

RESEARCH ARTICLE

Kochia scoparia fruit attenuates allergic airway inflammation in ovalbumin (OVA)-induced murine asthma model

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Abstract

Kochia scoparia fruit has been used in Asia for a long time. It possesses anti-inflammatory, antiallergic, and antipruritic actions. We investigated the role of a *K. scoparia* fruit ethanolic extract (KSEE) in allergic airway inflammation in a mouse asthma model. BALB/c mice were sensitized with ovalbumin (OVA) and, upon OVA aerosol challenge, developed airway eosinophilia, mucus hypersecretion, elevations in cytokine, chemokine, and immunoglobulin levels, and upregulation of MMP-9, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) expression. Intragastric administration of KSEE significantly attenuated OVA-induced influx of total leukocytes, eosinophils, neutrophils, macrophages, and lymphocytes into lungs, as well as attenuating levels of interleukin (IL)-4 and IL-5 in a dose-dependent manner. KSEE also significantly reduced the serum levels of total and OVA-specific immunoglobulin (Ig)E and OVA-specific IgG1 release into the airspace. Histological studies showed that KSEE inhibited OVA-induced lung tissue eosinophilia and airway mucus production. Moreover, in whole lung tissue lysates, immunoreactivity showed that KSEE markedly attenuated the OVA-induced increase in expression of ICAM-1, VCAM-1, and MMP-9. These results show that KSEE possesses protective effects against allergic airway inflammation, acts as an MMP-9 inhibitor, and induces a reduction in ICAM-1 and VCAM-1 expression.

Keywords: *Kochia scoparia* (L) schrad, asthma, cytokine, adhesion molecules, matrix metalloproteinase-9

Introduction

Allergic asthma is an immune-based inflammatory condition of the airway caused by exacerbated responses to inhaled allergens and characterized by reversible airway obstruction, increased mucus production and infiltration of eosinophils (Rodrigo et al., 2004; Galli et al., 2008; Medoff et al., 2008). Airway inflammation in asthma is a multicellular process involving mainly eosinophils, neutrophils, CD41 T lymphocytes, and mast cells, with eosinophilic infiltration being the most striking feature (Kay, 2005). Th2 cytokines are pivotal for B cell maturation, IgE synthesis, airway eosinophilia, and mucus secretion. The importance of Th2 cytokines in the pathogenesis of allergic asthma has been demonstrated by specific gene knockout and antibody neutralization studies in mouse asthma models (Taube et al. 2002; Foster et al. 1996; Kopf et al. 1993).

Matrix metalloproteinases (MMPs) are secreted by inflammatory and noninflammatory cells and have a

major role in extracellular matrix degradation, proteolytic modulation of biologically active proteins, and cell migration (Goetzl et al. 1996; Kelly et al. 2000; Kheradmand et al. 2002). MMP-9 induced by inflammatory stimuli and proinflammatory cytokines is generated in multiple cell types including alveolar macrophages and other resident pulmonary cells (Atkinson and Senior, 2003; Chakrabarti and Patel, 2005). MMP-9 (gelatinase B) has been implicated in the pathogenesis of asthma (Cataldo et al. 2000), idiopathic pulmonary fibrosis (Fukuda et al. 1998), chronic obstructive pulmonary disease (Russell et al. 2002), and acute lung injury (Lanchou et al. 2003). MMP-9 also induces the migration of eosinophils, lymphocytes, and neutrophils across basement membranes (Okada et al. 1997; Leppert et al. 1995; Delclaux et al. 1996). Recently, we demonstrated that the administration of an MMP inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes (Lee et al. 2001).

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Migration of inflammatory cells is regarded as a critical step in acute and chronic inflammation in lung tissue. Recruitment of leukocytes from the circulating blood into tissues is controlled by sequential activation of adhesion molecules and their ligands on leukocytes, epithelial cells and vascular endothelial cells (Wang and Doerschuk, 2002). It was reported that migration of inflammatory cell is caused by enhanced adhesion of leukocytes to epithelial cells via the expression of adhesion molecules (Rosseau et al., 2000). Over-expression of adhesion molecules such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on lung airway epithelium is associated with migration of inflammatory cell, critical for the pathogenesis of airway inflammatory diseases (Lee et al., 2003). Recent studies have revealed a relationship between the expression of adhesion molecules and MMP-9 in allergic airway inflammation (Kong et al. 2008). Lee et al. (2003) demonstrated that MMP inhibitors regulate inflammatory cell infiltration via reduction in adhesion molecules.

