Effects of spicatoside A isolated from the tuberous roots of *Liriope platyphylla* on ovalbumin-induced asthma in mice

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Abstract: The tuberous roots of *Liriope platyphylla* (Liriopis Tuber; LT) is traditionally used in Korean Medicine for treating colds, cough, and sputum production. In this study, we investigated the effect of spicatoside A isolated from LT methanol extract on ovalbumin (OVA)-sensitized/challenged asthmatic mice. For induction of allergic asthma, BALB/c mice were sensitized with OVA by an intraperitoneal injection at three times a week, and then challenged into the nasal cavities using a nebulizer. Spicatoside A at dose of 1 mg/kg body weight was treated in mice with an oral administration once daily for a week during OVA challenge. The concentrations of OVA-specific IgE, IL-4, IL-5, and IL-13 were measured in the sera or bronchoalveolar lavage fluids (BALF) of mice by enzyme-linked immunosorbent assay (ELISA). The numbers of total cells, macrophages, lymphocytes, neutrophils, and eosinophils were counted in BALFs using Diff-Quik staining, and histopathological changes of lung tissues were observed by hematoxylin and eosin (H&E), Periodic acid Schiff (PAS) and Masson's trichrome staining. The purity of spicatoside A was 98.1% with a white powder (yield: 465.6 mg). The treatment of spicatoside A in asthmatic mice significantly decreased the production of allergic mediator, OVA-specific IgE and Th2 cytokines, IL-4, IL-5, and IL-13 in sera and BALF. The numbers of inflammatory cells such as macrophages, lymphocytes, neutrophils and eosinophils in BALF of asthmatic mice were significantly reduced by the treatment of spicatoside A. Furthermore, the treatment of spicatoside A in asthmatic mice inhibited the structural damages of lung tissues with thickened bronchiolar epithelium and infiltration of inflammatory cells, the accumulation of mucus by the goblet cells hyperplasia and collagen in the bronchioles. These results suggest that spicatoside A of LT has a preventive effect on allergic asthma through the inhibition of lung inflammation and allergic response.

Keywords: Allergic asthma, spicatoside A, *Liriope platyphylla*, Liriopis Tuber, ovalbumin

INTRODUCTION

Asthma is a common chronic inflammatory disease of the airways characterized by acute symptoms of wheezing, coughing, chest tightness, shortness of breath, reversible airflow obstruction, and bronchospasm (NAEPP Coordinating Committee 2007). Asthma exacerbations are caused by a combination of genetic and environmental factors including allergens, air pollution, and other environmental chemicals. Treatment of asthma symptoms is usually with an inhaled short-acting beta-2 agonist and oral corticosteroids, while acute exacerbations are prevented with inhaled/intravenous corticosteroids and magnesium sulfate with long-acting beta agonists (LABA), leukotriene receptor antagonists, anticholinergic medications or mast cell stabilizer. However, the side effects, toxicity and resistance when administered over long periods or at high doses of these medications present a dilemma to the clinician, given the need to prevent the acute asthma attacks (Domingo *et al*., 2011). Therefore, safer preventive and therapeutic agents for asthma need to be developed despite the usefulness of current medications.

Patients with respiratory diseases such as asthma, particularly those dissatisfied with current treatment, are very likely to seek alternative treatments, and most patients use complementary medicines such as herbal medicines and nutritional supplements (Bielory 2004). This rising interest in alternative medical practices indicates a clear need for more thorough investigation into the safety and efficacy of herbal medicines with multiple therapeutic functions (Chung and Dumont 2011).

The tuberous roots (Liriopis Tuber; LT) of *Liriope platyphylla* Wang et Tang (Liliflorae) has been used for hundreds of years in Chinese medicine to treat dry cough with sticky sputum or phthisical cough with hemoptysis by moistening the lung, insufficiency of the stomach manifested as dry tongue and thirst, vexation and insomnia and constipation due to dryness of the bowels. In modern pharmacological studies, this plant's biological activities include anti-airway inflammation in asthma mice (Lee *et al*., 2005), anti-obesity-induced type II diabetes via inhibiting the fat accumulation and improving the glucose regulation in OLETF rats (Kim *et al*., 2012), immunomodulatory effect in LPS-stimulated mouse macrophages (Kim *et al*., 2012), anti-atopic dermatitis in IL-4/Luc/CNS-1 Tg mice and laxative

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effects on loperamide-induced constipation rats (Kim *et al*., 2013). LT has been reported to contain various compounds such as lupenone, lupeol, ursolic acid, beta-sitosterol, diosgenin, LP-A, LP-B (Jiang *et al*., 2007), beta-sitosterol-3-O-beta-D-glucopyranosile, palmic acid, ruscogenin, LP-C, LP-D, 25(S)-ruscogenin 1-O-beta-D-xylopyranoside-3-O-alpha-L-rhamnopyranoside (Jiang *et al*., 2011), dihydrobenzofuroisocoumarins and homoisoflavonoids (Tsai *et al*., 2013).

