

Author's Accepted Manuscript

Anti-allergic effect of α -cubebenoate isolated from *Schisandra chinensis* using in vivo and in vitro experiments

Kyoung-Pil Lee, Saeromi Kang, Soo-Jin Park, Jung-Min Kim, Jung-Min Lee, Ae-Yeon Lee, Hae-Young Chung, Young-Whan Choi, Young-Geun Lee, Dong-Soon Im



PII: S0378-8741(15)30064-7
DOI: <http://dx.doi.org/10.1016/j.jep.2015.07.049>
Reference: JEP9664

To appear in: *Journal of Ethnopharmacology*

Received date: 9 March 2015
Revised date: 29 July 2015
Accepted date: 31 July 2015

Cite this article as: Kyoung-Pil Lee, Saeromi Kang, Soo-Jin Park, Jung-Mi Kim, Jung-Min Lee, Ae-Yeon Lee, Hae-Young Chung, Young-Whan Choi, Young-Geun Lee and Dong-Soon Im, Anti-allergic effect of α -cubebenoate isolated from *Schisandra chinensis* using in vivo and in vitro experiments *Journal of Ethnopharmacology*, <http://dx.doi.org/10.1016/j.jep.2015.07.049>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Anti-allergic effect of α -cubebenoate isolated from *Schisandra chinensis* using in vivo and in vitro experiments

Kyoung-Pil Lee^a, Saeromi Kang^a, Soo-Jin Park^a, Jung-Min Kim^a, Jung-Min Lee^a, Ae-Yeon Lee^a, Hae-Young Chung^a, Young-Whan Choi^b, Young-Geun Lee^c, and Dong-Soon Im^{a,*}

^aMolecular Inflammation Research Center for Aging Intervention (MRCA) and College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea

^bDepartment of Horticultural Bioscience and ^cDepartment of Food Engineering, College of Natural Resources & Life Science, Pusan National University, Miryang-si, Gyeongsangnam 627-706, Republic of Korea

Running title: Anti-allergic effect of α -cubebenoate

*Corresponding author: Dong-Soon Im,
Laboratory of Pharmacology, College of Pharmacy, Pusan National University, 63 Beon-gil 2,
Busandaehag-ro, Geumjeong-gu, Busan 609-735, Republic of Korea,
Tel: +82-51-510-2817, Fax: +82-51-513-6754, E-mail: imds@pusan.ac.kr

Abstract

Aim of the study : In Oriental countries, the dried fruits of *Schisandra chinensis* are extensively used in traditional medicine to treat asthma, gonorrhea, and other diseases. Recently, α -cubebenoate was isolated as an anti-inflammatory component from *Schisandra chinensis*. In the present study, the authors examined the anti-allergic effect of α -cubebenoate using in vivo and in vitro experiments.

Materials and methods: α -Cubebenoate was isolated from an extract of *Schisandra chinensis* fruits. Antigen-induced degranulation and Ca^{2+} mobilization were measured in RBL-2H3 mast cells. In addition, BALB/c mice were sensitized with ovalbumin and aluminum hydroxide, and then challenged with ovalbumin for three consecutive days. α -Cubebenoate (1 mg/kg) was administered intraperitoneally 30 min before each ovalbumin challenge.

Results : In RBL-2H3 mast cells, α -cubebenoate inhibited antigen-induced degranulation and increase of intracellular Ca^{2+} concentrations. In the ovalbumin-induced asthma model, α -cubebenoate suppressed bronchiolar structural changes induced by ovalbumin challenge. Furthermore, α -cubebenoate strongly inhibited accumulations of eosinophils, macrophages, and lymphocytes in bronchoalveolar lavage fluid. α -Cubebenoate also suppressed Th2 cytokines (IL-4 and IL-13) and TGF- β 1 in lung tissues and in immune cells at the mRNA and protein levels.

Conclusion : α -Cubebenoate has an inhibitory effect on allergic inflammation and could be utilized as an agent for the treatment of asthma

Keywords : α -cubebenoate; anti-allergy; asthma; mast cells; *Schisandra chinensis*

1. Introduction

In the genus *Schisandra* (family Magnoliaceae), *Schisandra chinensis* (Turcz.) Baill. and *Schisandra repanda* have a history of medicinal use (Panossian and Wikman, 2008). *Schisandra chinensis* grows wild in China, Korea, Japan, and Russia, and has been used for more than 40 years as an anti-fatigue tonic by the medical profession in Russia (Mashkovskij, 2000). In Oriental countries, the dried fruits of *Schisandra chinensis* are extensively used in traditional medicine to treat asthma, gonorrhea, enuresis, dysentery, protracted diarrhea, thirst, diabetes, and other diseases (Liu, 1989; Panossian and Wikman, 2008). In atopic dermatitis and asthma models, *Schisandra chinensis* extract has been reported to have suppressive effects (Kang and Shin, 2012; Kim et al., 2014), and in a guinea pig model of asthma, Wuwei Dulong decoction (*Schisandra* fruit and earthworm decoction) was found to inhibit the infiltration and diffusion of inflammatory cells and to regulate leukotriene B₄ and interferon- γ (Li et al., 2009). *Schisandra* fruit is also the main active constituent of bakumijogon, an herbal formula used in traditional Chinese medicine, which suppresses atopic dermatitis-like symptoms by correcting Th1/Th2 imbalance (Makino et al., 2008). However, the active constituents responsible for the anti-allergic and anti-asthma effects of *Schisandra chinensis* fruits have not been identified. Previously, we isolated α -cubebenoate from *Schisandra chinensis* fruits and showed its anti-inflammatory effect on peritoneal macrophages (Kang et al., 2014).

Bronchial asthma is a common chronic immune disease of the lungs, and affects 4-5% of the population (Wills-Karp, 2004). Airway hyper-responsiveness, reversible airflow obstruction, and bronchial hyper-responsiveness are characteristic features of this disease (Busse and Rosenwasser, 2003), and its pathogenesis is associated with increased

inflammatory cell infiltration and excessive mucus secretion into airways (Wills-Karp, 2004). A variety of cell types, such as, lymphocytes (Th2), mast cells, and eosinophils are involved in asthma. Mast cells are the predominant cells of allergic responses, and when activated by allergen binding to IgE attach to high affinity receptors for IgE (FcεRI), they release cytokines, chemokines, leukotrienes, and secretory granules (Lane and Lee, 1996). T lymphocytes play a pivotal role in the pathogenesis by producing Th2 cytokines (IL-4 and IL-13) (Wills-Karp, 2004), which importantly drive IgE production by B cells and mucus hypersecretion (Wenzel, 2012). Transforming growth factor-β1 (TGF-β1) is also an important factor. It is involved in fibrosis and causes vascular malformation and the induces the proliferation and hypertrophy of airway smooth muscle cells, the hypertrophy of goblet cells, and the destruction of epithelial cells (Makinde et al., 2007; Wen et al., 2003).

Because of the traditional use of the dried fruits of *Schisandra chinensis* to treat asthma, we sought to determine whether α-cubebenoate has anti-allergic activity against ovalbumin-induced asthma in vitro using rat RBL-2H3 mast cells, and subsequently, we investigated its in vivo anti-allergic effects using an ovalbumin (OVA)-induced mouse model of asthma .

2. Materials and methods

2.1. Materials

The fruits of *Schisandra chinensis* (Turcz.) Baill were collected in September 2010 in Moonkyong (South Korea). A voucher specimen (accession no. SC-PDRL-2) has been deposited in the Herbarium at the Pusan National University. The plant was identified by one of the authors (Y.W. Choi). The isolation and structural determination of α -cubebenoate were described in a previous study (Kang et al., 2014).

