J. Microbiol. Biotechnol. (2006), **16**(11), 1747–1752

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Fractions of *Chamaecyparis obtusa* Display Antiallergic Effect in RBL2H3 Cells

CHOI, INGYU[1], KYUNGJONG KIM[2], YOUNGMI KIM[2], MI-JIN PARK[1], YUN-SIL LEE[3], AND DOOIL JEOUNG[2]*

[1]*Department of Forest Sciences, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*
[2]*School of Biological Sciences, College of Natural Sciences, Kangwon National University, Chunchon 200-701, Korea*
[3]*Laboratory of Radiation Effect, Division of Radiation Biology, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Korea*

Received: May 19, 2006
Accepted: August 8, 2006

Abstract Allergic inflammation results from stimulation of β-hexosaminidase secretion, increased calcium influx, and activation of MAPK pathways. Some fractions of *Chamaecyparis obtusa* decreased secretion of β-hexosaminidase, calcium influx, ROS, and phosphorylation of ERK. These results suggest that *Chamaecyparis obtusa* would be valuable for development of allergy therapeutics.

Key words: Allergic inflammation, β-hexosaminidase, calcium influx, *Chamaecyparis obtusa*, ERK, reactive oxygen species

Medicinal herbs have commonly been used in health care in many countries [3, 8, 12]. Aloe vera is a complex plant containing biologically active substances, and glycoprotein extracted from aloe vera exhibits strong antiinflammatory responses and antiallergic reactions [15]. *Chamaecyparis obtusa* is a conifer in the cypress family Cupressaceae, native to central Japan, which is commonly known as Hinoki Cypress or Hinoki. Beta-thujaplicin, a major component of essential oil of *Chamaecyparis obtusa*, exhibits antimicrobial activity [7], and suppresses ultraviolet B-induced apoptosis in keratinocytes, and its effect is dependent on the induction of metallothionein [1]. Mast cells and basophils play essential roles in the pathogenesis of allergic reactions such as atopic dermatitis and asthma. RBL-2H3 (rat basophilic leukemia) cells are mucosal mast cells that express the immunoglobulin receptor FcεRI. Stimulation of IgE-sensitized RBL-2H3 cells with specific antigen triggers a cascade of events, leading to degranulation, mediator release, activation of MAPK, tyrosine kinase and phospholipase C, ROS production calcium influx, and cytokine production [2, 14, 16]. This cascade of events suggests that mast cells are ideal targets for controlling various allergic diseases, including atopic dermatitis, allergic rhinitis, and asthma [9, 10, 13, 17]. Here, we report effects of fractions of *Chamaecyparis obtusa* on allergic inflammations.

*Corresponding author
Phone: 82-33-250-8548; Fax: 82-33-242-0459;
E-mail: jeoungd@kangwon.ac.kr

MATERIALS AND METHODS

Cell Lines and Cell Culture

RBL-2H3 cells were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle's medium containing heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, San Diego, CA, U.S.A.). Cultures were maintained in 5% CO_2 at 37°C.

Fractionation of Essential Oil

The essential oil from *Chamaecyparis obtusa* was fractionated by silica gel column chromatography in order to identify the active constituents of the essential oil. The essential oil (240 g) was loaded on a silica gel column (15×31 cm), which was eluted with a mixture of hexane and ethyl acetate (20:1, v/v) at a flow rate of 20 ml/min.

One-hundred ml each of effluents was collected in an Erlenmeyer flask, and the total number of flasks was 417. The effluent was divided into six groups according to the types visualized on the TLC plate by 50% sulfuric acid with a mixture of hexane and ethyl acetate (8:1. v/v) as developing solvent. After concentration by rotary vacuum evaporator, six fractions were labeled as A (#1–#54), B (#55–#79), C (#80–#137), D (#138–#213), E (#214–#274), and F (#275–#417). Finally, after the above elution, the residue adsorbed on silica gel was re-eluted with two

column-bed volumes of ethyl acetate, and the effluent was then labeled as G.

GC/MS Analysis

The active fractions of the essential oils were analyzed by gas chromatography/mass spectrometry to identify the constituents of active fractions. The GC (HP 6890) was equipped with an HP-5 MS column (30 m×0.25 mm×0.25 mm), and injector temperature and detector temperature were maintained at 250°C and 280°C, respectively. Helium was employed as the carrier gas at 1 ml/min flow rate. The initial oven temperature was maintained at 60°C for 10 min, and then the temperature was raised to 280°C at a rate of 5°C/min and finally held at the temperature for 10 min.