It has been reported that the extract and saponins of *Kochia scoparia* fruits have antinociceptive, anti-inflammatory, antiallergic, and antipruritic actions (Matsuda et al. 1997a; Matsuda et al. 1997b; Matsuda et al. 1998). The fruits of *K. scoparia* (Chenopodiaceae) have been used for the treatment of skin disease in Chinese medicine (Matsuda et al. 1997a; Matsuda et al. 1997b), as a remedy for diabetes mellitus, and for treating rheumatoid arthritis in the folk medicine of Korea. It has been reported that the fruit of *K. scoparia* has a peripheral antinociceptive effect mediated by anti-inflammatory action, and that its active component can be partially attributed to momordin (Matsuda et al. 1998). *K. scoparia* also demonstrated its anti-inflammatory action on inflammation induced by Freund's complete adjuvant in rats (Choi et al. 2002).

The objective of the present study was to determine the effects of KSEE on airway inflammation in an established murine model of asthma. An additional aim of the study was to evaluate the effect of KSEE on the expression of MMP-9, ICAM-1 and VCAM-1.

Materials and methods

Chemicals and reagents

Commercially available enzyme-linked immunosorbent assays kit such as IL-4 (KMC0042) and IL-5 (KMC0051) were purchased from Invitrogen (Carlsbad, CA) and total IgE (MCA2475KZZ) and OVA-specific IgG1 (107008) were supplied by Serotec (Oxford, UK). OVA, aluminum hydroxide, *o*-phenylenediamine dihydrochloride, Tissue Lysis/Extraction reagent plus protease inhibitor, and hematoxylin and eosin solution were purchased from Sigma-Aldrich (Sigma, St Louis, MO). Protein assay reagent was obtained from Bio-Rad Laboratories. Reagents used in the histological examination, such as Diff-Quik Stain reagents and periodic acid-Schiff reagents (PAS), were purchased from Dade Behring (B4132-1A; Deerfield, IL) and IMEB (San Marcos, CA).

Antibodies used in western blot, such as anti-ICAM, anti-VCAM, and anti-MMP-9, were purchased from Abcam (Cambridge, UK). The anti- β -actin antibody and horse-radish peroxidase-conjugated secondary antibody were obtained from Cell Signaling (Boston, MA) and Jackson Immuno Research (West Grove, PA), respectively. The enhanced chemiluminescence (ECL) kit was supplied by Amersham (Buckinghamshire, UK).

Preparation of *K. scoparia* ethanolic extract (KSEE)

K. scoparia were purchased in October 2008 from HMAX (Chungbuk, Korea). A voucher specimen was deposited at the Korean Institute of Oriental Medicine (KIOM), Daejeon, Korea. Fresh *K. scoparia* fruits were washed three times with tap water to remove salts, epiphytes, and sand, and then stored at -20°C . Frozen samples were lyophilized and homogenized in a grinder before extraction. The dried fructus of *K. scoparia* (200 g) was extracted three times with 70% EtOH ($2\text{ L} \times 3$ times) by sonication for 1 h. The extracted solution was filtered through filter paper and evaporated to dryness (7.06 g). The yield of ethanolic extract obtained was 3.5% of the original dry weight.

Animals and experimental procedure

Six-week-old pathogen-free female BALB/c mice were obtained from Orient Co. (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow ad libitum. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of Korea. Mice were divided into five groups (each of 6–7 animals), and airway inflammation was induced by ovalbumin (Grade III) in three of the groups, using the method described by Oh et al. (Oh et al. 2006). Briefly, mice were immunized via intraperitoneal injection with 20 μg chicken OVA and 2 mg aluminum hydroxide in 200 μL phosphate buffered saline (pH 7.4), on days 0 and 14. Mice were administered by oral gavage with PBS in Group 1 (Normal control, NC) and 2 (OVA sensitization/challenge, OVA), and Montelukast (10 mg/kg) in group 3 (Positive control, Mon) and KSEE (100 mg/kg or 200 mg/kg) in group 4 and 5 (KSEE groups) once daily from day 18 to 23. During oral administration, mice received an airway challenge with a 1% (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan) on days 21, 22, and 23 after initial sensitization except for NC mice. Animals were sacrificed 48 h after the last challenge (day 25) to characterize the suppressive effects of KSEE. In addition, experimental treatment was described in detail in Table 1 to make it easier to understand. Montelukast is a cysteinyl leukotriene 1 receptor antagonist (Lynch et al. 1999), and useful clinical responses to this agent have been obtained in patients with aspirin-sensitive asthma, nocturnal exacerbation of asthma, and allergic asthma. These data have resulted in successful commercialization

Table 1. Experimental procedure.