2.2. Extraction of α -cubebenoate

The dried fruits of *Schisandra chinensis* (1.0 kg) were ground to a fine powder. Then, they extracted successively with n-hexane, CHCl_3 , and MeOH at room temperature. The hexane extract (158 g) was in vacuo evaporated and 50 g was chromatographed on a Diaion HP20 (250-850 μm ; Sigma-Aldrich, St. Louis, MO) column (40 x 8 cm) using EtOH, chloroform, and hexane to obtain 7 fractions. The first fraction (17SCH1, 22.95 g) was separated on a silica gel column (80 x 6.5 cm) sequentially with 0.5% acetone, 25% acetone, and 25% MeOH in CHCl_3 to obtain 19 fractions. The 3rd Fraction (17SCH1IC, 430.2 mg) was separated on a Sephadex column (100 x 3.0 cm) using 50% MeOH in CHCl_3 to obtain 21 fractions, and the 2nd fraction (17SCH1ICIB, 161.9 mg) was separated on a silica gel column (60 x 2.0 cm) using 5 % acetone in CH_2Cl_2 to obtain 6 fractions. The 3rd fraction (17SCH1ICIBIC, 130.4 mg) was then separated on a Sephadex column (100 x 3.0 cm) using 50% MeOH in CH_2Cl_2 to obtain 3 fractions. Finally, the first fraction (17SCH1ICIBICIA, 101.9 mg) was separated on a silica gel column (73 x 3.0 cm) using 50% CH_2Cl_2 in hexane to yield α -cubebenoate (17SCH1ICIAIL, 20.8 mg) (Kang et al., 2014).

2.3. Determination of the structure of α -cubebenoate

α -Cubebenoate was identified by ^1H , ^{13}C , Dept, HSQC, and HMBC NMR spectra in CDCl_3 . Its elemental composition was ascertained to be $\text{C}_{17}\text{H}_{26}\text{O}_2$ from the molecular ion signal at m/z 262. ^1H , ^{13}C , and Dept NMR, which showed four primary (δ_{C} 19.19, 19.33, 19.55, 20.95), four secondary (δ_{C} 21.79, 30.82, 36.35, 67.34), six tertiary (δ_{C} 32.43, 39.89, 44.77, 45.05, 48.02, 122.78), and three quaternary carbons (δ_{C} 39.30, 141.24, 170.98) confirmed the finding. The presence of one double bond was evident based on these data. Because the elemental composition $\text{C}_{17}\text{H}_{26}\text{O}_2$ indicates that four unsaturations and the presence of a single double bond indicated the presence of three rings. The ^1H NMR of α -cubebenoate had one methyl singlet (2.02) and three methyl doublets at δ_{H} = 0.84 and δ_{H} = 0.75.

At δ_{H} = 5.62, one olefinic proton was detected. At δ_{H} = 1.65 and δ_{H} = 2.18 two cyclopropane protons. At δ_{H} = 1.59 and 1.73 and 1.61 two unusually high-field methylene proton signals were observed. This high-field shift was attributed to the presence of protons in the anisotropic range of a cyclopropane ring system. At δ_{H} = 4.49 and 4.39 the OCH_2 connected to the carboxylic group showed resonances. $^1\text{H}^1\text{H}$ -COSY revealed that the two methylene protons were on adjacent methylene groups. The other protons of each methylene group appeared in the aliphatic region: 1.54–1.60. At δ_{H} = 2.26 two protons belonging to one methylene group appeared as a doublet. Corresponding cross peaks were observed in the $^1\text{H}^1\text{H}$ -COSY. The connectivities of different groups were established by 2D NMR experiments. $^1\text{H}^1\text{H}$ -COSY revealed the presence of an isopropyl group due to identical cross peaks to the multiplet. This finding was further confirmed by HMBC. The connectivity of α -cubebenoate was deduced based on this information, and its configuration was established (structure in Fig 1-A) (Kang et al., 2014).

2.4. Animals

Six-week old male Balb/c (19–22 g) mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a Laboratory Animal facility in Pusan National University, and provided unrestricted amounts of food and water. The animal protocol used in this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU–IACUC) on their ethical procedures and scientific care, and it has been approved (Approval Number ED-PNU2012-0068).

2.5. Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), cultured at 37°C in a 5% CO₂ humidified incubator, and maintained in high glucose DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 µg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (Lee et al., 2015).

2.6. Measurement of intracellular Ca²⁺ concentrations

Cells were trypsin-digested, allowed to sediment, incubated in complete medium containing monoclonal anti-dinitrophenyl specific mouse IgE (0.5 µg/ml, DNP-IgE, D8406, Sigma-Aldrich) at 37°C for 2 h, resuspended in Hepes-buffered medium (HBM), containing 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, 2.5 mM probenecid, and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 µM of fura 2-AM. Intracellular Ca²⁺ concentrations ([Ca²⁺]_i) were estimated by measuring changes in fura-2

fluorescence at an emission wavelength of 510 nm and two excitation wavelengths (340 nm and 380 nm) every 0.1 sec using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities ($\lambda_{340}/\lambda_{380}$) at these two wavelengths were used as a surrogate of $[\text{Ca}^{2+}]_i$, as previously described (Lee et al., 2015; Park et al., 2014).

2.7. Measurement of degranulation

Degranulation was estimated by measuring β -hexosaminidase release according to the method announced by Dearman et al. (2005). RBL-2H3 cells (2×10^5 cells/well in 24-well plates) were sensitized with 0.5 $\mu\text{g}/\text{ml}$ of monoclonal anti-dinitrophenyl specific mouse IgE (DNP-IgE, D8406, Sigma-Aldrich) overnight at 37°C in a 5% CO_2 incubator. To remove DNP-IgE before stimulation, cells were washed twice with PIPES buffer (pH 7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl_2 , 0.1% BSA, and 1 mM CaCl_2 . Then, the cells were incubated in 500 μl PIPES buffer. Different concentrations of α -cubebenoate were then added to each well and incubated at 37°C for 30 min. Human dinitrophenyl albumin (10 $\mu\text{g}/\text{ml}$; DNP-hAb, A6661 Sigma-Aldrich) was then added, and cells were incubated for another 30 min at 37°C to induce degranulation. Aliquots (50 μl) of medium were then transferred to a 96-well microplate and incubated for 60 min with 50 μl of 1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (N9376, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5). Cells were lysed with an equal volume of 0.5% Triton X-100 at 37°C for 1 h. Total β -hexosaminidase activity in the cells was also measured. The reaction was terminated by adding 250 μl of 0.05 M sodium carbonate buffer (pH 10.0; 0.05 M Na_2CO_3 /0.05 M NaHCO_3). Absorbance (OD) at 410 nm was measured using a microplate reader. Release (%) was calculated by the ratio of released β -hexosaminidase activity in the media to total β -

hexosaminidase activity in the cells. In "spontaneous", neither DNP-HSA nor sample was added to cells to confirm the spontaneous β -hexosaminidase release from cells in conditions without sample. In "total", cells were lysated and the total amount of β -hexosaminidase contained in the cells were measured. In "sample", both DNP-HSA and samples were treated to cells to confirm the β -hexosaminidase release from cells (Lee et al., 2013).

ratio of β -hexosaminidase release (%) =

$$\left(\frac{\text{OD control or OD sample} - \text{OD spontaneous}}{\text{OD total}} \right) \times 100$$

2.8. Induction of asthma in BALB/c mice and drug administration

Mice were divided into three groups (n = 5), that is, a phosphate-buffered saline(PBS)-injected control group, an OVA-injected asthma group, and α -cubebenoate treated asthma group (1 mg/kg). Asthma was induced by the intraperitoneal injection of OVA (Sigma-Aldrich, A5503) and aluminum hydroxide (Sigma-Aldrich, 239186) at 1st day and 14th day. From 28th day, mice were exposed to nebulized OVA for 20 min for three consecutive days (Aoki et al., 2010). Two days later (32th day), bronchoalveolar lavage fluids (BALF) were collected from the lungs and immune cells were stained and counted (Lee et al., 2013). Five animals for each group were analyzed (n = 5).

