



Anti-inflammatory and anti-asthmatic effects of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on airway inflammation in a mouse model of allergic asthma

Mee-Young Lee^{a,c}, Ji-Eun Yuk^a, Ok-Kyoung Kwon^a, Hui-Seong Kim^{a,b}, Sei-Ryang Oh^a, Hyeon-Kyu Lee^a, Kyung-Seop Ahn^{a,*}

^a Immune Modulator Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Daejeon 305-600, Republic of Korea

^b Biomolecular Science, University of Science & Technology, Daejeon, Republic of Korea

^c Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine, P.O. Box 483, Yusong-gu, Daejeon 305-811, Republic of Korea

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ABSTRACT

Aim of the study: We investigated the efficacy of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract in the treatment of bronchial asthma in an ovalbumin (OVA)-induced asthmatic BALB/c mouse model.

Materials and methods: Female BALB/c mice were sensitized with intraperitoneal (i.p.) ovalbumin (OVA) on days 0 and 14, and were next given intranasal OVA on days 28–30. Randomized treatment groups of sensitized mice received VM EtOH extract, dexamethasone, or placebo, orally, from days 28 to 30.

Results: VM EtOH extract significantly inhibited increases in total immunoglobulin E (IgE) and cytokines IL-4 and IL-13 levels in serum and bronchoalveolar lavage fluid (BALF), and also effectively suppressed airway hyperresponsiveness (AHR), eosinophilia, and mucus hypersecretion, in mice with OVA-induced asthma.

Conclusions: The results suggest that VM EtOH extract and allied extracts could be useful herbal medicines for asthma treatment, and that VM may also be a valuable lead material for anti-asthma drug development.

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1. Introduction

Allergic asthma is a chronic inflammatory disease characterized by eosinophilic airway inflammation, mucus hypersecretion, and bronchial hyperresponsiveness (Busse and Lemanske, 2001). It is now well recognized that asthma is caused by Th2-driven inflammatory responses, which enhance airway eosinophilia and lung mucus production (Issekutz and Sapru, 2008). The inflammatory response is characterized by increases in eosinophils and mast cells, mucus secretion, and activation of T cells through increased production of Th2 cytokines such as interleukin-4 (IL-4), IL-5, and IL-13 (Elias et al., 2003; Lukacs, 2001; Wardlaw et al., 1988). Airway allergen exposure produces a Th2-dominated response by recruiting and activating inflammatory cells including eosinophils, and increases the levels of IL-4, IL-5, and IL-13 (Bisset and Schmid-Grendelmeier, 2005). IL-4 causes class switch-

ing of B cells, resulting in IgE synthesis, which is involved in mast cell degranulation by crosslinking with IgE receptors (Fish et al., 2005). IL-5 is a key cytokine in the growth and differentiation of bone marrow eosinophils (Sanderson, 1988, 1992; Yamaguchi et al., 1988) and eosinophil release into the peripheral circulation (Collins et al., 1995). In addition, IL-5 enhances eosinophil recruitment, activation, and survival at inflammation sites (Farahi et al., 2007; Fujisawa et al., 2000). Therefore, inhibiting or reducing IL-5 and eotaxin expression is a promising therapeutic strategy in eosinophil-mediated diseases. Although the precise etiology of asthma is still unknown, pathology is clearly multifactorial, including a possible genetic predisposition coupled with exposure to certain environmental triggers (Bukstein et al., 2006). Medicinal plants are alternatives to conventional therapies in many diseases. *Viola mandshurica* W. Becker (VM) is a Korean traditional herbal medicine used for treatment of tuberculosis, skin inflammation, enteritis (Lee and Kim, 2003), and inflammatory diseases (Yook et al., 1989). However, VM use has not yet been investigated in patients with allergic diseases. Therefore, in this study, we employed a bronchial asthma model to examine anti-allergic and anti-inflammatory effects of VM EtOH extract; the system employed was the ovalbumin (OVA)-induced asthmatic mouse model.

* Corresponding author at: Immune Modulator Research Center, Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Daejeon 305-600, Republic of Korea. Tel.: +82 42 860 4482; fax: +82 42 860 4309.

E-mail address: ksahn@kribb.re.kr (K.-S. Ahn).