Groups	Sensitization (Days 0 and 14)	Drug treatment (once daily from day 18 to 23)	Inhalation (Days 21, 22, and 23)	Sacrifice
NC (Group 1)	PBS (i.p.)	PBS (p.o.)	PBS	Day 25
OVA (Group 2)	OVA/Alum (i.p.)	PBS (p.o.)	1% OVA	Day 25
Mon (Group 3)	OVA/Alum (i.p.)	Montelukast (30 mg/kg, p.o.)	1% OVA	Day 25
KSEE-100 (Group 4)	OVA/Alum (i.p.)	KSEE (100 mg/kg, p.o.)	1% OVA	Day 25
KSEE-200 (Group 5)	OVA/Alum (i.p.)	KSEE (200 mg/kg, p.o.)	1% OVA	Day 25

OVA/Alum: 20 µg of OVA and 2 mg of aluminum hydroxide dissolved in 0.2 mL of PBS.

of the drug (Korenblat, 2001). In the present work, montelukast was used as a positive control.

Bronchoalveolar lavage fluid (BALF) samples were obtained and processed, and inflammatory cells counted as previously described (Oh et al. 2006; Lee et al. 2010). In brief, mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 48 h after the last challenge, and a tracheotomy was performed. The trachea was cannulated and the left bronchi were tied for histopathological examination. After instilling ice-cold PBS (0.5 mL) into a lung, BALF was obtained by three successive aspirations (total volume 1.5 mL) via tracheal cannulation (Djukanovic et al. 1990). BALF was centrifuged (250g, 4°C, for 5 min) and each supernatant was collected and stored at -70°C for cytokine or chemokine measurements. Cell pellets were assessed by counting the cells in at least five squares of a hemocytometer after exclusion of dead cells stained with trypan blue (Daigle and Simon, 2001). BALF (100 µL) was placed on a slide and centrifuged (200g, 10 min, at 4°C) to fix the cells, using a cytospin apparatus (Hanil Science Industrial, Seoul, Korea). After the slides were dried, cells were fixed and stained using Diff-Quik Stain reagents, according to the manufacturer's instructions.

Enzyme-linked immunosorbent assays (ELISAs)

The concentrations of IL-4 and IL-5 in BALF were measured using ELISA kits, according to their manufacturers' instructions. The ranges of detection of IL-4 and IL-5 are 0–1000 pg/mL and 0–500 pg/mL, respectively. The concentrations of total and OVA-specific-IgE and OVA-specific IgG1 in serum were measured using sandwich ELISAs. Antibodies were detected in the serum using isotype-specific secondary antibodies. After four washes, 200 µL of *o*-phenylenediamine dihydrochloride was added to each well. Plates were incubated for 10 min in the dark, and the absorbance was measured at 450 nm. Total and OVA-specific-IgE and OVA-specific-IgG1 concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE or IgG1.

Measurement of MMP-9 in lung tissue

Lung tissues were homogenized (1/10 w/v) in Tissue Lysis/Extraction reagent plus protease inhibitor (Sigma) to obtain extracts of lung tissues. After centrifugation (12,000g, 4°C, 10 min), protein concentration in supernatants was determined using a protein assay reagent (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions, and equal amounts of total protein

loaded for gelatin zymography (60 µg /lane). SDS-PAGE zymography was performed to determine the gelatinase activity according to Heussen and Dowdle (1980). Briefly, zymogram gels consist of 10% SDS-PAGE containing 1% gelatin as a substrate for MMPs. The gels were washed in 2.5% Triton X-100 for 1 h to remove SDS, and then incubated at 37°C for 16 h in developing buffer (1 M Tris-HCl, pH 7.5 with CaCl₂). Thereafter, gels were stained with 25% methanol/8% acetic acid containing Coomassie Brilliant Blue. Gelatinase activity was visualized as white bands on a blue background, representing areas of proteolysis of the gelatin substrate.

Western blot

Equal amounts of total lung protein (30 µg) were heated at 100°C for 5 min and then loaded onto 8% SDS-PAGE gels, followed by transfer to a nitrocellulose membrane (at 100V for 2 h). The membranes were blocked for 1 h with Tris-buffered saline containing 0.05% Tween 20 (TBST) plus 5% skim milk and were incubated with anti-ICAM (1:1000 dilution), anti-VCAM (1:1000 dilution), anti-MMP-9 (1:1000 dilution) and anti-β-actin (1:1000 dilution) overnight at 4°C. The membranes were washed three times with TBST, and then incubated with a 1:10000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were again washed three times with TBST, and then developed using an enhanced chemiluminescence kit.

Histology

Before the lungs were removed, the left lungs were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligation around the trachea. Lung tissue was fixed in 10% (v/v) neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 4 µm thickness, and stained with hematoxylin and eosin solution and PAS to estimate the inflammation and mucus production, respectively. The degree of cell infiltration in the airway was scored in a double-blind screen by two independent pathologists according to previous study (Myou et al., 2003). The peribronchiole and perivascular inflammation was evaluated by using a scoring of 0–5: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell-layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells; and 5, cells deep. Goblet cell hyperplasia in the airway epithelium was quantified based on a five-point system: 0, no goblet cells; 1, <25% of the epithelium; 2, 25–50% of the epithelium; 3, 50–75% of the epithelium; and 4, >75% of the epithelium. For each

mouse, five airway sections that were randomly distributed throughout the left lung were analyzed, and their average scores were calculated. Quantitative analysis of mucus production was performed using an image analyzer (Leica Microsystem Imaging Solution Ltd., Cambridge, UK).