Lungs were excised, removed, and fixed with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer. Tissue was embedded (Tissue-Tek; OCT compound; VWR International, West Chester, PA, USA), frozen, and sectioned at 7 μ m using a cryostat (Leica, CM3050 S, Heidelberg, Germany). Sections were thaw-mounted onto Superfrost microscope slides

(Fisher Scientific, Pittsburgh, PA, USA) and stored at -20°C until processed.

2.9. Histologic analysis of the lung and cell counting in BALF

Lung sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) (Aoki et al., 2014). Five animals for each group were analyzed (n = 5). Briefly, sections were hydrated to water and then were placed in aniline acetic block solution [mixture of Aniline (10 ml) and 3% acetic acid (90 ml)] for 25 min at room temperature. After rinsing in 95% ethanol and washing in water, the sections were stained in Schiff's reagent (Sigma-Aldrich, 3952) for 15 min. For H&E staining, sections were washed lukewarm tap water for 5 min and counterstained in hematoxylin solution, Gill No. 3 (Sigma-aldrich, GHS-3) for 90 sec. The sections were washed in running tap water and dehydrated. The sections were coverslipped with Permount (Fisher Scientific Inc, SP-15, Waltham, MA, USA).

These histological lung tissues were used to determine lung inflammation. Lung inflammation was scored by a treatment-blind observer. The degree of lung inflammation was evaluated on a subjective scale of 0-3, as described elsewhere (Kwak et al., 2003; Tournoy et al., 2000). A value of 0 was assigned when no inflammation was detectable, a value of 1 was adjudged for occasional cuffing with inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and a value of 3 was given when most bronchi or vessels were surrounded by a thick layer (more than five cells thick) of inflammatory cells. Total lung inflammation was defined as the average of lung inflammation scores. The lung sections per mouse were scored and inflammation scores were expressed as a mean value (Braber et al., 2010).

Cells in BALF attached to slide glass using a Cellspin (5 min, 500 rpm, Hanil Electric).

Cells were fixed methanol for 30 sec. May-Grunwald solution (Sigma-Aldrich, 32856) processed in the slide for 5 min. And then, Giemsa solution (Sigma-Aldrich, 32884) was processed in the slide for 12 min. The slides were rinsed and covered.

2.10. Reverse transcriptase-PCR

To determine the expressions of asthmatic markers in Th2 cells by RT-PCR, first strand cDNA was synthesized from total RNA isolated using Trizol reagent (Invitrogen, Waltham, MA, USA). Total RNAs were isolated from five animals for each group (n = 5). Synthesized cDNA products and primers for each gene were used for PCR using Promega Go-Taq DNA polymerase (Madison, WI, USA). Specific primers for TGF- β 1 (sense 5'-TTG CTT CAG CTC CAC AGA GA-3', antisense 5'-TGG TTG TAG AGG GCA AGG AC-3'), IL-4 (sense 5'-ACG GAG ATG GAT GTG CCA AAC-3', antisense 5'-AGC ACC TTG GAA GCC CTA CAG A), and IL-13 (sense 5'-CGG CAG CAT GGT ATG GAG TG-3', antisense 5'-ATT GCA ATT GGA GAT GTT GGT CAG-3') were used to amplify gene fragments. PCR was performed over 27 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and elongation at 72°C for 30 s in an Eppendorf Mastcycler PCR machine (Hamburg, Germany) (Youn et al., 2013). Specific primers for GAPDH (sense 5'-TTC ACC ACC ATG GAG AAG GC-3', antisense 5'-GGC ATG GAC TGT GGT CAT GA-3') were used and annealing was undertaken at 60°C. Aliquots of PCR products (7 μ L) were electrophoresed in 1.2% agarose gels and stained with ethidium bromide (Kang et al., 2014).

2.11. Measurement of cytokines (IL-4 and IL-13)

BALFs from five animals for each group were analyzed for IL-4 and IL-13 (n = 5). 96-well plates (NUNC, Penfield, NY, USA) were coated overnight at 4°C with capture antibody for IL-4 (eBioscience, cat. 14-7041, San Diego, CA, USA) or IL-13 (eBioscience, cat. 14-7041). Following washing, plates were blocked for 1 h at room temperature with blocking buffer. Standard dilutions of cytokines were prepared and added to wells with supernatants. Plates were incubated for 2 h at room temperature with shaking and washed five times. Biotinylated detection antibody for IL-4 (eBioscience, cat. 13-7042) or for IL-13 (eBioscience, cat. 33-7135) was then added and incubated for 1 h at room temperature with shaking. Plates were then washed five times and avidin-HRP was added for 30 min at room temperature with shaking. Plates were then washed fourteen times and incubated with substrate solution for 15 min at room temperature. Stop solution was then added and absorbance was read at 450 nM (Vo et al., 2014).

2.12. Statistics

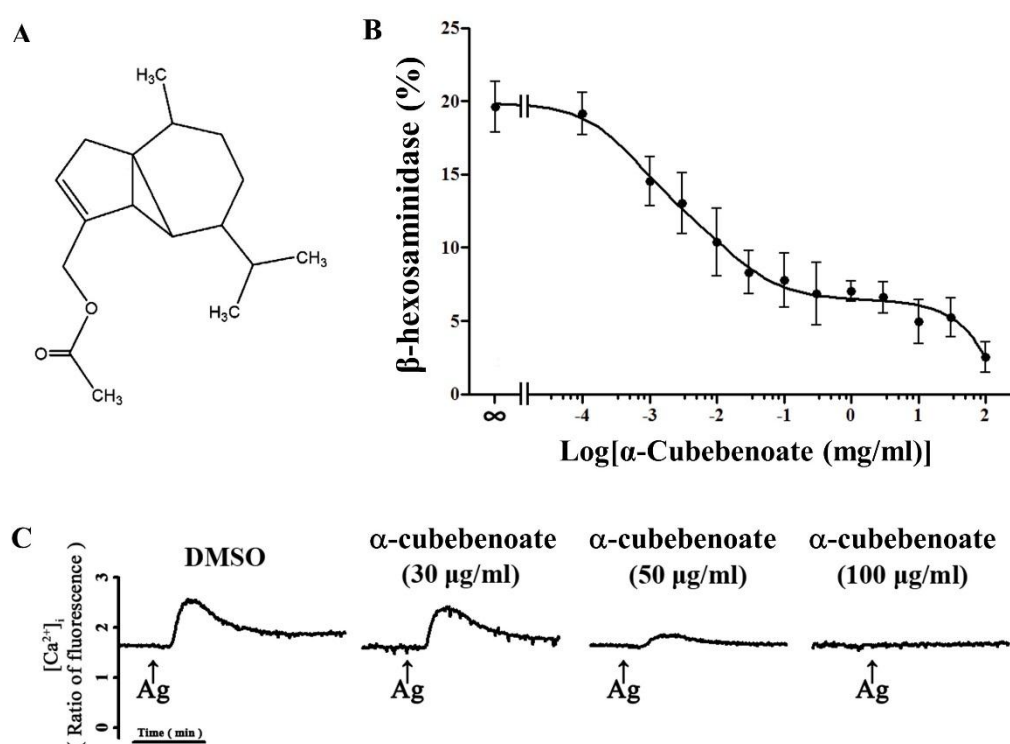
The results are expressed as the means $\pm$ SEs of the numbers of indicated determinations. Statistical significances of the differences were determined by analysis of variance (ANOVA) with turkey's post hoc, and statistical significance was accepted for p values < 0.05. Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA)

