



Anti-allergic effects of sesquiterpene lactones from *Saussurea costus* (Falc.) Lipsch. determined using *in vivo* and *in vitro* experiments

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ABSTRACT

Ethnopharmacological relevance: *Saussurea costus* (Falc.) Lipsch. root has been used in Asian traditional medicine for the treatment of asthma, rheumatism, and other conditions. *S. costus* extracts were shown to alleviate house dust mite-induced atopic-like dermatitis in Nc/Nga mice; besides, sesquiterpene lactones were isolated from *S. costus* extracts.

Aims of the study: We aimed to investigate the effects of sesquiterpene lactones (alantolactone, costunolide, and dehydrocostuslactone) in allergic asthma using female Balb/c mice and rat RBL-2H3 mast cells.

Materials and methods: Antigen-induced degranulation was assessed by measuring β -hexosaminidase activity *in vitro*. In addition, a murine ovalbumin-induced allergic asthma model was used to test the *in vivo* efficacy of sesquiterpene lactones.

Results: Sesquiterpene lactones inhibited antigen-induced degranulation, wherein dehydrocostuslactone > costunolide > alantolactone in potency. Administration of sesquiterpene lactones decreased the number of immune cells, particularly eosinophils, and reduced the expression and secretion of Th2 cytokines (IL-4 and IL-13) in the bronchoalveolar lavage fluid and lung tissues of mice with ovalbumin-induced allergic asthma. Histological studies showed that sesquiterpene lactones reduced inflammation and mucin production in the lungs. Similar to the *in vitro* study, dehydrocostuslactone showed the highest potency, followed by costunolide and alantolactone.

Conclusion: These findings provide evidence that sesquiterpene lactones might be potential anti-allergic therapeutics.

1. Introduction

Saussurea costus (Falc.) Lipsch. has traditionally been used for the treatment of asthma, rheumatism, abdominal pain, anorexia, nausea, and other conditions (Choi et al., 2012; Seo et al., 2015; Shah, 1982). Extracts of *S. costus* alleviated house dust mite-induced atopic-like dermatitis in Nc/Nga mice (Lim et al., 2014, 2015a). Three sesquiterpene lactones (alantolactone, costunolide, and dehydrocostuslactone) were isolated from *S. costus* and identified as anti-inflammatory components (structures in Fig. 1) (Seo et al., 2015; Seo and Shin, 2015). Sesquiterpene lactones showed anti-atopic effects *in vitro* in HaCaT human keratinocyte cell line (Lim et al., 2015b; Seo et al., 2015). In addition, they inhibited tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ)-induced chemokine production (Lim et al.,

2015b; Seo et al., 2015). However, the *in vivo* anti-allergic efficacy of sesquiterpene lactones has not been evaluated yet.

Given that the extracts of *S. costus* exhibited protective effects against atopic-like dermatitis (Lim et al., 2014, 2015a), and the isolated sesquiterpene lactones inhibited chemokine production in HaCaT keratinocyte cell line (Lim et al., 2015b; Seo et al., 2015), we aimed to investigate the effects of sesquiterpene lactones (alantolactone, costunolide, and dehydrocostuslactone) in allergic asthma using female Balb/c mice and rat RBL-2H3 mast cells.

Abbreviations: OVA, ovalbumin; IL-4, interleukin-4; IL-13, interleukin-13; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff; 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

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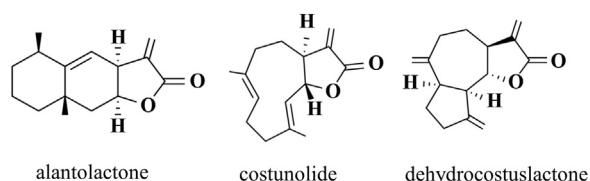


Fig. 1. Structures of alantolactone, costunolide, and dehydrocostuslactone.

2. Materials and methods

2.1. Materials

Alantolactone (SML0415, $\geq 98\%$ HPLC, molecular weight 232.32, Pubchem ID 329825410), costunolide (SML0417, $\geq 97\%$ HPLC, molecular weight 232.32, Pubchem ID 329825412), dehydrocostuslactone (42,575, $\geq 98\%$ GC, molecular weight 230.30, Pubchem ID 329756240) and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RBL-2H3 cells were cultured at 37°C in a 5% CO_2 -humidified incubator, and maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, and $50\text{ }\mu\text{g/mL}$ streptomycin (Lee et al., 2016a).