Statistical analysis

Data are expressed as means $\pm$ standard error (SEM). Statistical significance was determined using an analysis of variance followed by a multiple comparison test with a Bonferroni adjustment. *p*-Values of less than 0.05 were considered statistically significant.

Results

Effect of KSEE on OVA-induced eosinophilia and BALF composition

We examined changes in total cells, eosinophils, neutrophils, lymphocytes, and macrophages in BALF to determine the effects of KSEE on asthma. Suppression of eosinophilia by KSEE in OVA-challenged mice was estimated by counting cells recruited to the BALF 48 h after the last challenge. As shown in Figure 1, OVA induced a marked influx of leukocytes into BALF in the normal

control (NC) group treated with only PBS. However, OVA-induced mice treated with KSEE displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction. The inhibitory effect of KSEE treatment on numbers of total, lymphocytes, neutrophils, and eosinophils in BALF was more effective at 200 mg/kg than 100 mg/kg of KSEE. The positive drug control, montelukast, exerted similar suppressive effects on leukocyte influx into BALF.

Effect of KSEE on Th2 cytokines in BALF and on IgE and IgG1 in plasma

The levels of IL-4 and IL-5 were significantly increased in OVA-challenged mice compared with those in the NC group (Table 2). In contrast, administration of KSEE significantly decreased the increased levels of Th2 cytokines in BALF of OVA-induced asthmatic mice. In serum, total and OVA-specific IgE and IgG1 concentrations were elevated in asthmatic mice, whereas total and OVA-specific IgE and OVA-specific IgG1 were undetectable in the NC group. Intragastric administration of KSEE significantly reduced the serum total and OVA-specific IgE and OVA-specific IgG1 concentrations in OVA-induced asthmatic mice compared with the NC group.

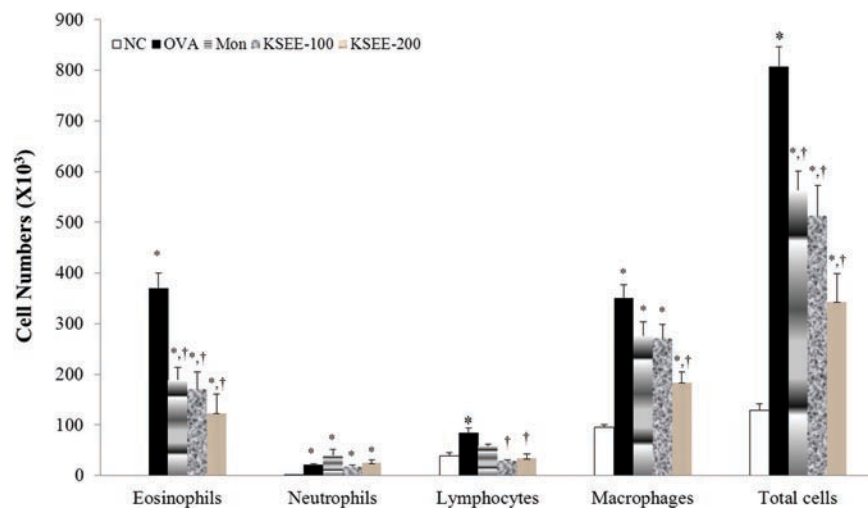


Figure 1. KSEE inhibits the recruitment of inflammatory cells in bronchoalveolar lavage fluid (BALF) of mice 48 h after the final OVA challenge. Cell numbers were determined using a light microscope to count cells in at least five squares of a hemocytometer after excluding dead cells using Trypan blue. Cells were isolated by centrifugation and stained with Diff-Quik® Stain reagent. NC, normal control mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Mon, Montelukast (30 mg/kg) + OVA-sensitized/challenged mice; KSEE-100, KSEE (100 mg/kg) + OVA-sensitized/challenged mice; KSEE-200, KSEE (200 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SEM. *Significantly different from NC, *p* < 0.05; †significantly different from OVA, *p* < 0.05.

Table 2. Effects of KSEE on Th2 cytokines in BLAF and on total IgE, OVA-specific IgE, and OVA-specific IgG1 in plasma.