Results

3.1 α -Cubebenoate inhibited antigen-induced degranulation and intracellular Ca^{2+} increases in RBL-2H3 mast cells

Fc ϵ RI exists on the membrane of mast cells and traps circulating IgE. Antigen exposure induces cross-linking of the cell-bound IgE, which results in degranulation (Lane and Lee, 1996). Degranulation induces release of prostaglandins, histamins and cytokines through increase of intracellular Ca^{2+} concentration. Degranulation in antigen-exposed mast cells is a key step in allergic response. Therefore, degranulation was used to test anti-allergic activity of α -cubebenoate. Degranulation was quantified by measuring β -hexosaminidase activity in buffer after antigen exposure. Antigen-induced degranulation was inhibited by α -cubebenoate in a concentration-dependent manner (Fig 1-B). Significant inhibition was observed above 1 ng/ml concentrations (Fig 1-B). Degranulation is evoked by $[\text{Ca}^{2+}]_i$ elevation after antigen-exposure in mast cells, and thus, we measured $[\text{Ca}^{2+}]_i$ after antigen-exposure. As shown in Fig. 1-C, exposure increased $[\text{Ca}^{2+}]_i$ and α -cubebenoate concentration-dependently inhibited this increase. The inhibitory concentration of α -cubebenoate for $[\text{Ca}^{2+}]_i$ increase was much higher than that required for degranulation, that is, 50 $\mu\text{g/ml}$ versus 0.01 $\mu\text{g/ml}$, respectively (Figs. 1-B and C)

Figure 1



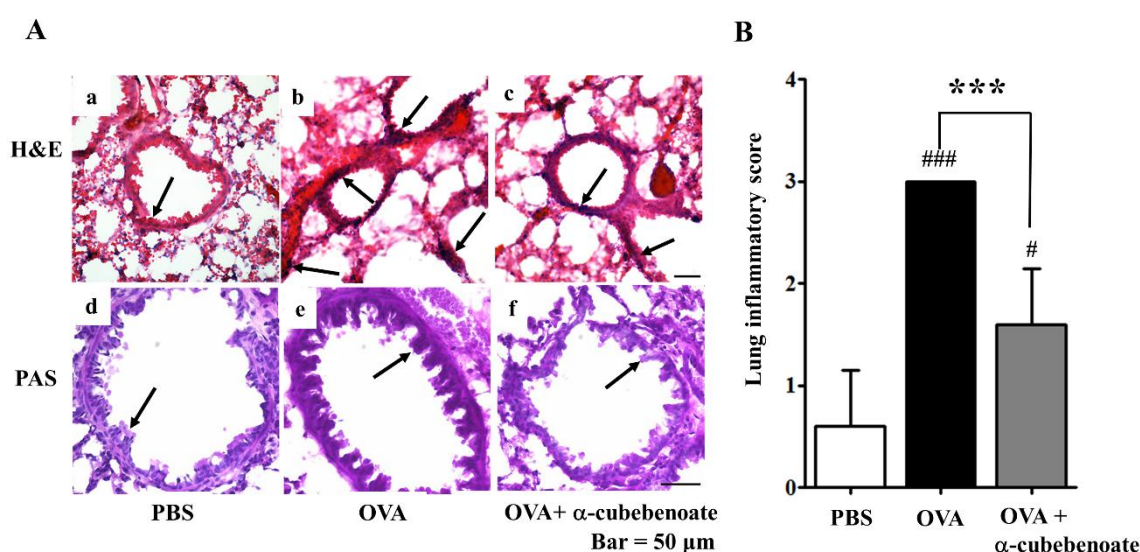
3.2 α -Cubebenoate inhibited asthma-induced structural changes in lungs

To confirm the in vitro inhibitory data of α -cubebenoate on antigen-induced degranulation in mast cells, we utilized a mouse model of OVA-induced asthma. Effective concentration of α -cubebenoate in peritoneal macrophages was 5-10 $\mu\text{g/mL}$ in vitro (Kang et al., 2014). In RBL2H3 cells, effective concentrations of α -cubebenoate were 0.1 $\mu\text{g/ml}$ for degranulation inhibition and over 50 $\mu\text{g/ml}$ for Ca^{2+} inhibition (Fig 1). Considering a whole body of mice as a compartment, 1 mg/kg intraperitoneal injection would be 20 $\mu\text{g}/20\text{ g}$, that is 1 $\mu\text{g/mL}$. In the previous study 1 mg/kg administration was significantly effective in vivo in an LPS-induced peritonitis model macrophages (Kang et al., 2014). Therefore, we used a dose of 1 mg/kg for the in vivo ovalbumin-induced asthma model.

Histologic analyses of lungs collected from mice 48 hr after final antigen challenge were

undertaken. The upper panels of Fig 2-A show representative H&E staining of lungs from PBS, OVA, and OVA + α -cubebenoate groups. The small black dots indicated by arrows are eosinophils and lymphocytes. In PBS group, eosinophils are rarely observed as arrow-indicated in the Fig 2-A-a. However, in OVA group, eosinophils are densely accumulated surrounding the bronchioles in Fig 2-A-b, indicating eosinophil infiltration in peribronchial regions. However, treatment with α -cubebenoate reduced the infiltration (Fig 2-A-c). That is, less accumulation of eosinophils is observable in OVA + α -cubebenoate group than OVA group (Fig 2-A-c). The degree of lung inflammation was further quantitatively analyzed on a subjective scale of 0-3, as described elsewhere (Kwak et al., 2003; Tournoy et al., 2000). Inflammatory score in OVA-treated group was 3, which is significantly higher than PBS group. And treatment with α -cubebenoate reduced the inflammation score (Fig 2-B).