Mass spectrometry (HP 5973) was used at EI mode, and the conditions were as follows: Ionization voltage, 70 eV; emission current, 40 μA, scan rate, 1 scan/s; scan range, 35–700 m/z ion; source temperature, 200°C. The chemical structure of each constituent was identified by comparing mass data of their peaks with standard library data.

Measurement of ROS

IgE-sensitized cells were incubated with 5 μM 2,7-dichlorodihydrofluorescein diacetate for 30 min. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. After incubation, cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss). The samples were excited with a 488 nm Ar laser and a 515 nm long-pass filter. About 20 cells were randomly selected from three separate experiments. To check the effect of each fraction on ROS production, cells were sensitized with DNP-specific IgE (100 ng/ml). The sensitized cells were then incubated with each fraction for 15 min in the presence of 5 μM 2,7-dichlorodihydrofluorescein diacetate. For the last 5 min of incubation, DNP-HSA was added and ROS measurement was carried out as above.

β-Hexosaminidase Secretion Assay

Degranulation was determined by measuring the release of β-hexosaminidase. RBL-2H3 cells were grown on 2% (w/v) BSA-coated 96-well plates (2×10^5 cells/well) and sensitized for 16 h with DNP-specific IgE (100 ng/ml). The growth medium was replaced with Tyrodes' assay buffer [119 mM NaCl, 4.74 mM KCl, 2.5 mM $CaCl_2$, 1.19 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose, 0.1% (w/v) BSA, pH 7.3]. The IgE-sensitized cells were reincubated with the above buffer for 15 min, and then treated with various fractions for 15 min. After treatment, the cells were stimulated with DNP-HSA (100 ng/ml) for 1 h. Following stimulation, the supernatant was incubated with an equal volume of substrate solution (1 mM *p*-nitrophenyl *N*-acetyl-beta-D-glucosamine in 0.05 M citrate buffer, pH 4.5) for 1 h at 37°C. The enzyme reaction was stopped by the addition of 0.05 M sodium bicarbonate buffer (pH 10.0) and the reaction product was measured at 450 nm. To determine the total amount of β-hexosaminidase released, the remaining cells were lysed by assay buffer containing 1% (v/v) Triton X-100 prior to incubation with substrate using the same procedure as for the determination of activity in the supernatant. The inhibitory effects of fractions on β-hexosaminidase secretion were compared with that of ketotifen fumarate.

Western Blot Analysis

The IgE-sensitized RBL-2H3 cells were treated with each fraction or ketotifen fumarate for 15 min and then stimulated with DNP-HSA (100 ng/ml) for 30 min. For Western blot analysis, cells were solubilized with lysis buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue, 10 mM NaF, 1% (v/v) protease inhibitor cocktail, and 1 mM sodium orthovanadate]. The samples were boiled for 5 min, and equal amounts of protein (20 μg/well) were analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membrane and subjected to immunoblotting. The antibodies and dilutions included anti-ERK (1:1,000), anti-phospho ERK(1:1,000), anti-Akt (1:1,000), and anti-phospho Akt (1:1,000). After extensive washing, blots were further incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibody at a 1:3,000 dilution for 1 h at room temperature and developed using the enhanced chemiluminescence kit (Amersham).

Calcium Influx

RBL-2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml) on 2% (w/v) BSA-coated 6-well plates. The growth medium was then replaced with assay buffer and the cells were exposed to 4 μM fluo-3-AM in the presence of 1% (v/v) fetal bovine serum and 0.04% Pluronic F-127 in the dark for 45 min. The fluo-3-loaded cells were washed three times with assay buffer, and then treated with each fraction for 5 min. The cells were immediately stimulated with DNP-HSA (100 ng/ml), and the change of fluo-3 fluorescence was monitored at an excitation wavelength of 488 nm and emission wavelength of 540 nm.