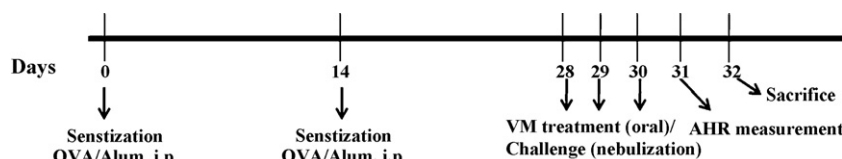


Fig. 1. Mouse model of airway inflammation and treatment with VM EtOH extract.

2. Materials and methods

2.1. Preparation of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract

VM was collected in the Kong-ju area of the Republic of Korea from March to May 2006. Fresh VM was washed three times with tap water to remove salts, epiphytes, and sand, and stored at -20°C . Frozen samples were lyophilized and homogenized in a grinder before extraction. Dried VM powder (1 kg) was extracted with 95% EtOH (1:10, w/v) at 20°C for 3 days and evaporated to dryness in vacuo. The yield of dried extract from starting crude material was 8.91%. Concentrated VM EtOH extract was freshly dissolved in PBS before use.

2.2. Animals

Specific pathogen-free female BALB/c mice, aged 6–8 weeks, which were routinely screened serologically for relevant respiratory pathogens, were purchased from the Orient Co. (Seoul, Korea). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and were 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 all animals handling followed the dictates of the National Animal Welfare Law of Korea.

2.3. Sensitization and airway challenge

Mice were used in groups ($n=5$ or $n=6$), and received the following treatments: (1) sham-sensitization plus challenge with

phosphate-buffered saline (PBS; ipNeb); (2) sensitization plus challenge with OVA (Sigma A5503; Sigma, St. Louis, MO) (ipNeb); (3) sensitization with OVA (i.p.) plus challenge with OVA (Neb) and VM EtOH extract (30 mg/kg) or dexamethasone (Sigma; 3 mg/kg), p.o. Briefly, mice were sensitized by intraperitoneal injection of $20\text{ }\mu\text{g}$ OVA emulsified in 2 mg aluminum hydroxide in $100\text{ }\mu\text{L}$ PBS buffer (pH 7.4), on days 0 and 14. Each mouse was challenged through the airway with OVA (1%, w/v, in PBS) for 20 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, and 30 after initial sensitization. Mice were orally administered 20, 30 or 50 mg/kg/day of VM EtOH extract each day from days 18 to 23 consecutively. Mice were sacrificed 48 h after the last challenge (thus on day 32) to characterize VM EtOH extract suppressive effects. A schematic diagram of this treatment schedule is shown in Fig. 1.

2.4. Measurement of airway hyperresponsiveness (AHR)

Twenty-four hours after the final aerosol challenge, AHR was assessed in conscious and unrestrained mice by means of whole-body plethysmography (OCP3000 instrument; Allmedicus, Seoul, Korea). Each mouse was placed in a plastic chamber and exposed to aerosolized PBS, followed by aerosols containing increasing concentrations of methacholine (6.25, 12.5 and 25 mg/mL; Sigma) in PBS, for 3 min at each exposure level. Bronchoconstriction was recorded for an additional 5 min after each methacholine dose. The highest Penh value obtained during each methacholine challenge was expressed as a proportion of the basal Penh value seen in response to PBS challenge.

2.5. Inflammatory cell counts in bronchoalveolar lavage fluid (BALF)

Mice were sacrificed with an overdose of 50 mg/kg of pentobarbital (Sigma) 48 h after the last challenge, and tracheotomies were