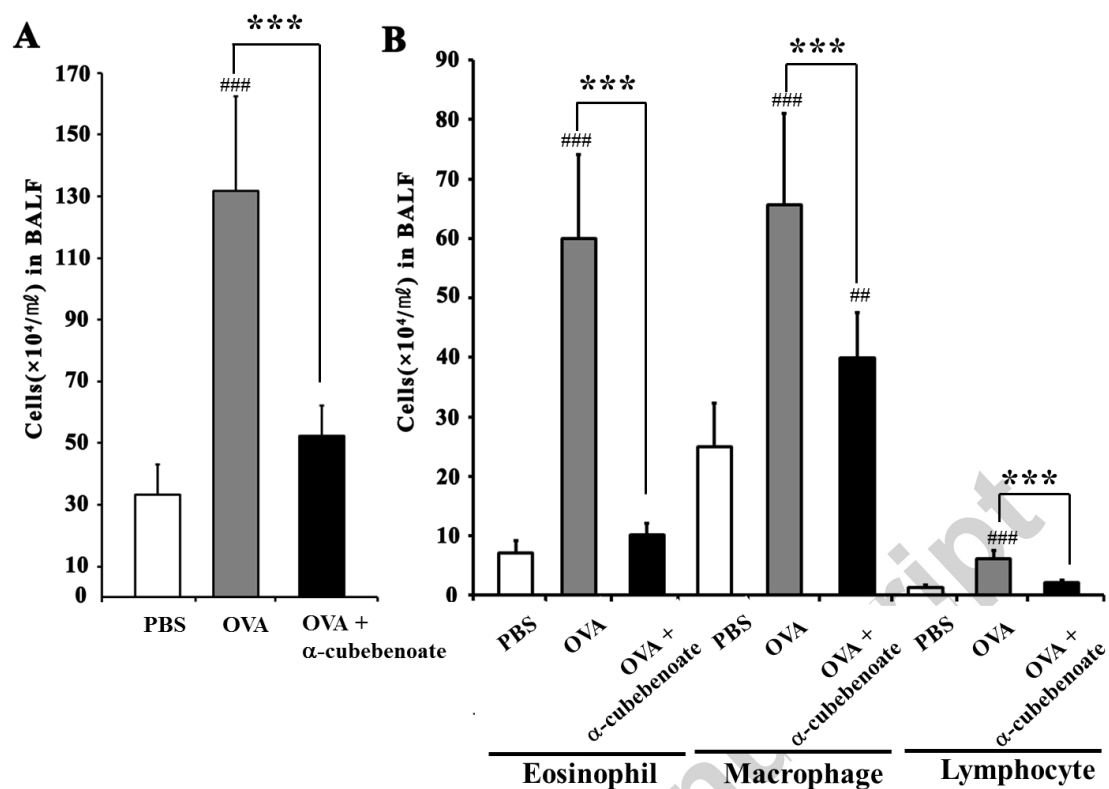
The lower panels of Fig 2-A show representative PAS staining of lungs from PBS, OVA, and OVA + α -cubebenoate groups. PAS staining was used to detect mucous glycoproteins (mucins) produced by goblet cells. In the PAS staining, secreted or stored mucin is stained as purple color. Comparing the Fig 2-A-d and e, it is obvious that OVA group showed darker purple surrounding the bronchiole and thicker than PBS group, meaning enhanced production of mucin in OVA-treated group. In contrast, Fig 2-A-f shows less and brighter purple staining in OVA + α -cubebenoate group than OVA group, suggesting inhibition of mucin production by α -cubebenoate. Therefore, PAS-stained mucin was strongly detected in the lungs of OVA-induced asthma mice, but treatment with α -cubebenoate reduced this staining (Fig 2-A lower panel).

Figure 2

3.3 α -Cubebenoate inhibited the asthma-induced accumulation of immune cells in BALF

Total cell numbers in BALF were increased as 395% in the OVA-induced asthma group compared to PBS control group (Fig 3-A). The increment of total cells in OVA group was inhibited by α -cubebenoate treatment about 81% (Fig 3-A). Further analyses of cell populations in BALF supported this observation (Fig 3-B). In particular, eosinophil numbers were increased as 848% in the BALF of the OVA group compared to PBS control group. This increment of eosinophils was inhibited in the OVA + α -cubebenoate group about 94% (Fig 3-B). Furthermore, BALF macrophage and lymphocyte counts in the OVA group were 263% and 22% increased, respectively compared to PBS group (Fig 3-B). In the OVA + α -cubebenoate group, the increased counts of macrophages and lymphocytes were inhibited about 63% and 82%, respectively (Fig 3-B).