RESULTS AND DISCUSSION

Fractions of *Chamaecyparis obtusa* Decrease β-Hexosaminidase Secretion from Antigen-Stimulated RBL Cells

RBL (rat basophilic leukemia) cells were employed to determine the effect of fractions of *Chamaecyparis obtusa* on the secretion of β-hexosaminidase, since β-hexosaminidase secretion is the hallmark of an allergic reaction resulting from allergen exposure. Antigen stimulation led to an

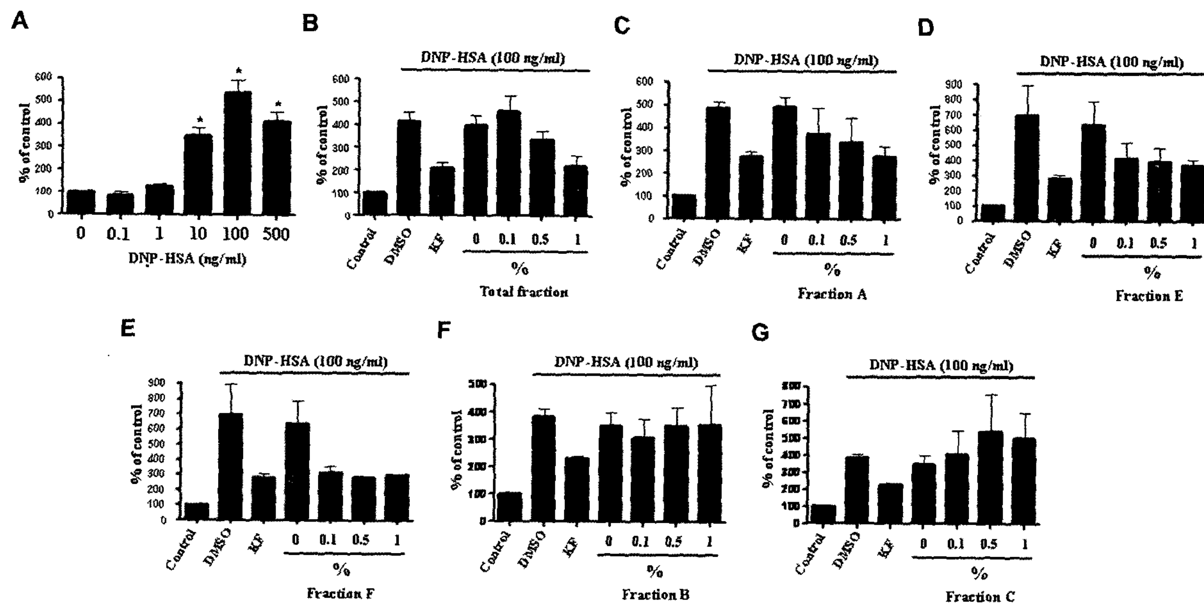

Fig. 1. Effects of various fractions of *Chamaecyparis obtusa* on β-hexosaminidase secretion from antigen-stimulated RBL-2H3 cells.
A. RBL-2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml), and stimulated with various concentrations of DNP-HSA (0, 0.1, 1, 10, 100, 500 ng/ml). The enzyme activities of β-hexosaminidase in the supernatant and solubilized cells were measured with *p*-nitrophenyl *N*-acetyl-beta-D-glucosamine. * P<0.01 compared with test without DNP-HSA challenge. RBL-2H3 cells were sensitized for 16 h with DNP-specific IgE (100 ng/ml), and treated with ketotifen fumarate (100 μM), or total fraction (**B**) or various fractions (**C–G**) of *Chamaecyparis obtusa* for 15 min. The significance between each control group and experimental group was analyzed by the student's *t* test. Asterisks denote statistical significance.