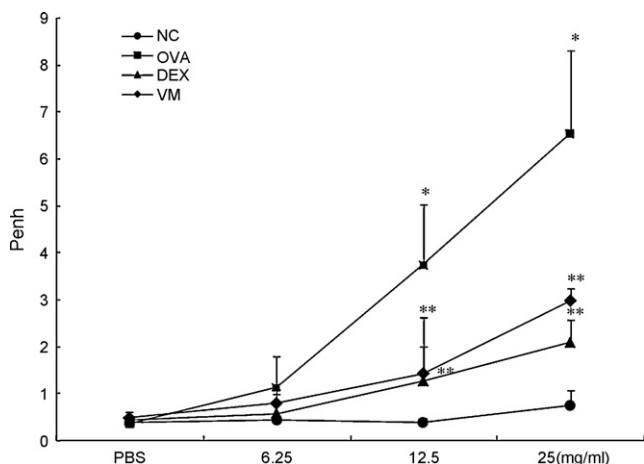


Fig. 2. Effect of VM EtOH extract on airway hyperresponsiveness (AHR). AHR was measured using plethysmography 24 h after the last ovalbumin (OVA) challenge, in mice given various doses of methacholine (6.25–25 mg/mL). NC, normal control (PBS only); OVA, OVA-sensitized/challenged mice; VM, VM EtOH extract (30 mg/kg orally) + OVA-sensitized/challenged mice; DEX, dexamethasone (3 mg/kg orally) + OVA-sensitized/challenged mice. Values are expressed as means $\pm$ SEMs. *Significant difference from NC and **significant difference from OVA, $P<0.05$.

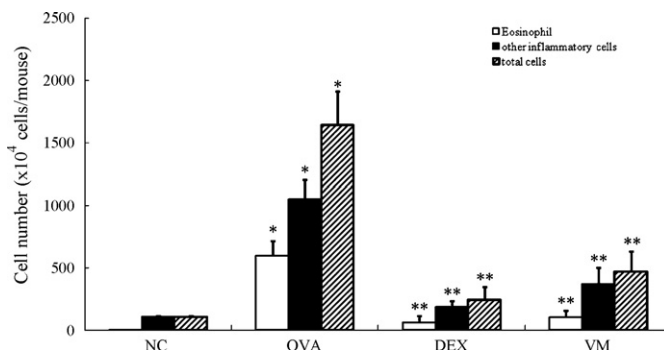


Fig. 3. Effect of VM EtOH extract on recruitment of inflammatory cells in bronchoalveolar lavage fluid (BALF) of mice 48 h after the last OVA challenge. Cell numbers was assessed by counting cells directly under a light microscope in at least five squares of a hemocytometer, after excluding dead cells staining with trypan blue. NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; VM, VM EtOH extract (30 mg/kg) + OVA-sensitized/challenged mice; DEX, dexamethasone (3 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as means $\pm$ SEMs ($n=6$ /group). *Significant difference from NC, $P<0.001$, and **significant difference from OVA, $P<0.005$.

