-known as an H-1 antagonist and a mast cell stabilizer,^{39,40)} showed an IC_{50} value of 16.5 µM (Fig. 2).

Interleukin-4 is a cytokine that regulates multiple biological processes during immune responses.⁴¹⁾ It can regulate proliferation, differentiation, and apoptosis in lymphocytes and in other cell types of hemological and nonhemological origin.⁴²⁾ One of the important effects of IL-4 is the regulation of T cell differentiation, and IL-4-driven Th2 cell differentiation is an important step during responses against parasitic infections.⁴³⁾ In contrast, alterations of IL-4 and its signaling machinery are believed to participate in the progression of diseases like autoimmunity, allergy, and cancer.⁴⁴⁾ (–)-Elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) and were further tested for inhibitory effect on IL-4 secretion and IL-4 mRNA/protein expression levels in antigen-induced RBL-2H3 cells. Treatment with (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) decreased the IL-4 secretion (IC_{50} values of 7.0, 6.7 µM, respectively) (Fig. 3), IL-4 mRNA expression (IC_{50} values of 16.5, 7.2 µM, respectively) (Fig. 4) and IL-4 protein expression (IC_{50} value of 17.0, 9.6 µM, respectively) (Fig. 5) levels. This inhibitory effect of (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) were as potent as that of the positive control compound, ketotifen, previously reported as an inhibitor of IL-4 expression¹⁴⁾ (Table 1). Whereas compounds **3**, **4**, and **5** showed no effect at concentrations tested (data not shown). Ketotifen inhibits IL-4 secretion (IC_{50} value of 6.0 µM) (Fig. 3C), IL-4 mRNA expression (IC_{50} value of 7.2 µM) (Fig. 4C), and IL-4 protein expression (IC_{50} value of 7.6 µM) (Fig. 5C) levels. Thujopsene-12-ol (**3**), cuparene (**4**) and γ -cuparene (**5**) revealed no significant effect at concentrations tested (IC_{50} values >100 µM). IL-4 derived from mast cells are believed to mediate late allergic symptoms.⁴¹⁾ A previous study reported that potentiation of anti-IgE-induced degranulation was augmented in mast cells cultured with IL-4.⁴⁵⁾ Also IL-4 derived from mast cells are believed to mediated late allergic symptoms,⁴¹⁾ and suppression of cytokine expression including IL-4 has been reported to attribute to the alleviation of allergic symptoms.¹⁴⁾ Thus, the inhibitory effects of (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) on mast cell degranulation might be explained by their abilities to suppress IL-4 expression. It is still unclear and needs to be tested how these compound (**1**) and (**2**) suppress IL-4 expression. A study has suggested that (–)-elema-1,3,11(13)-trien-12-ol (**1**) inhibits nitric oxide (NO) production⁴⁶⁾ which crucially depends on the

Table 1. Inhibitory Effect of Compounds from *Thujopsis dolabrata* on the Release of β -Hexosaminidase, IL-4 Secretion, IL-4 mRNA and Protein Expression in Antigen-Induced RBL-2H3 Cells

Compounds	IC_{50}^b (µM)			
	β -Hexosaminidase release	IL-4 secretion	IL-4 mRNA expression	IL-4 protein expression
(–)-Elema-1,3,11(13)-trien-12-ol (1)	27.4±1.6	7.0±0.9	16.5±1.0	17.0±1.2
Thujopsene (2)	25.1±0.9	6.7±0.2	7.2±0.6	9.6±0.1
Thujopsene 12-ol (3)	>100	>100	>100	>100
Cuparene (4)	>100	>100	>100	>100
γ -Cuparene (5)	>100	>100	>100	>100
Ketotifen ^{a)}	16.5±0.3	6.0±0.2	7.2±0.2	7.6±0.5

a) This compound was used as a positive control (Passante *et al.*, 2009). b) IC_{50} value represents the concentration giving 50% inhibitory measure of the effectiveness in antigen-induced RBL-2H3 cells. The values were determined in a semilogarithmic graph depicting the relationship between at least 4 different concentrations of compounds and the each percentage of inhibition. Each value represents the mean±S.D. from at least three independent experiments.

activity of nuclear factor kappa B (NF- κ B).⁴⁷⁾ Since NF- κ B is known as a key transcription factor for cytokines,^{48,49)} this pathway would be a plausible target to investigate the mode of action by of (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**).