2.3. Animals

Five-week-old female Balb/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea) and housed in the laboratory animal facility at Pusan National University. They were provided food and water *ad libitum*. The protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care Committee (PNU-IACUC) with respect to ethical issues and scientific care (Approval Number, ED-PNU-2017-1445).

2.4. Assessment of degranulation

Degranulation was evaluated by measuring β -hexosaminidase release (Lee et al., 2016a). Briefly, RBL-2H3 cells (2×10^5 cells/well in 24-well plates) were sensitized *via* incubation with $0.2\text{ }\mu\text{g/mL}$ monoclonal anti-dinitrophenyl mouse immunoglobulin E (DNP-IgE; D8406, Sigma-Aldrich) overnight at 37°C in a 5% CO_2 incubator. Before stimulation, cells were washed twice with piperazine- N,N' -bis-(2-ethanesulfonic acid) (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% bovine serum albumin (BSA), and 1 mM CaCl_2 to remove DNP-IgE. Cells were then incubated in $400\text{ }\mu\text{L}$ of PIPES buffer containing different concentrations of alantolactone, costunolide, or dehydrocostuslactone at 37°C for 30 min . Then human dinitrophenyl albumin ($1\text{ }\mu\text{g/mL}$; DNP-hAb; A6661, Sigma-Aldrich) was added, and cells were incubated for additional 15 min at 37°C to induce degranulation. Aliquots ($25\text{ }\mu\text{L}$) of the medium were then transferred to a 96-well microplate and incubated for 110 min with $25\text{ }\mu\text{L}$ of 5 mM 4-nitrophenyl N -acetyl- β -D-glucosaminide (N9376, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5). By adding $200\text{ }\mu\text{L}$ of 0.05 M sodium carbonate buffer (pH 10; 0.05 M $\text{Na}_2\text{CO}_3/0.05\text{ M}$ NaHCO_3), the reaction was terminated. Absorbance (optical density; OD) was measured at 405 nm using a microplate reader (Choi et al., 2016).

2.5. Induction of asthma in Balb/c mice and administration of sesquiterpene lactones

Six-week-old Balb/c mice (22 g) were divided into five groups ($n = 5$) as follows: phosphate-buffered saline (PBS)-injected control group, ovalbumin (OVA)-injected asthma group, OVA-injected plus alantolactone-treated group (10 mg/kg), OVA-injected plus dehydrocostuslactone-treated group (10 mg/kg), and OVA-injected plus costunolide-treated group (10 mg/kg). Asthma was induced by intraperitoneal injection of OVA ($50\text{ }\mu\text{g}$; A5503, Sigma-Aldrich) and aluminum hydroxide (1 mg ; 239,186, Sigma-Aldrich) on days 0 and 14. Mice were exposed to nebulized OVA for 20 min for three consecutive days from day 28 (Aoki et al., 2010). Each sesquiterpene lactone was administered intraperitoneally 30 min before OVA challenge. After two days (day 32), bronchoalveolar lavage fluid (BALF) was collected from the lungs, and immune cells in BALF were stained and counted (Choi et al., 2016).

On day 32, lungs were excised and fixed with 4% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer. Next, they were cryoprotected using 30% sucrose solution in 0.1 M phosphate buffer. Tissues were embedded in paraffin and sectioned at $4\text{ }\mu\text{m}$ thickness. Sections were then thaw-mounted onto Superfrost® microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at -20°C until further use.

2.6. Histological examination of the lungs and cell counting in BALF

Lung sections from mice of different experimental groups were prepared and examined. Briefly, left lungs were fixed in 10% formalin and embedded in paraffin. Sections ($4\text{ }\mu\text{m}$) were stained with periodic acid-Schiff (PAS) stain to identify mucus-secreting cells (goblet cells) in the airways, and were counterstained with hematoxylin (Lee et al., 2016a). Lung tissues from all five mice in each group were examined. For PAS staining, sections were deparaffinized, hydrated, and placed in periodic acid solution at room temperature for 15 min . After rinsing with water, sections were stained with Schiff's reagent (3952, Sigma-Aldrich) for 15 min , rinsed with running tap water, and stained with hematoxylin for 90 s . They were then rinsed, dehydrated, and cover-slipped.