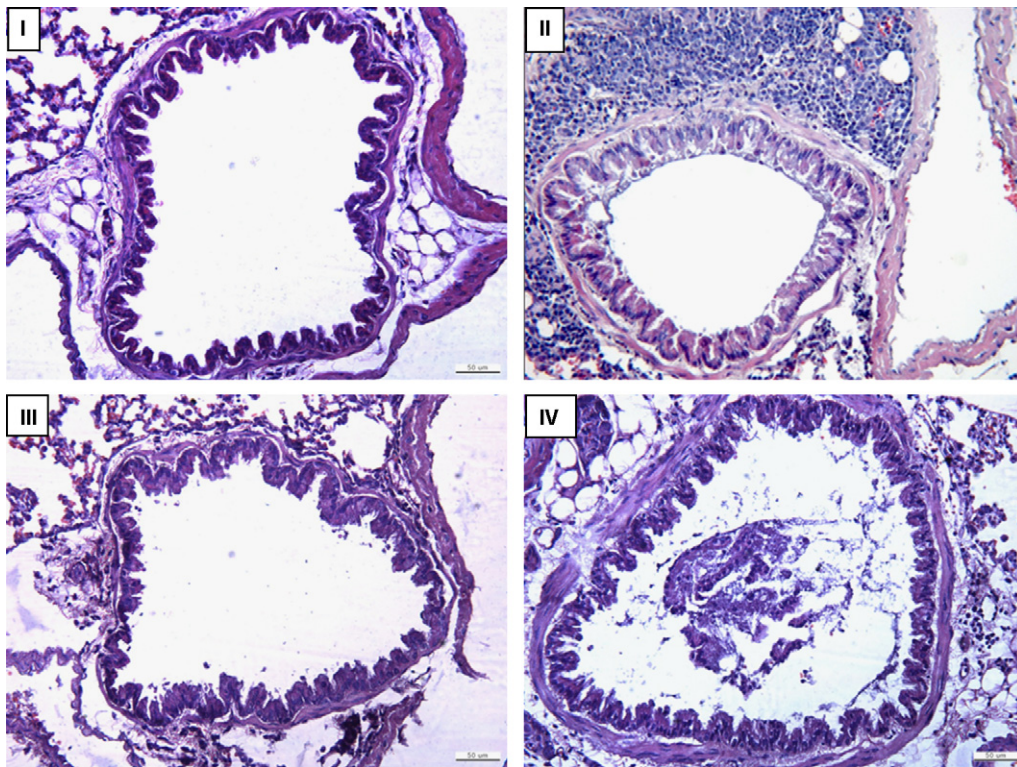


Fig. 4. Effect of VM EtOH extract on recruitment of leukocytes in lung tissue. (A) Lung tissues were fixed, sectioned at 4 μm thickness, and stained with H&E solution (magnification 200 $\times$). I, normal control mice (PBS only); II, OVA-sensitized/challenged mice; III, VM EtOH extract (30 mg/kg) + OVA-sensitized/challenged mice; IV, dexamethasone (3 mg/kg) + OVA-sensitized/challenged mice. *Significant difference from NC, $P < 0.005$, and **significant difference from OVA, $P < 0.05$.

performed. After ice-cold PBS (0.5 mL) was instilled into the lungs, bronchoalveolar lavage fluid (BALF) was obtained by three aspirations (total volume 1.5 mL) via tracheal cannulation (Djukanovic et al., 1990). Each BALF was centrifuged, and the supernatant collected and stored at -70°C . Total inflammatory cell numbers were assessed by counting cells in at least five squares of a hemocytometer, after exclusion of dead cells staining with trypan blue (Daigle and Simon, 2001). One hundred microliters of BALF was placed on a slide and centrifuged (200 $\times$ g, 4°C , 10 min) to fix the cells, using a cytospin machine (Hanil Science Industrial, Seoul, Korea). Cell pellets were suspended in 0.5 mL PBS, and 100 μL of each solution was spun onto a slide. After slides were dried, cells were fixed and stained using Diff-Quik[®] Stain reagents (B4132-1A; Dade Behring Inc., Deerfield, IL) according to the manufacturer's instructions.

2.6. Measurement of total IgE antibody in BALF

Serum was collected and stored at -70°C after centrifugation. Total IgE was measured by ELISA. Microtiter plates were coated with 100 μL /well IgE (2 $\mu\text{g}/\text{mL}$; Serotec, Oxford, UK) in PBS-tween 20. Antibodies were detected in BALF using isotype-specific secondary antibodies (anti-mouse IgE; Serotec, Oxford, UK). After washing four times, 200 μL of *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, USA) was added to each well. The plate was incubated for 10 min in the dark and absorbance measured at 450 nm. Total IgE concentrations were calculated from a standard curve generated using recombinant IgE purchased at 250 ng/mL (Serotec, Oxford, UK).

2.7. Measurement of Th2 cytokines in BALF

Enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's instructions. IL-5 and IL-13

contained in BALF were measured using specific mouse IL-5 and IL-13 ELISA kits (BioSource International, Camarillo, CA).

2.8. Lung tissue histopathology

After BALF was obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h. Tissues were next embedded in paraffin, cut into sections of 4 μm thickness, stained with H&E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and with periodic acid Schiff (PAS) stain (IMEB Inc., San Marcos, CA), to measure mucus production. Tissues were subsequently mounted and coverslipped using Dako-mounting medium (Dako-cytomation, Denmark, CA). The degree of airway inflammatory cell infiltration was scored in a double-blind manner by two independent investigators (Myou et al., 2003). Goblet-cell hyperplasia in airway epithelium was quantified based on a five-point system: 0, no goblet cells; 1, <25% of epithelium; 2, 25–50% of epithe-

Table 1

Effect of VM EtOH extract on total IgE in serum. Each sample was analyzed by ELISA ($n=5$ or $n=6$ /group). NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; VM20, 30 or 50, VM EtOH extract (20, 30 or 50 mg/kg) + OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as means $\pm$ SEMs ($n=5$ or $n=6$ /group).