Groups	IL-4 (pg/mL)	IL-5 (pg/mL)	Total IgE (ng/mL)	OVA-specific IgE (pg/mL)	OVA-specific IgG1 (ng/mL)
NC	63.6 $\pm$ 22.6	27.4 $\pm$ 9.1	79.0 $\pm$ 13.0	525.8 $\pm$ 70.8	0
OVA	101.3* $\pm$ 24.6	45.3* $\pm$ 10.7	401.9* $\pm$ 62.6	1130.1* $\pm$ 145.6	78.5* $\pm$ 14.2
Mon	45.9† $\pm$ 11.9	18.3† $\pm$ 7.2	278.63† $\pm$ 9.8	693.8† $\pm$ 59.8	48.8† $\pm$ 3.2
KSEE-100	76.6† $\pm$ 17.3	24.0† $\pm$ 6.0	291.0 $\pm$ 57.8	853.9† $\pm$ 211.3	41.9† $\pm$ 1.4
KSEE-200	48.4† $\pm$ 19.4	17.5† $\pm$ 6.1	185.6† $\pm$ 24.1	566.4† $\pm$ 99.9	40.1† $\pm$ 3.9

*Significantly different from NC, *p* < 0.05

†Significantly different from OVA, *p* < 0.05.

Effect of KSEE on inflammatory cell infiltration and mucus production in lung tissue of OVA-challenged mice

Because KSEE inhibits inflammatory cell recruitment into BALF, we next examined the antiasthmatic and antiremodeling effects of the drug. Lung tissue was collected 48 h after the final OVA challenge. In OVA-induced asthmatic lung tissue, we observed marked infiltration of inflammatory cells into perivascular and peribronchial connective tissue, compared with normal tissue. Moreover, most leukocytes were eosinophils. Eosinophilia in OVA-sensitized and -challenged mice treated with KSEE (100 or 200 mg/kg) was significantly attenuated compared with the level seen after OVA challenge. In addition, leukocytes in lung tissue were more effective at 200 mg/kg than 100 mg/kg of KSEE (Figure 2A and B). Mucus hypersecretion is a characteristic of airway remodeling. To determine whether KSEE suppressed mucus overproduction caused by goblet cell hyperplasia, we stained lung sections with PAS. In OVA-induced asthmatic mice, compared with the NC group, mucus overproduction was clearly observed as a violet color in bronchial airways. In contrast, the extent of mucus staining was markedly diminished in OVA-challenged mice treated with KSEE (Figure 2C and D). Our data thus confirm that KSEE significantly reduces the goblet cell hyperplasia and mucus hypersecretion characteristic of the airway remodeling process.

Effect of KSEE on MMP-9, ICAM-1, and VCAM-1 expression and MMP-9 activity in lung tissue of OVA-challenged mice

Gelatin zymography revealed that MMP-9 levels in the lung tissue were significantly increased after OVA challenge compared with those in the NC group. KSEE treatment reduced MMP-9 activity in lung tissue compared with that in the OVA-challenged group (Figure 3A). Consistent with the results obtained from the gelatin zymography and western blotting in lung tissue revealed that MMP-9 expression was increased in OVA-challenged mice compared with that in the NC group (Figure 3B and C). However, administration of KSEE significantly reduced MMP-9 expression in the lung tissue of OVA-challenged mice. Whereas OVA-challenged mice had increased MMP-9 activity, however in KSEE-treated mice was significantly decreased.

As shown in Figure 4, OVA-challenged mice showed a significant increase in the expression of adhesion molecules (ICAM-1 and VCAM-1) in lung tissue compared with expression in NC mice. In KSEE-treated mice, we found a significant reduction in the expression of ICAM-1 and VCAM-1 compared with that in OVA-challenged mice.

Discussion

Our present study using an OVA-induced murine model of allergic airway inflammation has revealed the

following typical pathophysiological features: airway inflammation, and increased levels of Th2 cytokines (IL-4 and IL-5), adhesion molecules (ICAM-1 and VCAM-1), IgE, and IgG1 levels in lungs. These features of asthma induced in our mouse model are similar to those found in human asthma and render this mouse model suitable for investigations of the pathophysiology and immunology of asthma. Our results revealed that KSEE treatment significantly reduced the levels of Th2-type cytokines such as IL-4 and IL-5, IgE, and IgG1 levels in BALF or serum. Moreover, KSEE suppressed the inflammatory cell infiltration and mucus secretion in lung tissue. In addition, KSEE suppressed the levels of adhesion molecules (ICAM-1 and VCAM-1), and MMP-9.