In this study, we isolated spicatoside A from LT methanol extract and investigated its anti-asthmatic effects on allergic and inflammatory responses in ovalbumin-sensitized/challenged asthmatic mice. The inhibitory activity of spicatoside A and ophiopogonin D derived from LT on the production/release of mucin from airway epithelial cells was recently reported (Park *et al*., 2014). However, little is known about the therapeutic effect of spicatoside A on asthma based on experimental evidence, although LT is reported to have anti-asthmatic property via inhibition of ovalbumin-induced allergic hyper responsiveness and Th2 cytokines production in mice (Kim *et al*., 2012). Therefore, in this study, we investigated the therapeutic effects of spicatoside A on airway inflammation in OVA sensitization/challenge-induced asthma mice.

MATERIALS AND METHODS

Isolation of spicatoside A

The tuberous roots of *L. platyphylla* (LT) were authenticated by Prof. J. H. Lee (Dongguk University, Gyeongju, Korea). A voucher specimen (12D1001-A01BKX1105) was deposited in the Herbarium of College of Korean Medicine, Dongguk University. The dried LT (43.0 kg) were extracted three times (X3) with hot MeOH under reflux and 6627.85 g of residue were produced. The MeOH extract was suspended in water and partitioned sequentially with equal volumes of *n*-hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). Each fraction was evaporated *in vacuo* to yield residues of *n*-hexane (31.22 g), CH_2Cl_2 (69.50 g), EtOAc (15.32g), *n*-BuOH (141.0g), and water (6070.81 g) extract. The CH_2Cl_2 fraction (66.02g) was chromatographed over a silica gel column using a gradient solvent system of hexane-EtOAc (gradient) and EtOAc saturated with H_2O: MeOH (gradient) to give 39 subfractions (C1-C39). Subfraction C31 was subjected to silica gel column chromatography (CC) eluting with a gradient solvent system of CH_2Cl_2: MeOH (gradient) to afford spicatoside A. The physico-chemical data including ^{1}H NMR, ^{13}C NMR of these compounds was identical with those reported in the literature (Zheng *et al*., 2003; Park *et al*., 2014).

Animals

Six-week-old male BALB/c mice (20±2 g) were purchased from Koatech Co. Ltd. (Gyeonggido, Korea). The animals were housed under controlled environmental conditions at a temperature of 22±3°C with a relative humidity of 55±5% and 12 h light/dark cycle throughout the study. The care and treatment of animals adhered to guidelines established by the Korean National Institute of Health at the Korean Academy of Medical Sciences for the care and use of laboratory animals and also by the Institutional Animal Care and Use Committee (IACUC) of Dongguk University.

Induction of asthma and drug administration

The mice were divided into five groups and given free access to a standard laboratory diet and water during the experimental period. OVA solution (1mg/ml in saline) and $AlOH_3$ (20 mg/ml in saline) were mixed in a 1 to 1 ratio and the mice were sensitized with an intraperitoneal injection at a dosage of 0.3mL/mice. For the second treatment, 0.1% OVA solution (antigen challenge) was injected peritoneally on days 7 and 14. In addition, a local challenge was performed three times (at two-day intervals) from day 21 to day 28 by instilling 0.1% OVA solution into the bilateral nasal cavities using a nebulizer. Spicatoside A at a dose of 1mg/kg body weight (bw) or ketotifen (an anti-histamine) as a reference drug, at a dose of 10mg/kg bw was orally administered to OVA-sensitized animals once daily for seven consecutive days during OVA challenge. Normal and OVA-sensitized/challenged animals were given saline alone on the same schedule. Blood samples were taken from each mouse by cardiac puncture under isoflurane anesthesia 24 h after the oral administration of OVA. Serum was prepared and frozen at -70°C prior to analysis. The lung tissues were removed from the body and histopathological changes were assessed.

Serological analysis

The concentrations of OVA-specific IgE (BD sciences, San Diego, CA, USA) and Th2 cytokines, IL-4, IL-5 and IL-13 (R&D Systems Inc., MN, USA) in the sera of mice were measured using commercially available enzyme immunoassay (EIA) kits or enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations. The concentration of each substance was calculated from the equations obtained from standard curve plots for the standard solutions in the kits.