Agent	Dose (mg/kg)	IgE production (ng/mL)	<i>P</i> value
NC		1.16 $\pm$ 0.61	
OVA		11.87 $\pm$ 4.74	$P=0.001^*$
Dex	3	3.39 $\pm$ 1.81	$P=0.004^{**}$
VM20	20	8.45 $\pm$ 2.89	$P=0.063$
VM30	30	5.20 $\pm$ 1.67	$P=0.001^{**}$
VM50	50	5.15 $\pm$ 2.14	$P=0.002^{**}$

Effect of VM EtOH extract on total IgE in serum.

* Significant difference from NC, $P < 0.005$.

** Significant difference from OVA, $P < 0.005$.

lium; 3, 50–75% of epithelium; 4, >75% of epithelium. For each mouse, five airway sections randomly distributed through the left lung were analyzed, and average scores were calculated. Quantitative analysis of mucus production was performed using an image analyzer (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK).

2.9. Image capture and photomicrography

Photomicrographs were captured using a Photometric Quantix digital camera running a Windows program and montages were assembled in Adobe Photoshop 7.0. Images were cropped and corrected for brightness and contrast, but were not otherwise manipulated.

2.10. Statistical analysis

Data are expressed as means $\pm$ standard errors of the mean (SEMs). Statistical significance was determined using Student's

two-tailed *t*-test to compare independent means, employing Microsoft Excel. The critical level for significance was set at $P < 0.05$.

3. Results

3.1. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on AHR

AHR was evaluated by calculation of Penh values (enhanced pauses) 24 h after final OVA challenge. The Penh value of the OVA-treated group was significantly higher than that of the PBS control group at any concentration between 6.25 and 25 mg/mL of methacholine. In the VM EtOH extract + OVA-challenged group, the Penh value was significantly reduced compared to that of the OVA-treated group. The positive control, mice given dexamethasone (widely used as an anti-asthmatic drug), showed an AHR decrease similar to that achieved using VM EtOH extract (Fig. 2).