Several cytokines, including IL-4, IL-5, IL-13, TNF- α , and IL-1 β are known to be present at elevated levels in asthmatic lungs and play critical roles in the development and maintenance of the asthmatic IgE-driven condition. Therefore, these Th2 cytokines and IgE levels are considered indicators of asthmatic condition. IL-4 plays an important role in B cell growth and differentiation, as well as in the secretion of IgE and IL-13, and regulates allergic inflammation by promoting Th2 cell differentiation, IgE synthesis and its receptor upregulation, VCAM-1 expression, and mucus hypersecretion (Seder et al. 1992; Pawankar et al. 1997; Moser et al. 1992; Dabbagh et al. 1999). IL-5 promotes eosinophilic inflammation and infiltration into airways (Okada et al. 1997). Eosinophilic leukocytes are prominent in the inflammation of allergic asthma, and are present not only in the airway wall but also in the sputum and BALF of uncontrolled asthma. Eosinophil transmigration into the airways is a multistep process orchestrated by Th2 cytokines (IL-4, IL-5, and IL-13) and coordinated by specific chemokines such as eotaxin in combination with adhesion molecules such as VCAM-1 (Lukacs, 2001; Jia et al. 1999). Moreover, eosinophil infiltration into the lungs is a primary inflammatory response in asthma, preceding hyperplasia and hypertrophy of the bronchial epithelium. Our results show that increased levels of eosinophils and other inflammatory cells in BALF are induced by OVA challenge and returned to normal levels by KSEE treatment, indicating that KSEE is an efficacious eosinophil-depleting agent. It has also been shown that IL-5 enables terminal differentiation and proliferation of eosinophil precursors and the eosinophil activation effect (Barnes, 2001). The reduction of eosinophil infiltrates found in the lung tissue of KSEE-treated group may be due to IL-5 reduction as it promotes the differentiation to and activation of eosinophils. Therefore, these results indicate that the suppressive effect of KSEE on inflammatory infiltration and mucus hypersecretion into the airway may be associated with an inhibitory effect of KSEE on the secretion of IL-5. An increased blood IgE level is closely associated with asthma and allergic inflammation in mouse models of asthma (Hessel et al. 1998). Our data show that serum levels of total and OVA-specific IgE in BALF were significantly reduced by KSEE pretreatment. Administration of montelukast induced