Inflammatory cell counts in bronchoalveolar lavage (BAL) fluid

To count the inflammatory cells in BAL fluids, mice were anesthetized with 50 mg/kg of pentobarbital (Hanlim Pharm., Seoul, Korea) and BAL fluids harvested by lavaging the lungs with saline delivered via a tracheal cannula (Chen and Jiang, 2011). The BAL fluids were deposited onto cytospin slides and stained with Diff-Quik (Dade Behring Inc., Deerfield, IL, USA). Differential cell counts were performed by two independent investigators.

Histological analysis of lung tissues

Mice were euthanized with a high dose of pentobarbital and exsanguinated after the final OVA challenge. The lung tissue was separated from the skin, muscle and soft tissue and immersed in freshly prepared 4% neutral buffered formalin for 48 h. The tissue was then rinsed in running tap water and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 5 days. After rinsing in tap water, the tissue was dehydrated through graded alcohol and embedded in paraffin. Serial sections (5-µm) were cut at the level of the incisive papillae and first and second palatal ridges. Ten sections were taken from every mouse. The sections were stained with hematoxylin and eosin (H&E), periodic acid stain (PAS), and Masson's trichrome.

STATISTICAL ANALYSIS

All data were analyzed in GraphPad PRISM 5.0 software (GraphPad Software, Inc., San Diego, CA, USA**)**. Data are expressed as means ± the standard error of mean (S.E.M.). The significance of treatment effects was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis; null hypotheses of no difference were rejected if p-values were less than 0.05.

RESULTS

Isolation of spicatoside A

Spicatoside A was purified from LT methanol extract. This process yielded a white powder of spicatoside A (purity: 98.0%, yield: 465.6 mg, fig. 1): ^{1}H-NMR (600 MHz, pyridine d_5): δ: 0.86 (3H, s, 18-CH_3), 1.07 (3H, d, J=7.2 Hz, 27-CH_3), 1.10 (3H, d, J=6.8 Hz, 21-CH_3), 1.35 (3H, s, 19-CH_3), 1.52 (3H, d, J=6.2 Hz, fucose-CH_3), 4.85 (1H, d, J=7.6 Hz, anomeric H), 5.28 (1H, d, J=7.6 Hz, anomeric H), 5.45 (1H, d, J=7.6 Hz, anomeric H). ^{13}C-NMR (150 MHz, pyridine d_5): δ: 83.4 (C-1), 37.8 (C-2), 68.7 (C-3), 44.2 (C-4), 140.2 (C-5), 124.7 (C-6), 32.7 (C-7), 33.4 (C-8), 50.9 (C-9), 43.3 (C-10), 24.1 (C-11), 40.4 (C-12), 41.0 (C-13), 57.4 (C-14), 32.3 (C-15), 81.8 (C-16), 64.8 (C-17), 17.3 (C-18), 15.3 (C-19), 41.0 (C-20), 16.8 (C-21), 113.2 (C-22), 31.4 (C-23), 28.6 (C-24), 34.9 (C-25), 75.2 (C-26), 17.1 (C-27), 100.9 (fuc-1), 79.3 (fuc-2), 83.4 (fuc-3), 72.7 (fuc-4), 71.5 (fuc-5), 17.6 (fuc-6), 106.7 (xyl-1), 75.6 (xyl-2), 78.7 (xyl-3), 71.1 (xyl-4), 67.7 (xyl-5), 105.4 (glc-1), 76.9 (glc-2), 79.1 (glc-3), 72.5 (glc-4), 78.8 (glc-5), 63.8 (glc-6).

Effect of spicatoside A on OVA-specific IgE production in OVA-induced asthma mice

To investigate the effect of spicatoside A on allergic immune response in vivo, the level of allergic mediator, OVA-specific IgE, was measured in the sera and the BAL fluids of OVA-sensitized/challenged asthma mice. The levels of OVA-specific IgE increased significantly in the sera (fig. 2A) and BAL fluids (fig. 2B) of OVA-sensitized/challenged mice compared with the normal group ($p<0.001$). Administration of spicatoside A (1 mg/kg) to OVA-sensitized/challenged mice for seven days significantly lowered the levels of OVA-specific IgE in the sera ($p<0.05$) and BAL fluids ($p<0.001$) compared with the OVA-control group (fig. 2). The administration of an antihistamine, ketotifen (10 mg/kg), significantly decreased the levels of OVA-specific IgE in the sera ($p<0.01$) and BAL fluids ($p<0.001$) in OVA-sensitized /challenged mice.