Lung inflammation was scored by a treatment-blind observer. Degree of lung inflammation was evaluated using a subjective scale of 0–3, as previously described (Kwak et al., 2003; Tournoy et al., 2000). Briefly, a score of 0 indicated no inflammation, 1 indicated occasional cuffing and presence of inflammatory cells, 2 indicated that most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and 3 indicated that most bronchi or vessels were surrounded by a thick layer (> 5 cells thick) of inflammatory cells. Total lung inflammation was defined as the average lung inflammation score. Five lung sections per mouse were evaluated, and the average inflammation score was calculated (Braber et al., 2010). Mucin-secreting cells in the airways were stained with PAS and counted in the large-caliber preterminal bronchi in at least two lung sections per mouse. The length of the basal lamina of bronchi was measured using ImageJ software (National Institute of Health).

Cells in BALF were attached to a glass slide using a Cellspin® centrifuge (5 min , 500 rpm ; Hanil Electric) and fixed in methanol for 30 s . Then, they were successively processed with May-Grünwald solution (32,856, Sigma-Aldrich) for 8 min and Giemsa solution (32,884, Sigma-Aldrich) for 12 min . Slides were then rinsed and covered.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Gene expression of asthmatic markers in type 2 T helper (Th2) cells was measured by RT-PCR. Total RNA was isolated from all five mice in each group using TRIzol reagent (Invitrogen, Waltham, MA, USA). cDNA was first synthesized from the total RNA. The synthesized cDNA

products, primers for each gene, and Promega GoTaq® DNA polymerase (Madison, WI, USA) were used. Specific primers and PCR conditions were previously described (Lee et al., 2016a). PCR was performed as follows: 27 cycles of denaturation at 95°C for 30 s, annealing at 49–53°C for 30 s, and elongation at 72°C for 30 s in an Eppendorf® Mastercycler® PCR machine (Hamburg, Germany). Aliquots (7 µL) were electrophoresed on 1.2% agarose gel and stained with StaySafe™ nucleic acid gel stain (Real Biotech Corporation, Taipei, Taiwan) (Lee et al., 2016b).

2.8. Measurement of cytokine (IL-4 and IL-13) levels

Interleukin (IL)–4 and IL-13 levels in BALF isolated from five mice per group were measured using enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates (NUNC, Penfield, NY, USA) were coated overnight at 4°C with a capture antibody specific for IL-4 (cat. 14–7041-68, eBioscience, San Diego, CA, USA) or IL-13 (cat. 14–7043-68, eBioscience). After washing, plates were blocked for 1 h at room temperature with a blocking buffer. Serial dilutions of standard cytokines were prepared and added to the appropriate wells; alternatively, supernatants of BALF were added to the wells. Plates were incubated for 2 h at room temperature with shaking and then washed five times. Biotinylated detection antibody specific for IL-4 (cat. 33-7042-68C, eBioscience) or IL-13 (cat. 33-7135-68B, eBioscience) was then added and incubated for 1 h at room temperature with shaking. Then plates were washed five times, and avidin-horseradish peroxidase (HRP) was added at room temperature for 30 min with shaking. Plates were then washed 14 times and incubated with the substrate solution at room temperature for 15 min. Then a stop solution was added, and the absorbance was measured at 450 nm (Lee et al., 2016a).

2.9. Statistical analysis

Results are expressed as means ± standard errors (SEs). Differences among groups were tested for statistical significance using analysis of variance (ANOVA) followed by Turkey's *post hoc* test. *P* values < 0.05 indicated statistical significance. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Sesquiterpene lactones inhibited antigen-induced degranulation in RBL-2H3 mast cells

Mast cells play an important role in the initiation and development of allergic responses. Fc epsilon RI (FcεRI) on the cell membranes of mast cells traps circulating IgEs. This antigen exposure induces cross-linking of the cell-bound IgE, resulting in degranulation (Lane and Lee, 1996). Degranulation of histamine, prostaglandins, and leukotrienes from antigen-exposed mast cells is a key step in allergic responses. Rat basophilic RBL-2H3 leukemia cells, a tumor analog of mast cells with high FcεRI surface expression, were used to determine whether sesquiterpene lactones could inhibit antigen-induced degranulation. Degranulation was assessed by measuring β-hexosaminidase activity in the medium after antigen exposure, as previously described (Lee et al., 2016a). All three sesquiterpene lactones inhibited antigen-induced release of β-hexosaminidase in a concentration-dependent manner (Fig. 2). Alantolactone and costunolide inhibited degranulation at concentrations higher than 10 µM (Fig. 2). Dehydrocostuslactone was the most potent and efficacious among the three sesquiterpene lactones (Fig. 2). And there was no cytotoxicity observed as previously reported (Lim et al., 2015b; Seo et al., 2015).