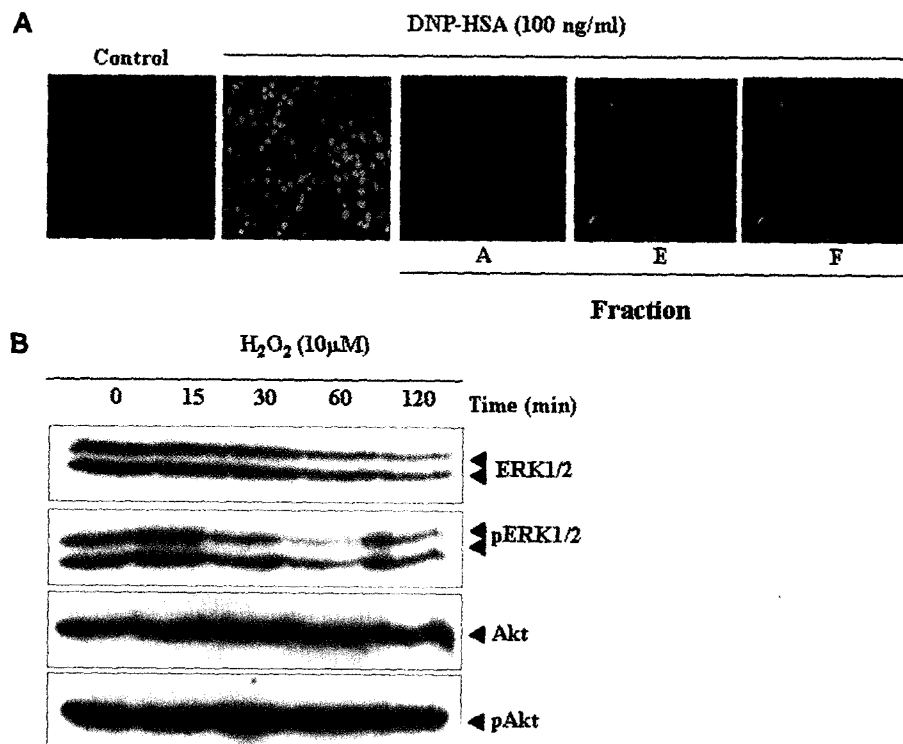

Fig. 2. Fractions of *Chamaecyparis obtusa* decrease ROS from antigen-stimulated RBL-2H3 cells, and ROS increases phosphorylation of ERK.
A. The IgE-sensitized RBL-2H3 cells were treated with ketotifen fumarate (100 μM) or total fraction or each fraction for 15 min. The cells were then incubated with 5 μM 2,7-dichlorodihydrofluorescin diacetate. For the last 5 min of incubation, DNP-HSA (100 ng/ml) was added. After incubation, cells were immediately observed by a laser scanning confocal microscope (LSM410, Carl Zeiss). **B.** RBL-2H3 cells were treated with H_2O_2 (10 μM) for various time intervals. At each time point, cell lysates were prepared and Western blot analysis was carried out.

increased secretion of β-hexosaminidase from RBL-2H3 cells in a dose-dependent manner (Fig. 1A). Next, we determined the effects of fractions of *Chamaecyparis obtusa* on β-hexosaminidase secretion from antigen-stimulated cells. For this, total or each fraction was added to IgE-sensitized RBL cells for 15 min, and followed by stimulation with DNP-HSA (100 ng/ml). Fraction alone without antigen stimulation did not affect β-hexosaminidase secretion (data not shown). However, total and fractions A, E, and F of *Chamaecyparis obtusa* significantly decreased β-hexosaminidase secretion from antigen-stimulated cells (Figs. 1B–1G). Ketotifen fumarate, an antiallergic drug, also decreased the β-hexosaminidase secretion. These results suggest that fractions of *Chamaecyparis obtusa* inhibit allergic inflammation.

Fractions of *Chamaecyparis obtusa* Decrease ROS from Antigen-Stimulated RBL-2H3 Cells, and ROS Increases Phosphorylation of ERK

RBL-2H3 cells release ROS in response to various stimuli [11, 18]. We therefore examined whether fractions of *Chamaecyparis obtusa* affected ROS production from antigen-stimulated RBL-2H3 cells. Total and each fraction of *Chamaecyparis obtusa* significantly decreased ROS from antigen-stimulated RBL-2H3 cells (Fig. 2A). Next, the downstream target of ROS was determined. Addition of H_2O_2 (10 μM) increased phosphorylation of ERK (Fig. 2B), suggesting that ERK is a downstream target of ROS.

Fractions of *Chamaecyparis obtusa* Decrease Calcium Influx and Phosphorylation of ERK in Antigen-Stimulated RBL Cells

RBL-2H3 cells increase phosphorylation of ERK in response to antigen stimulation [14]. Therefore, we examined whether fractions of *Chamaecyparis obtusa* affected phosphorylation of signaling molecules. Antigen stimulation led to increased phosphorylation of ERK, and ketotifen fumarate decreased the phosphorylation in antigen-stimulated RBL cells (Fig. 3A). We did not observe activation of p38 MAPK or Akt (data not shown). Fractions A, E, and F significantly decreased the phosphorylatin of ERK in antigen-stimulated RBL cells (Fig. 3B). The inhibition of ERK by PD98059