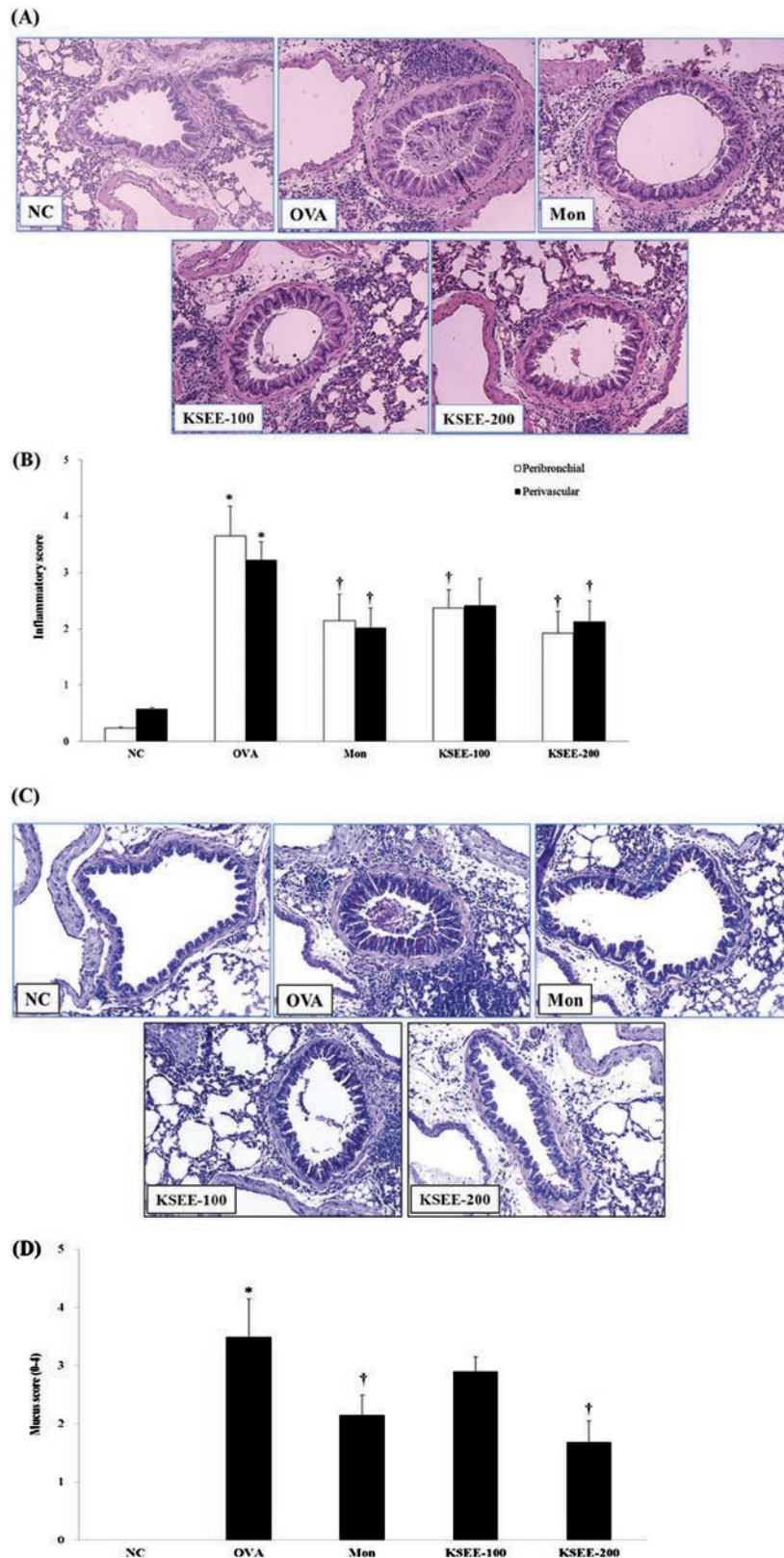


Figure 2. KSEE inhibits the recruitment of inflammatory cells and reduces mucus production in lung tissue of mice 48 h after the final OVA challenge. (A) Lung tissues were fixed, sectioned at 4 μ m thickness and stained with H&E solution (magnification $\times 200$). (B) Scoring of the extent of inflammation *via* quantitative analysis of inflammatory cell infiltration in lung sections was performed as described in the Materials and Methods section. (C) Histological examination of mucus secretion in lung tissue 48 h after the last OVA challenge. Lung tissue was fixed, sectioned at 4 μ m thickness and stained with periodic acid-Schiff (PAS) to assess mucus production (magnification $\times 200$). (D) Scoring of mucus production in lung sections. NC, normal control mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Mon, Montelukast (30 mg/kg) + OVA-sensitized/challenged mice; KSEE-100, KSEE (100 mg/kg) + OVA-sensitized/challenged mice; KSEE-200, KSEE (200 mg/kg) + OVA-sensitized/challenged mice.

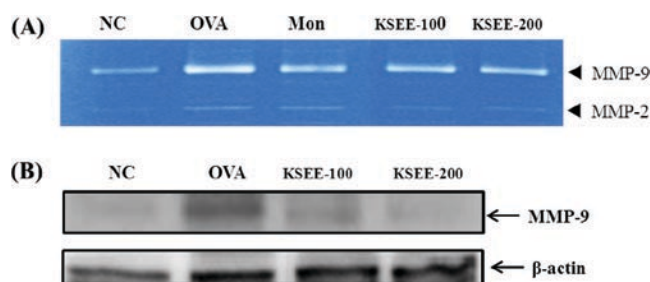


Figure 3. KSEE decreases the MMP-9 activity and protein expression in lung tissues of mice 48h after the final OVA challenge. (A) The protein of supernatants was loaded for gelatin zymography (60 μ g/lane). SDS-PAGE zymography was performed according to the method of Heussen and Dowdle (1980). (B) MMP-9 protein expression. (C) Relative unit of MMP-9 protein expression. Relative unit is expressed as relative ratios of MMP-9 to β -actin. NC, normal control mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Mon, Montelukast (30 mg/kg) + OVA-sensitized/challenged mice; KSEE-100, KSEE (100 mg/kg) + OVA-sensitized/challenged mice; KSEE-2, KSEE (200 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SEM. *Significantly different from NC, $p < 0.05$; †significantly different from OVA, $p < 0.05$.