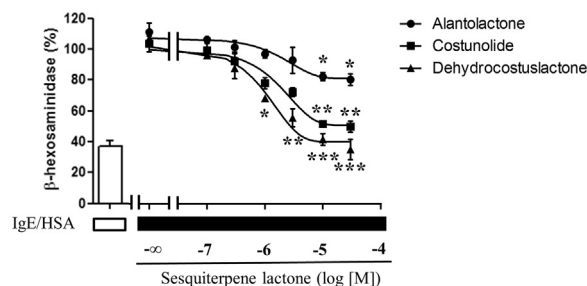


Fig. 2. Effects of alantolactone, costunolide, and dehydrocostuslactone on antigen-induced β-hexosaminidase release in RBL-2H3 mast cells. RBL-2H3 cells were sensitized for 18 h with anti-DNP IgE and then challenged with DNP human serum albumin (HSA). Alantolactone, costunolide, or dehydrocostuslactone was administered at the indicated concentrations 30 min before antigen challenge. Open and filled rectangular bars mean without or with IgE/HSA, respectively. The samples without IgE and HSA mean basal degranulation from the cells and samples with IgE and HSA mean positive control of antigen-induced degranulation. The results are presented as means ± SEs of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the HSA-treated group.

3.2. Sesquiterpene lactones inhibited eosinophil accumulation in BALF of OVA-induced mice

The inhibitory effects of sesquiterpene lactones against antigen-induced degranulation in RBL-2H3 mast cells were confirmed in an OVA-induced asthma mouse model. Cell number and distribution of cell populations in BALF were analyzed. The total cell number in BALF increased to 297% in the OVA-induced asthma group compared to that in the PBS-treated control group (Fig. 3). Costunolide and dehydrocostuslactone significantly inhibited OVA-induced increase in total cell number by 53.3% and 48.7%, respectively; in addition, alantolactone resulted in a non-significant decrease in total cell number by 36.2% (Fig. 3-B). Further analyses of cell populations in BALF supported this finding (Fig. 3-B). Especially, OVA-induced increase in BALF eosinophil number was significantly inhibited by alantolactone, costunolide, and dehydrocostuslactone by 58.9%, 61.8%, and 48.2%, respectively (Fig. 3-B). Lymphocyte counts were lesser than eosinophil counts. OVA administration resulted in a significant increase in lymphocyte counts, which were reduced by sesquiterpene lactone treatment (Fig. 3-B). However, BALF macrophage counts did not significantly increase in the OVA group and were not altered by sesquiterpene lactone treatment (Fig. 3-B).

3.3. Sesquiterpene lactones inhibited inflammation and mucin secretion in the lungs of OVA-induced asthmatic mice

Histological examination of lung samples was performed two days after final antigen challenge. Fig. 4-A shows representative PAS/hematoxylin-stained lung sections from mice in the PBS, OVA, and OVA + sesquiterpene lactone groups. Eosinophils appear as small, navy blue dots, as indicated by the red arrows. In the PBS control group, eosinophils were rarely observed (Fig. 4-A). In the OVA group, however, eosinophils densely surrounded the bronchioles (Fig. 4-A). Treatment with sesquiterpene lactones reduced eosinophil infiltration (Fig. 4-A). Moreover, lung inflammation was semi-quantitatively evaluated using a subjective scale of 0–3, as previously described (Choi et al., 2016; Kwak et al., 2003; Tournoy et al., 2000). The average inflammation score in the OVA-treated group was approximately 3, whereas treatment with costunolide and dehydrocostuslactone significantly reduced the inflammation score (Fig. 4-B). Alantolactone treatment tended to decrease the inflammation score; however, it did not reach statistical significance (Fig. 4-B).

PAS was used to stain mucous glycoproteins (mucins), which are produced by goblet cells. Secreted or stored mucins are stained purple with PAS. As shown in Fig. 4-A, dark violet-stained cells surrounded the bronchioles in the OVA group, as indicated by the green arrows.