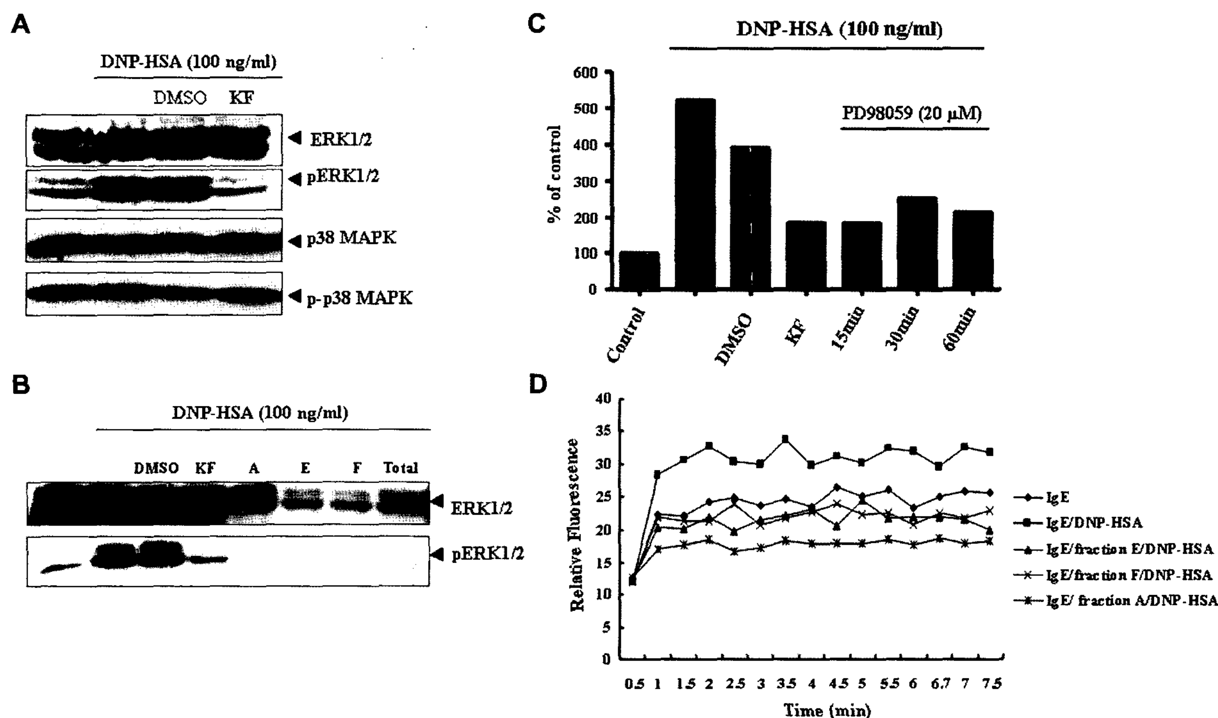

Fig. 3. Fractions of *Chamaecyparis obtusa* decrease phosphorylation of ERK and calcium influx.

A. The IgE-sensitized RBL-2H3 cells were treated with or without ketotifen fumarate (100 μM) for 15 min. Then, the cells were stimulated with DNP-HSA (100 ng/ml). After stimulation, protein extracts were prepared and subjected to Western blot analysis using anti-ERK, anti-phospho ERK, anti-p38 MAPK, or anti-phospho p38 MAPK antibodies. KF denotes ketotifen fumarate. **B.** The IgE-sensitized RBL-2H3 cells were treated with ketotifen fumarate (100 μM) or each fraction for 15 min. After treatment, the cells were stimulated with DNP-HSA (100 ng/ml). Western blot analysis was carried out as above. **C.** The IgE-sensitized RBL-2H3 cells were treated with ketotifen fumarate (100 μM) for 15 min or PD98059 for various time intervals. The cells were then stimulated with DNP-HSA (100 ng/ml). Secretion of β-hexosaminidase from antigen-stimulated RBL-2H3 cells was measured as described. **D.** Ca^{2+} responses in RBL-2H3 cells were measured by using fluo-3-AM (4 μM). The IgE-sensitized RBL-2H3 cells were loaded with fluo-3-AM (4 μM). After 1 h of incubation, the cells were then treated with or without each fraction for 15 min. The cells were immediately placed on the FLIPR (Molecular Device) and stimulated with DNP-HSA (100 ng/ml).