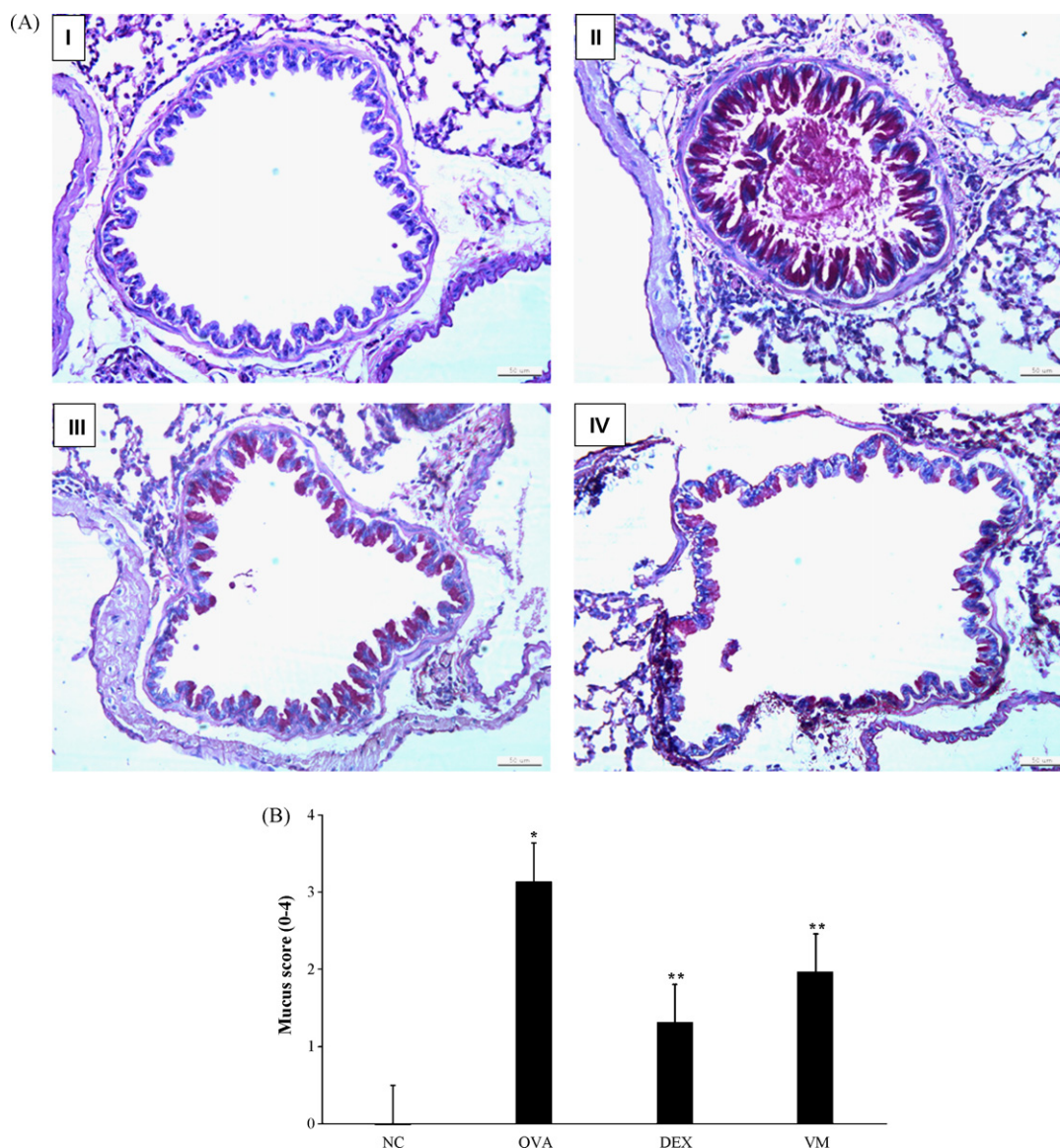


Fig. 5. Effect of VM EtOH extract on mucus production. (A) Lung tissues were fixed, sectioned at 4 μ m thickness, and stained with periodic acid Schiff (PAS) reagent to assess mucus production (magnification 200 $\times$). (B) Scoring of mucus production in lung sections was based on the method of Myou et al. I and NC, normal control mice (PBS only); II and OVA, OVA-sensitized/challenged mice; III and VM, VM EtOH extract (30 mg/kg) + OVA-sensitized/challenged mice, IV and DEX, dexamethasone (3 mg/kg) + OVA-sensitized/challenged mice; *Significant difference from NC, $P < 0.005$ and **significant difference from OVA, $P < 0.05$.

3.2. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on OVA-induced eosinophilia in BALF

To evaluate suppression by VM EtOH extract of eosinophilia in OVA-challenged mice, cells recruited to the BALF were counted 48 h after the last challenge. As shown in Fig. 3, OVA caused a marked leukocyte influx into BALF in the PBS control group. Eosinophils constituted less than 1% of total cells in PBS-treated mice. However, eosinophil levels dramatically increased, to form more than 86.3% of total leukocytes, in the BALF of OVA-challenged mice. In mice treated with VM EtOH extract, cell migration was significantly attenuated. A $71.3 \pm 39.6\%$ decrease in total cells and an 82.9% drop in eosinophils were seen, compared to the OVA-treated control group. A positive drug control, dexamethasone, showed a similar suppressive effect on leukocyte influx into BALF.

3.3. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on OVA-induced eosinophilia in lung tissue

To measure the anti-inflammatory or anti-remodeling effects of VM EtOH extract, lung tissues were collected 48 h after the last OVA challenge. In OVA-challenged mice, leukocytes infiltrated into peribronchiole and perivascular connective tissue; most leukocytes were eosinophils (Fig. 4II). In mice given VM EtOH extract and challenged with OVA, infiltration of eosinophil-rich leukocytes was significantly attenuated compared to what was seen in OVA-treated mice (Fig. 4III).