In this report it was demonstrated that (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) dose-dependently inhibited the release of β -hexosaminidase, the secretion of IL-4 and IL-4 mRNA/protein expression levels in antigen-induced RBL-2H3 cells without causing significant cytotoxicity at concentrations tested. Based on our investigation, (–)-elema-1,3,11(13)-trien-12-ol (**1**) and thujopsene (**2**) might be used as potential candidates for allergic disorders.

Acknowledgement This work was supported by Mid-career Researcher Program through National Research Foundation of Korea (NRF Grant No.: 2011-0027501) funded by the MEST.

REFERENCES

- 1) Etzel RA. How environmental exposures influence the development and exacerbation of asthma. *Pediatrics*, **112**, 233–239 (2003).
- 2) Bornehag CG, Sundell J, Weschler CJ, Sigsgaard T, Lundgren B, Hasselgren M, Hägerhed-Engman L. The association between asthma and allergic symptoms in children and phthalates in house dust: a nested case-control study. *Environ. Health Perspect.*, **112**, 1393–1397 (2004).
- 3) Rönmark E, Bjerg A, Perzanowski M, Platts-Mills T, Lundbäck B. Major increase in allergic sensitization in schoolchildren from 1996 to 2006 in northern Sweden. *J. Allergy Clin. Immunol.*, **124**, 357–363, e351–e315 (2009).
- 4) Beasley R, Crane J, Lai CK, Pearce N. Prevalence and etiology of asthma. *J. Allergy Clin. Immunol.*, **105**, S466–S472 (2000).
- 5) Cookson W, Moffatt M. Making sense of asthma genes. *N. Engl. J. Med.*, **351**, 1794–1796 (2004).
- 6) Goswami A, S Raghavan S, Ghotekar LH. Postcoital haemoperitoneum: a cause for shock. *Emerg. Med. Australas.*, **16**, 170–171 (2004).
- 7) Sur R, Hall J, Cavender D, Malaviya R. Role of Janus kinase-2 in IgE receptor-mediated leukotriene C4 production by mast cells. *Biochem. Biophys. Res. Commun.*, **390**, 786–790 (2009).
- 8) Passante E, Frankish N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflamm. Res.*, **58**, 737–745 (2009).
- 9) Marquardt DL. Histamine. *Clin. Rev. Allergy*, **1**, 343–351 (1983).
- 10) Barsumian EL, Isersky C, Petrino MG, Siraganian RP. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.*, **11**, 317–323 (1981).
- 11) Taurog JD, Fewtrell C, Becker EL. IgE mediated triggering of rat basophil leukemia cells: lack of evidence for serine esterase activation. *J. Immunol.*, **122**, 2150–2153 (1979).
- 12) Han EH, Park JH, Kim JY, Chung YC, Jeong HG. Inhibitory mechanism of saponins derived from roots of *Platycodon grandiflorum* on anaphylactic reaction and IgE-mediated allergic response in mast cells. *Food Chem. Toxicol.*, **47**, 1069–1075 (2009).
- 13) Schwartz LB, Austen KF, Wasserman SI. Immunologic release of beta-hexosaminidase and beta-glucuronidase from purified rat serosal mast cells. *J. Immunol.*, **123**, 1445–1450 (1979).
- 14) Matsubara M, Masaki S, Ohmori K, Karasawa A, Hasegawa K. Differential regulation of IL-4 expression and degranulation by anti-allergic olopatadine in rat basophilic leukemia (RBL-2H3) cells. *Biochem. Pharmacol.*, **67**, 1315–1326 (2004).
- 15) Lynch SM, Austen KF, Wasserman SI. Release of arylsulfatase A but not B from rat mast cells by noncytolytic secretory stimuli. *J. Immunol.*, **121**, 1394–1399 (1978).
- 16) Pernis AB, Rothman PB. JAK-STAT signaling in asthma. *J. Clin. Invest.*, **109**, 1279–1283 (2002).
- 17) Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, Ferrara P. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. *FEBS Lett.*, **401**, 163–166 (1997).