(20 μM) decreased the secretion of β-hexosaminidase from antigen-stimulated RBL-2H3 cells (Fig. 3C), suggesting that the activation of ERK is necessary for the secretion of β-hexosaminidase. It is known that antigen stimulation leads to increase in calcium influx. We, therefore, examined whether each fraction affected calcium influx in antigen-stimulated RBL-2H3 cells, and found that antigen-stimulated RBL-2H3 cells showed significant increase in calcium influx, whereas fractions of *Chamaecyparis obtusa* showed opposite effects (Fig. 3D). Furthermore, the inhibition of ERK by PD98059 significantly decreased calcium influx in antigen-stimulated RBL-2H3 cells (data not shown), suggesting that the activation of ERK is required for calcium influx.

GC/MS Analysis of Fractions of *Chamaecyparis obtusa*
Table 1 summarized the major constituents of A, D, E, and F fractions identified by GC/MS analysis. The major constituents of A fraction were sabinene (15.46%), limonene (11%), alpha-pinene (7.06%), beta-myrcene (6.34%), gamma-terpinene (5.75%), thujopsene (4.85%), and cardinene. The content of monoterpenes in the fraction A was higher than those of sesquiterpenes.

Fraction D was mainly composed of terpinene-4-ol (53.01%), delta-cadinene (9.55%), delta-selinene (6.33%), nerolidol (3.81), and alpha-cedrol (5.1%).

In fraction E, eudesmol (21.32%), alpha-cedrol (19.66%), terpinene-4-ol (10.52%), elemol (6.69%), and terpineol (4.94%) were detected. Alpha-cedrol and eudesmol comprised 40% of the fraction E, and elemol, which was not detected in the fraction D, was identified in this fraction.

In fraction F, the major constituents identified by GC/MS included elemol (33%), 2-naphthalenemethanol (22.23%), gamma-eudesmol (13.26%), alpha-terpineol (3.62%), and borneol L (2.62%).

Even though there was quantitative difference between the fractions, alpha-cedrol was detected in three fractions; D, E, and F. In addition, elemol and eudesmol were identified in both fractions E and F. Terpinene-4-ol comprised a large portion of the fraction D. It should be mentioned that linalool and alpha-terpineol have been reported to be the most active antibacterial components of tea tree oil [4, 5], which could possibly influence the bacterial cell wall.

In this study, various fractions of *Chamaecyparis obtusa* showed potential antiallergic functions by decreasing secretion of β-hexosaminidase, calcium influx, ROS, and phosphorylation of ERK. We also identified constituents of various fractions. Therefore, it would be necessary to further examine the effect of constituents of each fraction on allergic inflammation.

Acknowledgements

This work was supported by grants (0103026-1-2) from the Basic Research Program of the Korea Science & Engineering Foundation, from Korea Research Foundation and Vascular Research Center, (0805011-1-1) from Korea Research Foundation, (FPR02A8-31-110) from 21C Frontier Functional Proteomics Project from the Korean Ministry of Science & Technology, and (A050260) from the Ministry of Health and Welfare of Korea.

Table 1. The major constituents of active fractions identified by GC/MS analysis.

Fraction	Constituents	Area (%)	Fraction	Constituents	Area (%)
A	Alpha-pinene	7.06	E	1-Octen-3-ol	2.75
	Sabinene	15.46		Terpineol	4.49
	Beta-myrcene	6.34		Linalool	1.74
	Delta-3-carene	5.04		2-Cyclohexen-1-ol	1.85
	Alpha-terpinene	2.49		Exo-methyl-camphenilol	0.90
	1-Methyl-2-(1-methyl)-benzene	3.44		Terpinene-4-ol	10.52
	Limonene	11.00		Elemol	6.69
	Gamma-terpinene	5.78		Alpha-cedrol	19.66
	Thujopsene	4.85		Gamma-eudesmol	13.17
	(+)-Epi-bicyclosesquiphellandrene	3.54		Alpha-cadinol	9.84
	Epizonaren	2.45		(+)-Delta-selinene	1.00
	Gamma-cadinene	2.18		Alpha-eudesmol	8.15
	Delta-cadinene	2.95	F	Trans-sabinene hydrate	2.59
	13-Methyl-17-norkaur-15-ene	5.78		Cis-sabinene hydrate	2.16
D	Linalool	2.50		2-Cyclohexen-1-ol	1.33
	Terpinene-4-ol	53.08		Borneol L	2.62
	Nerolidol	3.81		Alpha-terpinene	3.62
	Alpha-cedrol	5.10		Elemol	33.09
	Delta-selinene	6.33		Alpha-cedrol	1.91
	Delta-cadinene	9.55		Gamma-eudesmol	13.26
	Levomenol	4.32		2-Naphthalenemethanol	22.33