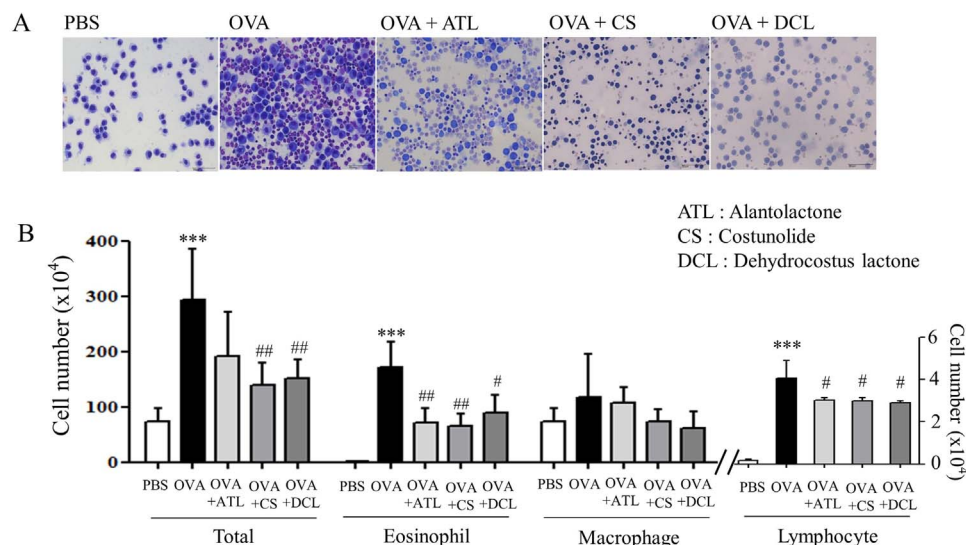


Fig. 3. Effects of alantolactone, costunolide, and dehydrocostuslactone on immune cell accumulation in BALF of OVA-induced asthmatic mice. Mice ($n = 5$ per group) were sensitized with OVA twice by intraperitoneal injection on days 0 and 14, and later challenged with nebulized OVA on days 28, 29, and 30. Each sesquiterpene lactone was intraperitoneally administered at 10 mg/kg 30 min before OVA challenge. Cells in BALF were stained using May-Grunwald stain and counted (A). Total cell counts in BALF of PBS-treated control mice, OVA-induced asthmatic mice (OVA), and sesquiterpene lactone-treated (10 mg/kg) OVA groups and counts of eosinophils, macrophages, and lymphocytes in BALF samples obtained from these four groups (B). Values represent the means $\pm$ SEs ($n = 5$). *** $p < 0.001$ vs. the PBS-treated group, # $p < 0.05$, ## $p < 0.01$ vs. the OVA-treated group.

However, sections from mice in the OVA + sesquiterpene lactone-treated groups showed fewer stained cells, compared to the OVA group, which indicated inhibition of mucin production by sesquiterpene lactones (Fig. 4-A). In addition, mucin production was semi-quantitatively analyzed by counting PAS-positive cells per mm of the bronchioles (Fig. 4-C) (Aoki et al., 2013). Rare staining was observed in the PBS group; however, approximately 100 PAS-positive cells/mm were detected in the OVA-treated group. Sesquiterpene lactones significantly reduced PAS-positive cells (Fig. 4-C). Similar to the *in vitro* degranulation inhibition experiment (Fig. 2), dehydrocostuslactone was the most potent, followed by costunolide and alantolactone (Fig. 4-C).

3.4. Sesquiterpene lactones inhibited the production of Th2 cytokines in lung tissues

Th2 cytokines play key roles in the pathogenesis of asthma (Fish et al., 2005; Locksley, 2010). In particular, IL-4, IL-5, and IL-13 induce eosinophil recruitment and activation, mucus hypersecretion by epithelial cells, goblet cell metaplasia, and proliferation of smooth muscle cells (Fish et al., 2005; Locksley, 2010). Therefore, we measured the mRNA levels of IL-4, IL-5, and IL-13 in BALF and lung tissues. The

mRNA levels of IL-4, IL-5, and IL-13 increased by 1702, 1595, and 1584%, respectively, in the BALF of mice from the OVA group; however, these elevations were inhibited by sesquiterpene lactones (Fig. 5-A). Sesquiterpene lactones inhibited the mRNA expression of IL-4 by 33.9%, 61.4%, and 87.2%; of IL-5 by 51.4%, 60.1%, and 59.9%; and of IL-13 by 62.7%, 65.3%, and 65.6% (Fig. 5-A). Moreover, as shown in Fig. 5-B, the mRNA levels of IL-4, IL-5, and IL-13 increased by 3635, 1776, and 629%, respectively, in the lung tissues of mice from the OVA group; however, sesquiterpene lactones inhibited the mRNA expression of IL-4 by 31.5%, 39.2%, and 78.8%; of IL-5 by 47.8%, 48.7%, and 73%; and of IL-13 by 56.7%, 59.7%, and 65.4% (Fig. 5-B).