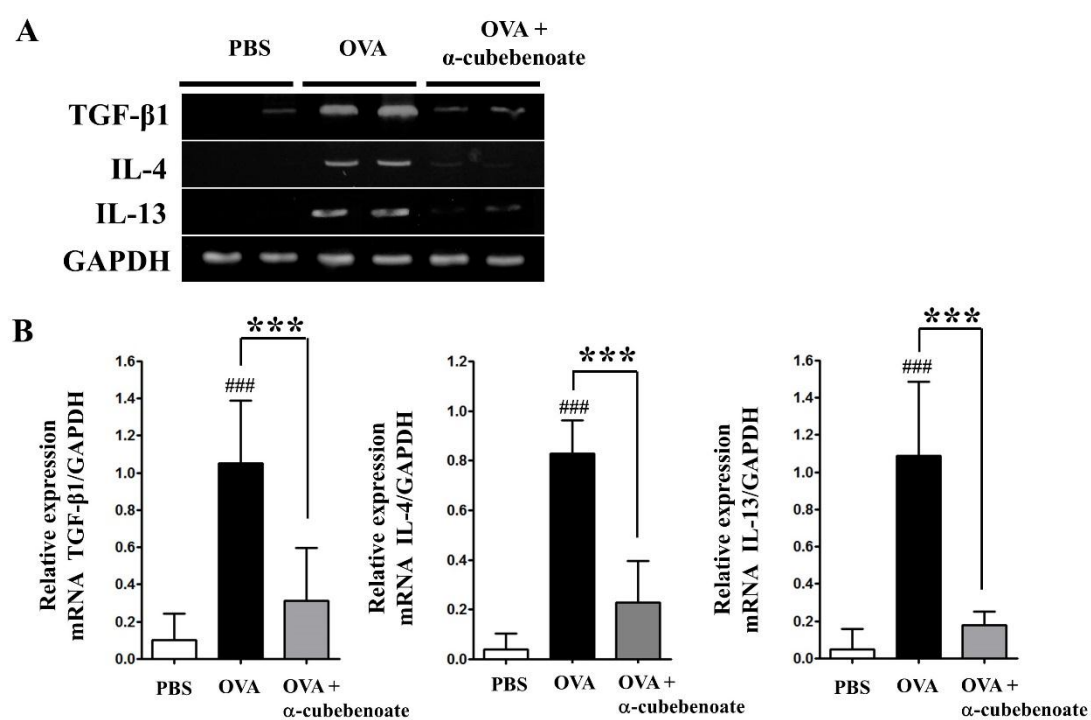
Figure 3

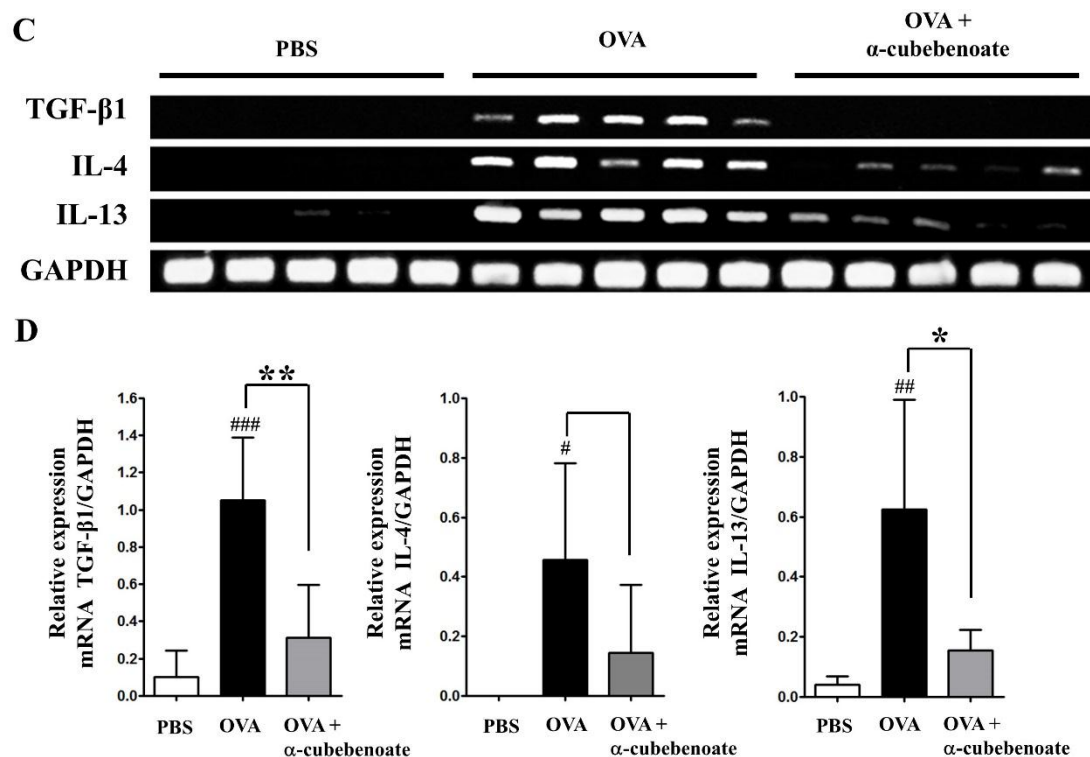


α - Cubebenoate inhibited the production of Th2 cytokines in BALF

The cytokines of Th2 cells play key roles in the pathogenesis of asthma (Fish et al., 2005; Locksley, 2010). IL-4 and IL-13 are related to eosinophil recruitment and activation, goblet cell metaplasia, mucus hyper-secretion in epithelial cells, and smooth muscle proliferation (Fish et al., 2005; Locksley, 2010), whereas TGF- β 1 is related to goblet cell hypertrophy and the destruction of epithelial cells (Duvernelle et al., 2003). Therefore, we measured changes in the mRNA levels of IL-4, IL-13, and TGF- β 1 in lung tissues and in immune cells of BALF. As shown in Fig 4, the mRNA levels of IL-4, IL-13, and TGF- β 1 were elevated 1033%, 2159%, and 2216%, respectively in the lung tissues and 1402%, 24506%, 1522%, respectively in BALF immune cells in the OVA group, but these elevations of IL-4, IL-13, and TGF- β 1 were markedly inhibited by 78%, 74%, and 88%, respectively in the lung tissues and by 95%, 68%, and 81%, respectively in immune cells in the OVA + α -cubebenoate group (Fig 4).

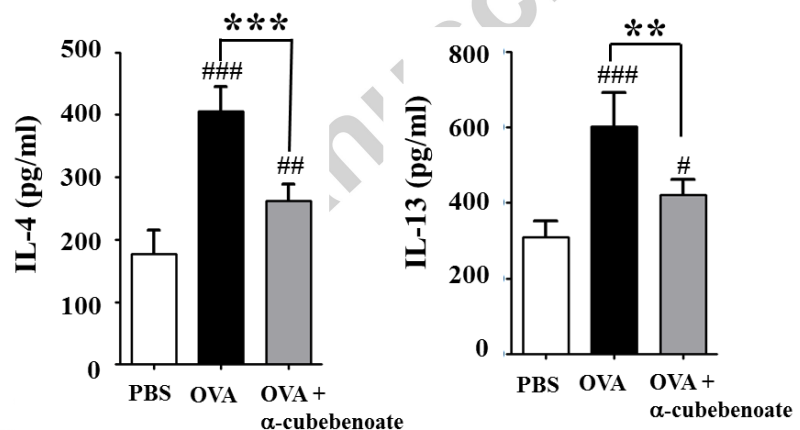
Figure 4





To confirm the effect of α -cubebenoate on the expressions of Th2 cytokines, we measured the protein levels of IL-4 and IL-13 in BALF by ELISA. As shown in Fig 5, IL-4 and IL-13 levels were increased as 229% and 222%, respectively, in the OVA group compared to the levels in control group (Fig 5). Again these increases were partly inhibited by the treatment of α -cubebenoate (Fig 5). IL-4 was 63% reduced by α -cubebenoate and IL-13 was 75% reduced by α -cubebenoate (Fig 5)

Figure 5



3. Discussion

The dried fruits of *Schisandra chinensis* are used traditionally to treat asthma in the Orient (Liu, 1989; Panossian and Wikman, 2008). Extracts of *Schisandra chinensis* fruits or mixtures containing *Schisandra chinensis* fruits have been shown to possess anti-allergic and anti-asthma effects. For example, *Schisandra chinensis* extract was found to have a suppressive effect in an atopic dermatitis model and in an OVA-induced asthma model (Kang and Shin, 2012; Kim et al., 2014), and a decoction of *Schisandra* fruit and earthworm inhibited the infiltration and diffusion of inflammatory cells and regulated leukotriene B₄ and interferon- γ in a guinea pig model of asthma (Li et al., 2009). Furthermore, *Schisandra* fruit is the main constituent of bakumijiogan, a herbal formula used in traditional Chinese medicine to suppress atopic dermatitis-like symptoms by correcting Th1/Th2 imbalance (Makino et al., 2008). However, the constituents of *Schisandra* fruit responsible for its anti-allergy and anti-asthma effects have not been identified. Nevertheless, gomisin N and schisandrin from *Schisandra chinensis* fruit have been previously studied with respect to their anti-allergic effects, and gomisin N was shown to have an anti-allergic effect and to inhibit inflammatory cytokine expression in mouse bone marrow-derived mast cells (Chae et al., 2011). In addition, the anti-allergic effects of schizandrin has been demonstrated on OVA-induced airway inflammation in a murine asthma model (Lee et al., 2010) and on passive cutaneous anaphylaxis and scratching behavior in mice (Lee et al., 2007). Furthermore, the effect of schizandrin on the expression of thymic stromal lymphopoietin has been reported in the HMC-1 human mast cell line (Moon et al., 2012).

In a previous study, we identified α -cubebenoate in the extract of the dried fruits of *Schisandra chinensis* and described its anti-inflammatory effects in peritoneal macrophages

(Kang et al., 2014). The present study shows that α -cubebenoate has anti-allergic effects in vitro and in vivo. In RBL-2H3 mast cells, α -cubebenoate inhibited antigen-induced degranulation and antigen-induced $[Ca^{2+}]_i$ increases. In a previous study, schizandrin was found to inhibit in vitro degranulation in compound 48/80-induced rat peritoneal mast cells and in IgE-induced RBL 2H3 cells (Lee et al., 2007). Mast cell degranulation is triggered by an increase in $[Ca^{2+}]_i$ (Lee et al., 2007), and thus, our observation of the inhibition of antigen-induced increases in $[Ca^{2+}]_i$ by α -cubebenoate is interesting from the mechanistic perspective.

IL-4 is an important contributor to the pathogenesis of allergic diseases because it acts on B cells to facilitate IgE production (Fish et al., 2005). IL-13 also plays critical roles in asthma by promoting the productions of Th2 cytokines in immune cells (Fish et al., 2005; Locksley, 2010), and has also been reported to be necessary for mucous metaplasia in several mouse models of asthma (Grunig et al., 1998; Tyner et al., 2006; Wills-Karp et al., 1998). On the other hand, TGF- β 1 is produced in airways by inflammatory cells, such as, eosinophils, lymphocytes, and macrophages, which infiltrate bronchial mucosa, and by structural cells of the airway wall, such as, fibroblasts and epithelial, endothelial, and smooth muscle cells (Duvernelle et al., 2003). Increased levels of TGF- β 1 have been reported in BALF samples from asthmatic patients (Akdis et al., 2004; Howell and McAnulty, 2006). In the present study, α -cubebenoate inhibited the inductions of Th2 cytokines, such as, IL-4 and IL-13, which are responsible for IgE production, eosinophil accumulation, and mucus hypersecretion (Jiang et al., 2000), in our OVA-induced mouse model. Furthermore, this inhibitory effect was observed in BALF immune cells and in lung tissues at the mRNA and protein levels. Furthermore, α -cubebenoate decreased the expression of TGF- β 1 at the mRNA level in lung tissue and immune cells of the OVA-induced asthma mouse model. These

findings regarding the inhibition of cytokine expression by α -cubebenoate, suggest that α -cubebenoate might have anti-asthma effects in vivo. Moreover, our lung histology findings indicate that α -cubebenoate reduces eosinophil counts in lung tissue and mucin secretion by goblet cells, and thus, suggest that α -cubebenoate might suppress histopathologic changes in lungs, by inhibiting IL-4 and IL-13 levels in lung tissues.