REFERENCES

1. Baba, T., H. Nakano, K. Tamai, D. Sawamura, K. Hanada, I. Hashimoto, and Y. Arima. 1998. Inhibitory effect of beta-thujaplicin on ultraviolet B-induced apoptosis in mouse keratinocytes. *J. Invest. Dermatol.* **110:** 24–28.
2. Cambier, J. C. 1995. Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *J. Immunol.* **155:** 3281–3285.
3. Cho, J., B. Sung, M. Lim, H. Kim, S. Lee, and H. Lee. 2004. Acaricidal components of medicinal plant oils against *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*. *J. Microbiol. Biotechnol.* **14:** 631–634.
4. Gustafson, J., Y. Liew, and S. Chew. 1998. Effects of tea tree oil on *Escherichia coli*. *Lett. Appl. Microbiol.* **26:** 194–198.
5. Halcün, L. and K. Milkus. 2004. *Staphylococcus aureus* and wounds: A review of tea tree oil as a promising antimicrobial. *Am. J. Infect. Contr.* **32:** 402–408.
6. Hampton, M. B., A. J. Kettle, and C. C. Winterbourn. 1998. Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood* **92:** 3007–3014.
7. Ishimatsu, S., Y. Ohga, T. Ishidao, and H. Hori. 2003. Antimicrobial activity of hinokitiol against *Legionella pneumophila*. *J. UOEH.* **25:** 435–439.
8. Kim, H., D. Yoon, S. Lee, S. Baek, G. Han, Y. Kho, and C. Lee. 2005. Screening and biotransformation of interleukin-1β converting enzyme production inhibitors from *Arctii fructus*. *J. Microbiol. Biotechnol.* **15:** 269–273.
9. Kobayashi, T., T. Miura, T. Haba, M. Sato, I. Serizawa, H. Nagai, and K. Ishizaka. 2000. An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. *J. Immunol.* **164:** 3855–3861.
10. Matsuda, H., N. Watanabe, G. P. Geba, J. Sperl, M. Tsudzuki, J. Hiroi, M. Matsumoto, H. Ushio, S. Saito, P. W. Askenase, and C. Ra. 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.* **9:** 461–466.
11. Matsui, T., Y. Suzuki, K. Yamashita, T. Yoshimaru, M. Suzuki-Karasaki, S. Hayakawa, M. Yamaki, and K. Shimizu. 2000. Diphenyleneiodonium prevents reactive oxygen species generation, tyrosine phosphorylation, and histamine release in RBL-2H3 mast cells. *Biochem. Biophys. Res. Commun.* **276:** 742–748.
12. Park, K. and H. Lee. 2005. *In vitro* antiviral activity of aqueous extracts from Korean medicinal plants against influenza virus type A. *J. Microbiol. Biotechnol.* **15:** 924–929.
13. Pawankar, R., M. Okuda, H. Yssel, K. Okumura, and C. Ra. 1997. Nasal mast cells in perennial allergic rhinitics exhibit increased expression of the Fc epsilonRI, CD40L, IL-4, and IL-13, and can induce IgE synthesis in B cells. *J. Clin. Invest.* **99:** 1492–1499.
14. Reth, M. 1989. Antigen receptor tail clue. *Nature* **338:** 383–384.
15. Ro, J. Y., B. C. Lee, J. Y. Kim, Y. J. Chung, M. H. Chung, S. K. Lee, T. H. Jo, K. H. Kim, and Y. I. Park. 2000. Inhibitory mechanism of aloe single component (alprogen) on mediator release in guinea pig lung mast cells activated with specific antigen-antibody reactions. *J. Pharmacol. Exp. Ther.* **292:** 114–121.
16. Wange, R. L. and L. E. Samelson. 1996. Complex complexes: Signaling at the TCR. *Immunity* **5:** 197–205.
17. Williams, C. M. and S. J. Galli. 2000. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* **192:** 455–462.
18. Yoshimaru, T., Y. Suzuki, T. Matsui, K. Yamashita, T. Ochiai, M. Yamaki, and K. Shimizu. 2002. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* **32:** 612–618.