To confirm the effects of sesquiterpene lactones on the expression of Th2 cytokines, the levels of IL-4 and IL-13 in BALF were measured by ELISA. IL-4 and IL-13 levels increased in the OVA group, compared to the PBS control group (Fig. 6). OVA-induced increase in IL-13 levels was significantly inhibited by sesquiterpene lactones (Fig. 6). In addition, costunolide and dehydrocostuslactone tended to reduce IL-4 levels; however, these differences did not reach statistical significance (Fig. 6).

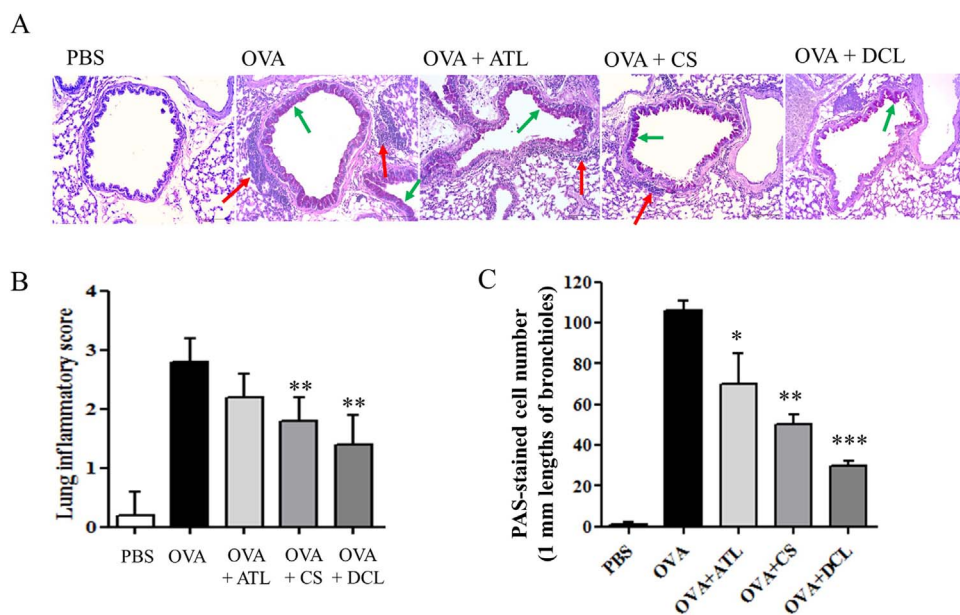
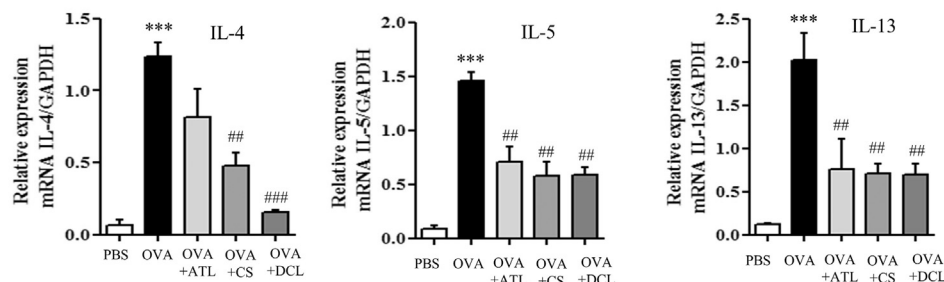