4. Conclusion

The present study shows that α -cubebenoate from *Schisandra chinensis* fruits exerts anti-allergy effects by inhibiting antigen-induced degranulation and $[Ca^{2+}]_i$ increases in mast cells and by suppressing Th2 cytokine secretion in BALF and TGF- β 1 expression in the lung tissues and BALF from OVA-induced asthma mice. We believe these findings indicate α -cubebenoate could be utilized to develop anti-allergic and anti-asthma treatments.

Acknowledgement

This research was supported by the High Value-added Food Technology Development Program (Grant no. 111135-03-2-SB030) of the Korean Ministry for Food, Agriculture, Forestry, and Fisheries and by the Korean National Research Foundation funded by the Korean government (MSIP) (Grant no. 2009-0083538).

Accepted manuscript

Abbreviations used are: OVA, ovalbumin; IL-4, interleukin-4; IL-13, interleukin-13; TGF- β 1, transforming growth factor beta-1; BALF, bronchoalveolar fluid; PAS, periodic acid-schif; H&E, hematoxylin and eosin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ELISA, enzyme-linked immunosorbent assay; DNP, dinitrophenyl; DNP-hAb, human dinitrophenyl albumin; PBS, phosphate-buffered saline; PCR, polymerase-chain reaction; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

Figure Legends

Figure 1. Effects of α -cubebenoate on antigen-induced β -hexosaminidase release and $[Ca^{2+}]_i$ increases in RBL-2H3 mast cells

In A, structure of α -cubebenoate. In B, RBL-2H3 cells were sensitized for 18 hr with anti DNP-IgE and then challenged with DNP human serum albumin. α -Cubebenoate at the indicated concentrations was treated an hour before antigen challenge. Results are presented as means $\pm$ SEs of three independent experiments. In C, RBL-2H3 cells were sensitized 2 h in complete medium with anti DNP-IgE. The cells were loaded with Fura-2 AM for 45 min, washed twice with HBM. The cells were pre-incubated with α -cubebenoate at the indicated concentrations for 10 min, and then treated with DNP human serum albumin. Each arrow indicates that the antigen was added. Representative traces from three independent experiments.

Figure 2. Protective effect of α -cubebenoate on airway inflammation.

In A, each panel shows lung tissues from each corresponding group; a and d from PBS group, b and e from OVA group and c and f from α -cubebenoate-treated OVA group. a, b, and c show H&E-stained sections and d, e, and f show PAS-stained sections. In the H&E staining, the accumulation of eosinophils surrounding the bronchiole is observable as small black dots. In PBS group, eosinophils are rarely observed as arrow-indicated. In OVA group, eosinophils are densely accumulated surrounding the bronchioles as arrow-indicated. In OVA + α -cubebenoate group, the accumulation of eosinophils is less than OVA group. Therefore, α -cubebenoate treatment improved airway inflammation compared with OVA mice. In PAS

staining, mucin is stained as purple color. In OVA group, darker and thicker purple color is observed surrounding the bronchiole compare to PBS group. In OVA + α -cubebenoate group, less and brighter purple staining is observed than OVA group. Five mice were used for each group. Bar = 50 μ m.

In B, degree of lung inflammation was evaluated from H&E staining. Data of the histological analysis are reported as scores. Scoring method is described in method section in accordance with Tournoy et al., 2000 and Kwak et al., 2003 (Kwak et al., 2003; Tournoy et al., 2000). The values shown are means $\pm$ SEs (n=5). Statistical significance: # $p < 0.05$, and ### $p < 0.001$ vs. PBS controls. *** $p < 0.001$ vs. the OVA-treated group.

Figure 3. Effect of α -cubebenoate on immune cell counts in BALF of OVA-induced asthmatic mice.

Five mice were used for each group. Mice were sensitized with OVA twice by intraperitoneal injection (1st and 14th days) and were later challenged with nebulized OVA on days 28, 29, and 30. α -Cubebenoate was administrated by intraperitoneal injection (1 mg/kg) on these days 30 min before OVA challenge. Cells in BALF were stained using the May-Grunwald method and counted. (A) Total cell counts in the BALF of PBS control mice, OVA-induced asthmatic mice (OVA), and α -cubebenoate-treated asthma mice (OVA + α -cubebenoate). (B) Cells counts of eosinophils, macrophages, and lymphocytes in the BALF of these three groups. The values shown are means $\pm$ SEs (n=5). Statistical significance: ## $p < 0.01$, and ### $p < 0.001$ vs. PBS controls. *** $p < 0.001$ vs. the OVA-treated group.

Figure 4. α -Cubebenoate inhibited Th2 cytokines and TGF- β 1 expressions at the mRNA level

in lung tissues and immune cells in the OVA-induced mouse model of asthma.

Five mice were used for each group. A, the expressions of TGF- β 1, IL-4, and IL-13 mRNAs in the lung tissues of the OVA-induced model. B, mRNA levels were quantified as ratios of GAPDH mRNA levels. C, the expressions of TGF- β 1, IL-4, and IL-13 mRNAs in BALF immune cells. Five lanes for each group were made with samples from five different mice. D, mRNA levels were quantified as ratios of GAPDH mRNA levels. The values shown are means $\pm$ SEs (n=5). Statistical significance: ### p < 0.001 vs. PBS controls. ** p < 0.01, and *** p < 0.001 vs. OVA-treated mice group.

Figure 5. The effect of α -cubebenoate on the secretions of IL-4 and IL-13 in BALF

ELISA results for IL-4 and IL-13 were obtained in BALFs from five mice for each group. The values shown are means $\pm$ SEs (n=5). Statistical significance: # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. PBS controls. ** p < 0.01, and *** p < 0.001 vs. OVA-treated mice.

References

- Akdis, C.A., Blaser, K., Akdis, M., 2004. Genes of tolerance. *Allergy* 59, 897-913.
- Aoki, H., Hisada, T., Ishizuka, T., Utsugi, M., Ono, A., Koga, Y., Sunaga, N., Nakakura, T., Okajima, F., Dobashi, K., Mori, M., 2010. Protective effect of resolvin E1 on the development of asthmatic airway inflammation. *Biochem Biophys Res Commun* 400, 128-133.
- Aoki, H., Mogi, C., Okajima, F., 2014. Ionotropic and metabotropic proton-sensing receptors involved in airway inflammation in allergic asthma. *Mediators Inflamm* 2014, 712962.
- Braber, S., Henricks, P.A., Nijkamp, F.P., Kraneveld, A.D., Folkerts, G., 2010. Inflammatory changes in the airways of mice caused by cigarette smoke exposure are only partially reversed after smoking cessation. *Respir Res* 11, 99.
- Busse, W.W., Rosenwasser, L.J., 2003. Mechanisms of asthma. *J Allergy Clin Immunol* 111, S799-804.
- Chae, H.S., Kang, O.H., Oh, Y.C., Choi, J.G., Keum, J.H., Kim, S.B., Kim, Y.S., Mun, S.H., Shin, D.W., Han, S.H., Kwon, D.Y., 2011. Gomisin N has anti-allergic effect and inhibits inflammatory cytokine expression in mouse bone marrow-derived mast cells. *Immunopharmacol Immunotoxicol* 33, 709-713.
- Duvernelle, C., Freund, V., Frossard, N., 2003. Transforming growth factor- β and its role in asthma. *Pulm Pharmacol Ther* 16, 181-196.
- Fish, S.C., Donaldson, D.D., Goldman, S.J., Williams, C.M., Kasaian, M.T., 2005. IgE generation and mast cell effector function in mice deficient in IL-4 and IL-13. *J Immunol* 174, 7716-7724.
- Grunig, G., Warnock, M., Wakil, A.E., Venkayya, R., Brombacher, F., Rennick, D.M.,