3.4. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on airway goblet-cell hyperplasia and mucus production

Mucus hypersecretion is a characteristic of airway remodeling. To evaluate possible suppression by VM EtOH extract of mucus

overproduction caused by goblet-cell hyperplasia, we stained lung sections with PAS and scored percentages of stained areas in each experimental group. In OVA-treated mice, mucus overproduction was clearly observed as a violet color in bronchial airway tissue slides, compared to minimal staining in tissues of PBS-treated mice. In contrast, mucus expression was markedly diminished in mice given VM EtOH extract and challenged with OVA (Fig. 5A). The mucus area was scored as 3.13 ± 0.34 (point units) in OVA-treated mice compared with PBS-treated mice, and the area was significantly decreased to 1.43 ± 0.23 in VM EtOH extract + OVA-treated animals, which was even lower than that of the positive control, dexamethasone + OVA-treated mice (Fig. 5B). This showed that VM EtOH extract significantly reduced goblet-cell hyperplasia and the mucus hypersecretion characteristic of the airway remodeling process.

3.5. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on the release of IgE into serum

Total serum IgE (20, 30 and 50 mg/kg) was measured by ELISA in each experimental group 48 h after the last OVA challenge. Serum IgE levels were dramatically elevated in OVA-treated mice compared with PBS-treated animals. Treatment with VM EtOH extract reduced IgE levels compared with the OVA-challenged group but doses of both 30 and 50 mg/kg were effective (Table 1).

3.6. Effect of *Viola mandshurica* W. Becker (VM) ethanolic (EtOH) extract on cytokine levels

To determine the effect of VM EtOH extract on cytokine release in mice with OVA-induced asthma, the levels of cytokines (IL-5 and IL-13) in BALF were measured by ELISA 48 h after the last OVA challenge. OVA induced significant BALF cytokine elevation, to 274.9 ± 54.3 pg/mL (IL-5) and $41.9.6 \pm 8.1$ pg/mL (IL-13), compared with control BALF. In the VM EtOH extract-treated group, cytokine elevation was significantly suppressed; there was a 173.2 ± 23.1 pg/mL decrease in IL-5 and a 25.6 ± 4.7 pg/mL drop in IL-13, compared to the OVA-challenged group. Dexamethasone also caused significant reduction in both IL-5 and IL-13 levels, compared to control values. These results demonstrate that VM EtOH extract reduced IL-5 and IL-13 concentrations in the BALF of asthmatic mice, as did dexamethasone (Fig. 6).

4. Discussion and conclusion

The objective of this study was to evaluate the effect of an EtOH extract of *Viola mandshurica* W. Becker (the VM EtOH extract) on asthma induced by OVA in a mouse model. As discussed previously, herbal medicine including VM EtOH extract is frequently used in the Orient, but both therapeutic efficacies and mechanisms are unclear. This report is the first to examine the effects of VM EtOH extract in the OVA-induced allergic asthma mouse model. Before investigating such effects in detail, using VM EtOH extract at 30 mg/kg, we made a preliminary study of the effects of VM EtOH extract (given at 20, 30, and 50 mg/kg) on serum IgE levels, to ascertain an optimal dosage (data not shown). Doses of both 30 and 50 mg/kg were effective, and the lower dosage was chosen for the principal study. The present study has demonstrated that VM EtOH extract exhibited an anti-asthmatic effect by suppressing elevations in levels of total IgE, IL-5, and IL-13; eosinophilia in both serum and BALF; and mucus overproduction in lung tissues, in OVA-induced asthmatic mice. Asthma is characterized by airway eosinophilia, edema, mucus hypersecretion, bronchial epithelial injury, and hyperreactivity. Disease pathogenesis includes contributions from several cell types including airway epithelial cells, eosinophils, macrophages, dendritic cells, T-helper type 2 (Th2)