- 18) Hershey GK. IL-13 receptors and signaling pathways: an evolving web. *J. Allergy Clin. Immunol.*, **111**, 677–690, quiz, 691 (2003).
- 19) Aalberse RC. Structural features of allergenic molecules. *Chem. Immunol. Allergy*, **91**, 134–146 (2006).
- 20) Snapper CM, Paul WE. B cell stimulatory factor-1 (interleukin 4) prepares resting murine B cells to secrete IgG1 upon subsequent stimulation with bacterial lipopolysaccharide. *J. Immunol.*, **139**, 10–17 (1987).
- 21) Morita Y, Matsumura E, Okabe T, Fukui T, Ohe T, Ishida N, Inamori Y. Biological activity of beta-dolabrin, gamma-thujaplicin, and 4-acetyltopolone, hinokitiol-related compounds. *Biol. Pharm. Bull.*, **27**, 1666–1669 (2004).
- 22) Okabe T, Saito K, Fukui T, Iinuma K. Antibacterial activity of hinokitiol against methicillin-resistant *Staphylococcus aureus* (MRSA). *Mokuzai Gakkaishi*, **40**, 1233–1238 (1994).
- 23) Saito K, Okabe T, Fukui T, Inamori Y. Antibacterial activity of hinokitiol against methicillin-resistant *Staphylococcus aureus* (MRSA). 2. Applications to the disinfections of hospital environments. *Mokuzai Gakkaishi*, **43**, 882–891 (1997).
- 24) Inamori Y, Sakagami Y, Morita Y, Shibata M, Sugiura M, Kumeda Y, Okabe T, Tsujibo H, Ishida N. Antifungal activity of hinokitiol-related compounds on wood-rotting fungi and their insecticidal activities. *Biol. Pharm. Bull.*, **23**, 995–997 (2000).
- 25) Son DJ, Park YH, Kim YM, Chung NH, Lee HS. Antiplatelet activity of *Thujopsis dolabrata* var. *hondai*-derived component against platelet aggregation. *J. Microbiol. Biotechnol.*, **15**, 425–427 (2005).
- 26) Ahn YJ, Lee SB, Lee HS, Kim GH. Insecticidal and acaricidal activity of carvacrol and beta-thujaplicine derived from *Thujopsis dolabrata* var. *hondai* sawdust. *J. Chem. Ecol.*, **24**, 81–90 (1998).
- 27) Oh I, Yang WY, Park J, Lee S, Mar W, Oh KB, Shin J. *In vitro* Na⁺/K⁺-ATPase inhibitory activity and antimicrobial activity of sesquiterpenes isolated from *Thujopsis dolabrata*. *Arch. Pharm. Res.*, **34**, 2141–2147 (2011).
- 28) Inoue K. Complement activation and biological activities. *Nippon Saikungaku Zasshi*, **31**, 535–555 (1976).
- 29) Nam KW, Noh JK, Kim SK, Lee SJ, Kim KH, Oh KB, Shin J, Mar W. Essential oil of *Thujopsis dolabrata* suppresses atopic dermatitis-like skin lesions in NC/Nga mice. *Biomol Ther*, **19**, 102–108 (2011).
- 30) Maurer B, Grieder A. Sesquiterpenoids from costum root oil (*Saussurea lappa* CLARKE). *Helv. Chim. Acta*, **60**, 2177–2190 (1977).
- 31) Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br. J. Cancer*, **56**, 279–285 (1987).
- 32) Schwartz LB, Austen KF. Enzymes of the mast cell granule. *J. Invest. Dermatol.*, **74**, 349–353 (1980).
- 33) Matsuda H, Wang Q, Matsuhira K, Nakamura S, Yuan D, Yoshikawa M. Inhibitory effects of thunberginols A and B isolated from *Hydrangeae Dulcis* Folium on mRNA expression of cytokines and on activation of activator protein-1 in RBL-2H3 cells. *Phytomedicine*, **15**, 177–184 (2008).
- 34) Powner DJ, Hodgkin MN, Wakelam MJ. Antigen-stimulated activation of phospholipase D1b by Rac1, ARF6, and PKCalpha in RBL-2H3 cells. *Mol. Biol. Cell*, **13**, 1252–1262 (2002).
- 35) Braga M, Quecchia C, Cavallucci E, Di Giampaolo L, Schiavone C, Petrarca C, Di Gioacchino M. T regulatory cells in allergy. *Int. J. Immunopathol. Pharmacol.*, **24** (Suppl.), 55S–64S (2011).