- Sheppard, D., Mohrs, M., Donaldson, D.D., Locksley, R.M., Corry, D.B., 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282, 2261-2263.
- Howell, J.E., McAnulty, R.J., 2006. TGF- β : its role in asthma and therapeutic potential. *Curr Drug Targets* 7, 547-565.
- Jiang, H., Harris, M.B., Rothman, P., 2000. IL-4/IL-13 signaling beyond JAK/STAT. *J Allergy Clin Immunol* 105, 1063-1070.
- Kang, S., Lee, K.P., Park, S.J., Noh, D.Y., Kim, J.M., Moon, H.R., Lee, Y.G., Choi, Y.W., Im, D.S., 2014. Identification of a novel anti-inflammatory compound, α -cubebenoate from *Schisandra chinensis*. *J Ethnopharmacol* 153, 242-249.
- Kang, Y.H., Shin, H.M., 2012. Inhibitory effects of *Schizandra chinensis* extract on atopic dermatitis in NC/Nga mice. *Immunopharmacol Immunotoxicol* 34, 292-298.
- Kim, H., Ahn, Y.T., Kim, Y.S., Cho, S.I., An, W.G., 2014. Antiasthmatic effects of *schizandrae fructus* extract in mice with asthma. *Pharmacogn Mag* 10, S80-85.
- Kwak, Y.G., Song, C.H., Yi, H.K., Hwang, P.H., Kim, J.S., Lee, K.S., Lee, Y.C., 2003. Involvement of PTEN in airway hyperresponsiveness and inflammation in bronchial asthma. *J Clin Invest* 111, 1083-1092.
- Lane, S.J., Lee, T.H., 1996. Mast cell effector mechanisms. *J Allergy Clin Immunol* 98, S67-71.
- Lee, B., Bae, E.A., Trinh, H.T., Shin, Y.W., Phuong, T.T., Bae, K.H., Kim, D.H., 2007. Inhibitory effect of schizandrin on passive cutaneous anaphylaxis reaction and scratching behaviors in mice. *Biol Pharm Bull* 30, 1153-1156.
- Lee, K.P., Kang, S., Noh, M.S., Park, S.J., Kim, J.M., Chung, H.Y., Je, N.K., Lee, Y.G., Choi, Y.W., Im, D.S., 2015. Therapeutic effects of s-petasin on disease models of asthma and peritonitis. *Biomol Ther (Seoul)* 23, 45-52.

- Lee, K.P., Kang, S., Park, S.J., Choi, Y.W., Lee, Y.G., Im, D.S., 2013. Anti-allergic and anti-inflammatory effects of bakkenolide B isolated from *Petasites japonicus* leaves. *J Ethnopharmacol* 148, 890-894.
- Lee, M.Y., Seo, C.S., Lee, N.H., Ha, H., Lee, J.A., Lee, H., Lee, K.Y., Shin, H.K., 2010. Anti-asthmatic effect of schizandrin on OVA-induced airway inflammation in a murine asthma model. *Int Immunopharmacol* 10, 1374-1379.
- Li, X.H., Tu, X.Y., Zhang, D.X., Xu, J.F., Wang, W.Y., Zhang, Y., Du, Y.M., 2009. Effects of wuwei dilong decoction on inflammatory cells and cytokines in asthma model guinea pigs. *J Tradit Chin Med* 29, 220-223.
- Liu, G.T., 1989. Pharmacological actions and clinical use of fructus schizandrae. *Chin Med J (Engl)* 102, 740-749.
- Locksley, R.M., 2010. Asthma and allergic inflammation. *Cell* 140, 777-783.
- Makinde, T., Murphy, R.F., Agrawal, D.K., 2007. The regulatory role of TGF- β in airway remodeling in asthma. *Immunol Cell Biol* 85, 348-356.
- Makino, T., Hamanaka, M., Yamashita, H., Mizukami, H., 2008. Effect of bakumijiogan, an herbal formula in traditional Chinese medicine, on atopic dermatitis-like skin lesions induced by mite antigen in NC/Jic mice. *Biol Pharm Bull* 31, 2108-2113.
- Mashkovskij, M.D., 2000. Medicinal Drugs-Manual on Pharmacotherapy for Doctors., vol. 1., 14th ed. New Wave Publishing House, Moscow. pp134-135
- Moon, P.D., Jeong, H.J., Kim, H.M., 2012. Effects of schizandrin on the expression of thymic stromal lymphopoietin in human mast cell line HMC-1. *Life Sci* 91, 384-388.
- Panossian, A., Wikman, G., 2008. Pharmacology of *Schisandra chinensis* Bail.: an overview of Russian research and uses in medicine. *J Ethnopharmacol* 118, 183-212.
- Park, S.J., Lee, K.P., Im, D.S., 2014. Action and Signaling of Lysophosphatidylethanolamine

- in MDA-MB-231 Breast Cancer Cells. *Biomol Ther (Seoul)* 22, 129-135.
- Tournoy, K.G., Kips, J.C., Schou, C., Pauwels, R.A., 2000. Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 30, 79-85.
- Tyner, J.W., Kim, E.Y., Ide, K., Pelletier, M.R., Roswit, W.T., Morton, J.D., Battaile, J.T., Patel, A.C., Patterson, G.A., Castro, M., Spoor, M.S., You, Y., Brody, S.L., Holtzman, M.J., 2006. Blocking airway mucous cell metaplasia by inhibiting EGFR antiapoptosis and IL-13 transdifferentiation signals. *J Clin Invest* 116, 309-321.
- Vo, V.A., Lee, J.W., Shin, S.Y., Kwon, J.H., Lee, H.J., Kim, S.S., Kwon, Y.S., Chun, W., 2014. Methyl p-Hydroxycinnamate Suppresses Lipopolysaccharide-Induced Inflammatory Responses through Akt Phosphorylation in RAW264.7 Cells. *Biomol Ther (Seoul)* 22, 10-16.
- Wen, F.Q., Liu, X., Manda, W., Terasaki, Y., Kobayashi, T., Abe, S., Fang, Q., Ertl, R., Manouilova, L., Rennard, S.I., 2003. TH2 Cytokine-enhanced and TGF- β -enhanced vascular endothelial growth factor production by cultured human airway smooth muscle cells is attenuated by IFN- γ and corticosteroids. *J Allergy Clin Immunol* 111, 1307-1318.
- Wenzel, S.E., 2012. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 18, 716-725.
- Wills-Karp, M., 2004. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 202, 175-190.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258-2261.
- Youn, C.K., Park, S.J., Lee, M.Y., Cha, M.J., Kim, O.H., You, H.J., Chang, I.Y., Yoon, S.P., Jeon, Y.J., 2013. Silibinin Inhibits LPS-Induced Macrophage Activation by Blocking p38 MAPK in RAW 264.7 Cells. *Biomol Ther (Seoul)* 21, 258-263.