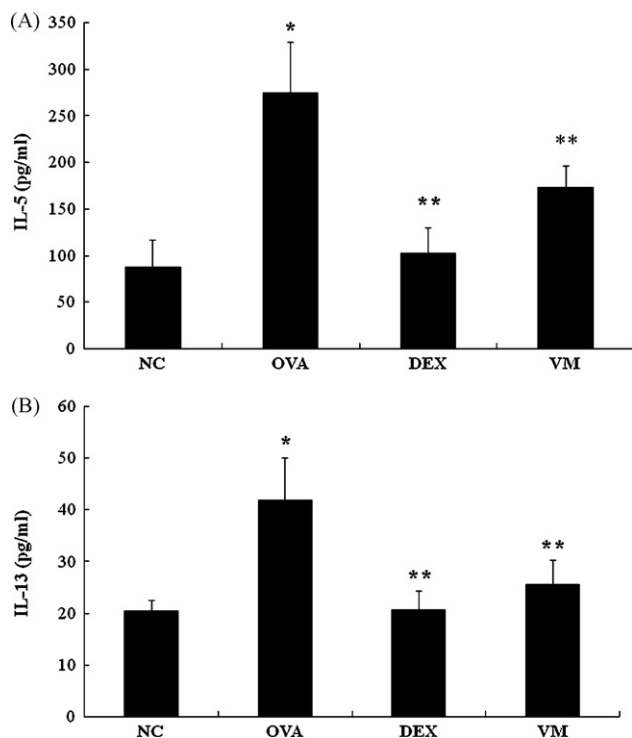


Fig. 6. Effect of VM EtOH extract on BALF cytokine levels. (A) interleukin (IL)-5 level in BALF, (B) IL-13 level in BALF. NC, normal control mice (PBS only); OVA, OVA-sensitized/challenged mice; VM, VM EtOH extract (30 mg/kg) + OVA-sensitized/challenged mice; Dex, dexamethasone (3 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as means $\pm$ SEMs ($n = 5$ or $n = 6$ /group). *Significant difference from NC, $P < 0.005$ and **significant difference from OVA, $P < 0.05$.

cells, IgE-secreting B cells, and mast cells, as well as changes in BALF (Herrick and Bottomly, 2003).

AHR is a measure of the bronchial constriction commonly found in asthmatics. In our study, the effect of VM EtOH extract on airway responsiveness was obtained by monitoring the enhanced pause (P_{enh}) for 150 s, as shown in Fig. 1. The finding that AHR was significantly reduced by VM EtOH extract could be explained, in part, by reduction in both IgE overexpression and cytokine levels. In addition, AHR has been associated with subepithelial fibrosis, which is observed as airway wall thickening during the remodeling process (Hoshino et al., 1998).

BALF analysis is widely accepted to be a good measure of epithelial lining fluid components, and such analysis can perhaps provide the most faithful reflection of protein composition in pulmonary airways (Lewis et al., 2007; Pounds et al., 2008). BALF is a biofluid that mirrors expression of secreted pulmonary proteins, products of activated cells, and outcomes of destructive processes (Leroy et al., 2008; Lewis et al., 2007). It is well known that Th-lymphocytes play a key role in regulation of immune and inflammatory reactions, through the release of cytokines (Barnes, 1998; Mosmann et al., 1986; Mosmann and Sad, 1996). Th2 lymphocytes play an important role in initiation and progression of allergic diseases, including asthma, by releasing IL-4, IL-5, and IL-13 (Brightling et al., 2002). These Th2 cytokines induce inflammatory responses, including airway infiltration and eosinophil activation, IgE production, and mucus secretion (Ngoc et al., 2005; Wills-Karp, 2004). We found that, compared with PBS-treated mice, VM EtOH extract-treated animals showed significantly reduced eosinophilia in both BALF and lung tissue. This effect of VM EtOH extract treatment may result from a decrease in the levels of Th2 cytokines responsible for eosinophil recruitment into the asthmatic lung. In this study, VM EtOH extract caused significant reductions in goblet-cell hyperplasia and mucus production in OVA-treated mice. Infiltrating airway eosinophils also affected mucus secretion by epithelial goblet cells (Shimizu et al., 2003). Our findings imply that mucus hypersecretion was attenuated by the suppressive effects of VM EtOH extract on cytokine production and eosinophilia.

In summary, we have shown that VM EtOH extract effectively suppressed elevations in IgE and cytokine levels, eosinophilia, AHR, and mucus overproduction, in mice with OVA-induced asthma. Our results suggest that VM EtOH extract could be efficacious in immunological and pharmacological modulation of allergic asthma. VM EtOH extract has real potential as a herbal medicine for asthma treatment, and is also a strong candidate for use in anti-asthmatic drug combinations.

Acknowledgments

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