Fig. 4. Protective effects of alantolactone, costunolide, and dehydrocostuslactone against airway inflammation and mucin production. (A) Panels show PAS/hematoxylin-stained sections of lung tissues from the PBS group, OVA group, and sesquiterpene lactone-treated (10 mg/kg) OVA groups. Sections from mice in the OVA and sesquiterpene lactone-treated OVA groups showed accumulation of eosinophils (small navy blue dots) around the bronchioles, whereas eosinophils were rarely observed in the PBS group. Moreover, eosinophils densely accumulated around the bronchioles in the OVA group (red arrows); however, eosinophil accumulation was less obvious in the OVA + 10 mg/kg sesquiterpene lactone groups than in the OVA group, which suggested that sesquiterpene lactones reduced airway inflammation. (B) Lung inflammation was semi-quantitatively evaluated; histological findings were scored as described in the Materials and Methods section and by Tournoy et al. (2000) and Kwak et al. (2003). Values represent the means $\pm$ SEs ($n = 5$). ** $p < 0.01$ vs. the OVA-treated group. (C) Mucous production was evaluated by counting the number of PAS-positive cells (green arrows) per mm of bronchiole ($n = 5$ per group). Bar = 50 μ m. ** $p < 0.01$, *** $p < 0.001$ vs. the OVA-treated group.

A. BALF



B. Lung

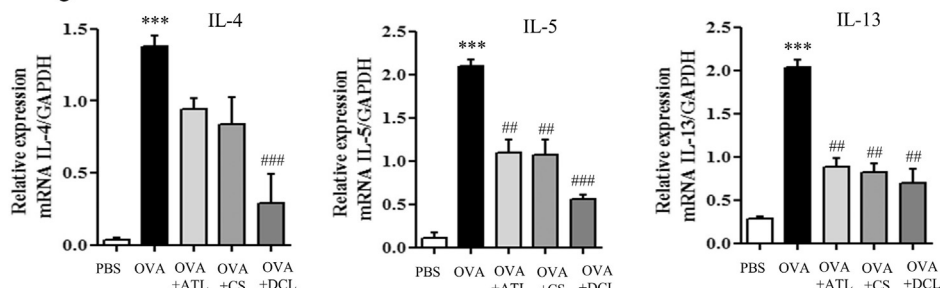


Fig. 5. Alantolactone, costunolide, and dehydrocostuslactone inhibited Th2 cytokine expression at the mRNA level in the lung tissues of OVA-induced asthmatic mice (A) mRNA expression of IL-4, IL-5, and IL-13 in the BALF cells of OVA-treated mice. (B) mRNA expression of IL-4, IL-5, and IL-13 in the lung tissues of OVA-treated mice. mRNA levels of IL-4, IL-5, and IL-13 were quantified as ratios to GAPDH mRNA level. Each lane represents one of five different mice. Values represent the means $\pm$ SEs ($n = 5$). *** $p < 0.001$ vs. the PBS-treated group, ## $p < 0.01$, ### $p < 0.001$ vs. the OVA-treated group.

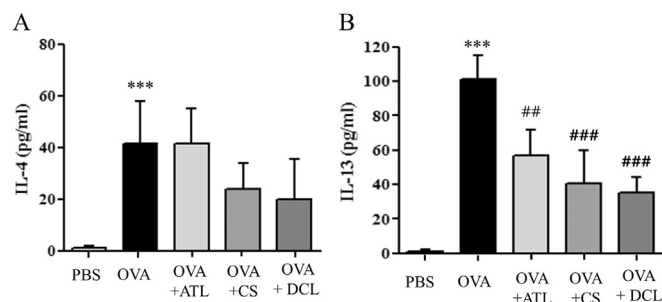


Fig. 6. Effects of dehydrocostuslactone, costunolide, and alantolactone on IL-4 and IL-13 levels in BALF. ELISA was used to measure IL-4 and IL-13 levels in BALF samples ($n = 5$ per group). Values represent the means $\pm$ SEs ($n = 5$). *** $p < 0.001$ vs. the PBS-treated group, ## $p < 0.01$, ### $p < 0.001$ vs. the OVA-treated group.

4. Discussion

Sesquiterpene lactones have been isolated from numerous genera of the Compositae family (Rungeler et al., 1999). They have been shown to exhibit antimicrobial, antitumor, and anti-inflammatory effects in the central nervous system and cardiovascular system, as well as anti-allergic effects (Picman and Picman, 1985; Rungeler et al., 1999; Salapovic et al., 2013; Wen et al., 2002). However, the anti-allergic effects have not been fully elucidated.