- 36) McGuirk P, Higgins SC, Mills KH. The role of regulatory T cells in respiratory infections and allergy and asthma. *Curr. Allergy Asthma Rep.*, **10**, 21–28 (2010).
- 37) Passante E, Ehrhardt C, Sheridan H, Frankish N. RBL-2H3 cells are an imprecise model for mast cell mediator release. *Inflamm. Res.*, **58**, 611–618 (2009).
- 38) Reyes HR, Orozco AR, Nafarrate SS. IL-4, IL-5 and IL-13 expression during *in vitro* immune response to fungal allergens in sensitized subjects. *Rev. Alerg. Mex.*, **55**, 181–188 (2008).
- 39) Joly F, Bessou G, Benveniste J, Ninio E. Ketotifen inhibits paf-acether biosynthesis and beta-hexosaminidase release in mouse mast cells stimulated with antigen. *Eur. J. Pharmacol.*, **144**, 133–139 (1987).
- 40) Schoch C. *In vitro* inhibition of human conjunctival mast-cell degranulation by ketotifen. *J. Ocul. Pharmacol. Ther.*, **19**, 75–81 (2003).
- 41) Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol. Rev.*, **77**, 1033–1079 (1997).
- 42) Zamorano J, Wang HY, Wang LM, Pierce JH, Keegan AD. IL-4 protects cells from apoptosis *via* the insulin receptor substrate pathway and a second independent signaling pathway. *J. Immunol.*, **157**, 4926–4934 (1996).
- 43) Yanagida M, Fukamachi H, Ohgami K, Kuwaki T, Ishii H, Uzumaki H, Amano K, Tokiwa T, Mitsui H, Saito H, Iikura Y, Ishizaka T, Nakahata T. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood*, **86**, 3705–3714 (1995).
- 44) Urban JF Jr, Noben-Trauth N, Donaldson DD, Madden KB, Morris SC, Collins M, Finkelman FD. IL-13, IL-4 α , and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *Nippostrongylus brasiliensis*. *Immunity*, **8**, 255–264 (1998).
- 45) Hua X, Chason KD, Patel JY, Naselsky WC, Tilley SL. IL-4 amplifies the pro-inflammatory effect of adenosine in human mast cells by changing expression levels of adenosine receptors. *PLoS ONE*, **6**, e24947 (2011).
- 46) Matsuda H, Toguchida I, Ninomiya K, Kageura T, Morikawa T, Yoshikawa M. Effects of sesquiterpenes and amino acid-sesquiterpene conjugates from the roots of *Saussurea lappa* on inducible nitric oxide synthase and heat shock protein in lipopolysaccharide-activated macrophages. *Bioorg. Med. Chem.*, **11**, 709–715 (2003).
- 47) Xie QW, Cho H, Kashiwabara Y, Baum M, Weidner JR, Elliston K, Mumford R, Nathan C. Carboxyl terminus of inducible nitric oxide synthase. Contribution to NADPH binding and enzymatic activity. *J. Biol. Chem.*, **269**, 28500–28505 (1994).
- 48) Andreacos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM. Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. *Blood*, **103**, 2229–2237 (2004).
- 49) Li-Weber M, Giaisi M, Treiber MK, Krammer PH. The anti-inflammatory sesquiterpene lactone parthenolide suppresses IL-4 gene expression in peripheral blood T. *Eur. J. Immunol.*, **32**, 3587–3597 (2002).