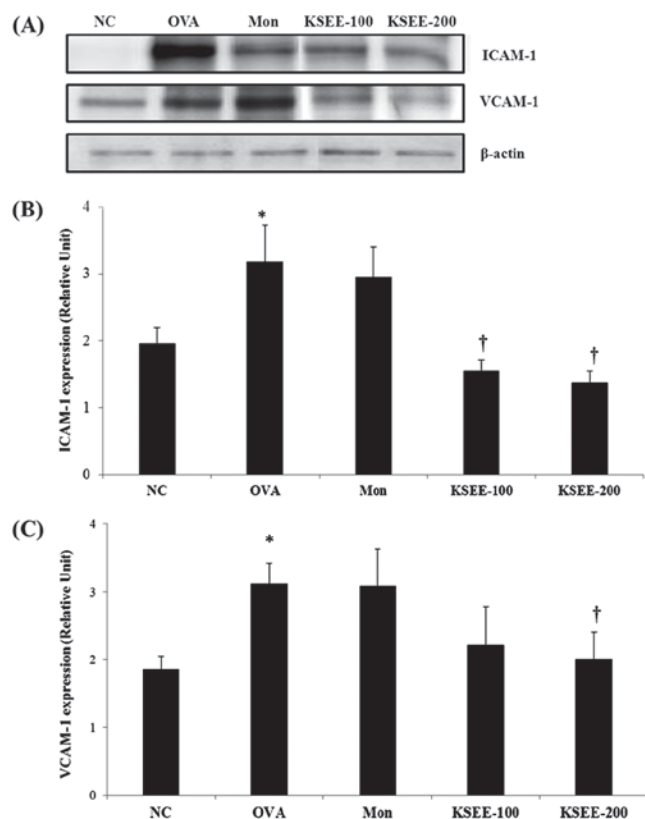


Figure 4. KSEE decreases the ICAM-1 and VCAM-1 proteins expressions in lung tissues of mice 48h after the final OVA challenge. (A) ICAM-1 and VCAM-1 protein expression on the gels. (B) Relative unit of ICAM-1 protein expression. (C) Relative unit of VCAM-1 protein expression. Relative unit are expressed as relative ratios of ICAM-1, and VCAM-1 to β -actin. NC, normal control mice treated with PBS only; OVA, OVA-sensitized/challenged mice; Mon, Montelukast (30 mg/kg) + OVA-sensitized/challenged mice; KSEE-100, KSEE (100 mg/kg) + OVA-sensitized/challenged mice; KSEE-2, KSEE (200 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean $\pm$ SEM. *Significantly different from NC, $p < 0.05$; †significantly different from OVA, $p < 0.05$.

anti-inflammatory effects such as the reduction in the numbers of inflammatory cells in BALF, inflammatory cells infiltration into lung tissue and levels of cytokines and IgE are explained in this study. These results support the conclusion that KSEE suppressed the generation of a Th2-type immune response, including IgE elaboration, in this animal model of asthma. A positive control, montelukast, which has been widely used as an antiasthmatic drug, showed similar effects with KSEE.

KSEE has long been used as herbal medicine in Asia. Recent studies were reported anti-inflammatory effects of KSEE in *in vivo* and *in vitro* experiments (Matsuda et al., 1997a; Choi et al., 2002; Shin et al., 2004). Anti-inflammatory effects of KSEE have been associated with active ingredients of KSEE such as saponin (Xia et al., 2002, Xia et al., 2003). The results of current study were similar to the previous studies of KSEES. KSEE induces the decreases in the number of inflammatory cells such as eosinophils, neutrophils, lymphocytes and macrophages in BALF. These results were confirmed by histopathological examination, which showed that KSEE inhibited inflammatory cell infiltration in lung tissue. Thus, the results indicate that KSEE has anti-inflammatory effects in allergic asthma.

Adhesion molecules and chemokines are expressed by various airway cells including eosinophils, bronchial epithelium cells, endothelial cells, T cells, mast cells, and alveolar macrophages (Wardlaw et al. 1994; Bisset and Schmid-Grendelmeier, 2005). Expression of ICAM-1 or VCAM-1 is modulated by Th2 cytokines in airway inflammation (Dustin et al. 1986; Osborn et al. 1989; Hirata et al. 1998). The expression of ICAM-1 and VCAM-1 is modulated by cytokines such as IL-1 β , IL-4, and IL-13. Our data demonstrate that KSEE inhibition dampened the levels of these inflammatory cytokines (Th2 type) and IgE levels in OVA-challenged mice. These findings suggest that IL-4 could be involved in the reduction of blood IgE levels by decreasing the expression of adhesion molecules and altering IgE class switching. Therefore, ICAM-1 and VCAM-1 may play an important role in the therapeutic effect of KSEE treatment in our OVA-induced mouse asthma model via cytokine production.

A recent study revealed a relationship between the expression of adhesion molecules and MMP-9 in allergic airway inflammation (Kong et al. 2008). MMP inhibitors reduced inflammatory cell infiltration through reduction of ICAM-1 and VCAM-1 expression in a murine allergy model and MMP-9 may be a potential therapeutic target for asthma (Lee et al. 2003). Therefore, we evaluated the activity of MMP-9 and protein expression in lung tissue in our OVA-induced mouse asthma model. In current study, KSEE treatment induced the reduction in MMP-9, ICAM-1 and VCAM-1 proteins expressions in lung tissue. These results are in good agreement with previous studies on investigating anti-inflammatory effects against airway inflammation of allergic asthma (Lee et al., 2003; Kong et al., 2008).

In conclusion, our data provide evidence that KSEE might be responsible for the protective effects of this

agent against airway inflammation. Given the efficacy demonstrated in this model, KSEE might prove to be a novel and effective phytochemistry for asthma.

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Conflicts of interest: The authors declare no conflicts of interest.

Declaration of interest

The authors declare no conflicts of interest.

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