S. costus extract was found to suppress chemokine production in TNF- α - and IFN- γ -stimulated HaCaT cells (Lim et al., 2014, 2015a). In addition, it showed inhibitory effects against histamine production in phorbol 12-myristate 13-acetate (PMA) and A23187-stimulated MC/9 mast cells (Lim et al., 2014). Furthermore, in a house dust mite-induced atopic dermatitis model, *S. costus* extract decreased dermatitis scores and plasma IgE levels (Lim et al., 2014). In addition, it suppressed the mRNA expression of the p110 catalytic subunit of phosphatidylinositol 3-kinase (PI3K) in skin tissues from Nc/Nga mice (Lim et al., 2015a).

Subsequently, sesquiterpene lactones isolated from *S. costus* extract were shown to suppress chemokine production in HaCaT cells (Lim et al., 2015b; Seo et al., 2015). The phosphorylation and activation of signal transducer and activator of transcription-1 (STAT1), which is critically involved in atopic dermatitis-related signaling, were inhibited by *S. costus* extract and alantolactone, but not by costunolide and

dehydrocostuslactone (Lim et al., 2015b). In addition, alantolactone decreased regulated on activation, normal T cell expressed and secreted (RANTES/CCL5) and IL-8 production in TNF- α - and IFN- γ -stimulated keratinocytes via inhibition of STAT1 signaling (Lim et al., 2015b). Moreover, alantolactone was shown to suppress inducible cyclooxygenase-2 (COX-2) expression by downregulating nuclear factor kappa-B (NF- κ B), mitogen-activated protein kinase (MAPK), and activator protein-1 (AP-1) via the myeloid differentiation primary response gene 88 (MYD88) signaling in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages (Chun et al., 2012).

Three sesquiterpene lactones markedly reduced TNF- α - and IFN- γ -induced mRNA expression of chemokines (thymus- and activation-regulated chemokine [TARC/CCL17], macrophage-derived chemokine [MDC/CCL22], and IL-8) (Seo et al., 2015). Alantolactone markedly suppressed the mRNA expression of RANTES in HaCaT cells, whereas costunolide and dehydrocostuslactone exhibited weak inhibitory effects on RANTES expression (Seo et al., 2015). It is noteworthy that these chemokines are elevated in the serum and skin lesions of patients with atopic dermatitis (Casas et al., 2011; Pease, 2011).

Therefore, alantolactone has been considered the most potent sesquiterpene lactone isolated from *S. costus* extract with regard to suppression of chemokine production in keratinocytes and anti-inflammatory effects in macrophages. However, in the present study, we found that dehydrocostuslactone was the most potent sesquiterpene lactone regarding degranulation inhibition and anti-asthmatic efficacy, followed by costunolide and alantolactone. Therefore, at least in allergic asthma, dehydrocostuslactone might be the active component responsible for the anti-allergic effects of *S. costus* extract. The amounts of alantolactone, costunolide, and dehydrocostuslactone in *S. costus* extract were found to be 0.01, 17.32, and 28.26 mg/g, respectively (Seo et al., 2015).

The molecular mechanisms responsible for the anti-inflammatory effects of sesquiterpene lactones were investigated using helenaline as a representative member. Helenalin directly alkylated the p65 subunit of NF- κ B (Lyss et al., 1997; Rungeler et al., 1999; Rungeler et al., 1998). As sesquiterpene lactones have an α -methylene- γ -lactone group, which reacts with free sulfhydryl (SH) or amino (NH₂) groups in proteins through Michael addition reaction, direct alkylation of the cysteine residues of p65 NF- κ B leads to inhibition of DNA binding (Castro et al., 2000). The anti-inflammatory activity of other sesquiterpene lactones (diversifolol, helenalin, and

(diversifolin, helenalin, parthenolide, tectroside, and tirtundin) has been studied with regard to their effects on NF- κ B and the enzymes involved in the arachidonic acid pathway (Castro et al., 2000; Hehner et al., 1998; Kim et al., 2013; Rungeler et al., 1998; Tornhamre et al., 2001). Therefore, similar mechanisms might mediate the anti-allergic effects of dehydrocostuslactone. However, because asthma is not a simple inflammatory response, but involves Th2-mediated chronic inflammatory immune responses, additional targets other than NF- κ B might be involved. Additionally, although the three sesquiterpene lactones have the same α -methylene- γ -lactone group, they showed different anti-allergic effects in mast cell degranulation in vitro and allergic asthma in vivo. Therefore, further studies are required to elucidate the precise mechanisms of sesquiterpene lactone actions in allergic asthma.

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