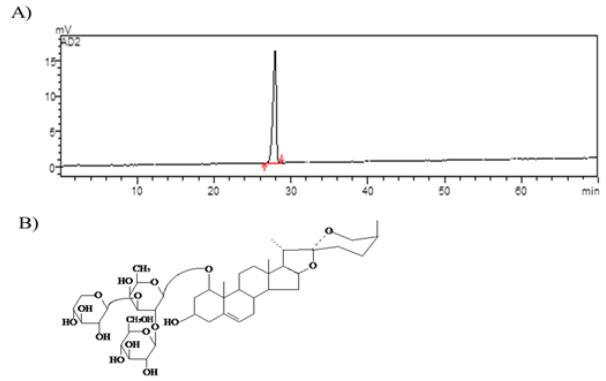

Fig. 1: HPLC analysis of spicatoside A from LT methanol extract, and its chemical structure. HPLC conditions. Column ZORBAX Eclipse Plus C18 (5 µm, 250 mm × 4.6 mm), mobile phase; A. water, B Acetonitrile with gradient of 41-44%, flow rate; 1 ml/min; detection; ELSD.

Effect of spicatoside A on the production of Th2 cytokines in OVA-induced asthma mice

We examined the effect of spicatoside A on the release of Th2 cytokines, IL-4, IL-5, and IL-13 in the BAL fluids of OVA-sensitized/challenged mice. The levels of IL-4, IL-5, and IL-13 in the BAL fluids significantly increased in OVA-sensitized/challenged mice compared with the normal group (fig. 3). The administration of spicatoside A (1 mg/kg) in OVA-sensitized/challenged mice significantly decreased the serum levels of IL-4 ($p<0.05$), IL-5 ($p<0.05$), and IL-13 ($p<0.001$) compared with the OVA-control group. Ketotifen administration significantly lowered the IL-13 level ($p<0.001$) in OVA-sensitized /challenged mice, but did not significantly decrease IL-4 and IL-5.

Effect of spicatoside A on the inflammatory cells in BAL fluids in OVA-induced asthma mice

To investigate the effect of spicatoside A on lung inflammation in OVA-sensitized/challenged asthma mice, inflammatory cells (macrophages, lymphocytes, neutrophils, and eosinophils) were counted in BAL fluids. Total cells, lymphocytes, neutrophils, and eosinophils were significantly higher in BAL fluids of OVA-sensitized/challenged asthma mice compared with the control group (fig. 4). The numbers of cells significantly decreased following the administration of spicatoside A (1 mg/kg). Ketotifen also significantly reduced the numbers of these inflammatory cells. There was no

difference in the numbers of macrophages in BAL fluids among groups.

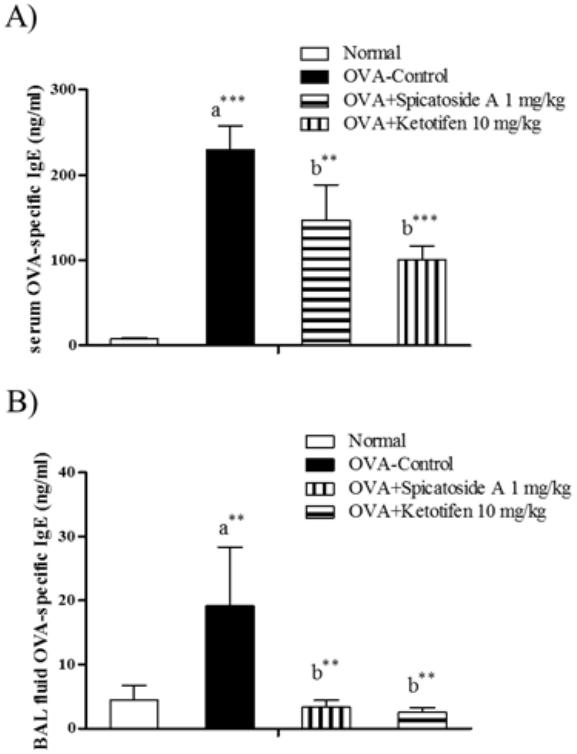

Fig. 2: Effect of spicatoside A on the levels of OVA-specific IgE in the sera (A) and BAL fluids (B) of OVA-sensitized/challenged asthma mice. The levels of OVA-specific IgE were measured by ELISA. Data are expressed as means ± S.E.M. of 5 mice per group. **P<0.01, and ***P<0.001 vs. normal (a) or OVA-Control group (b).

Effect of spicatoside A on histopathological changes of lung tissues in OVA-induced asthma mice

To investigate the effect of spicatoside A on the histopathological changes to lung tissues from asthma, the lung tissues were stained with H&E, PAS and trichrome. In the H&E stain (fig. 5A), the lung tissue of OVA-sensitized/challenged asthma mice displayed the typical asthma pathological features, including infiltration of numerous inflammatory cells around the bronchiole, peribronchial, vascular region, thickened airway epithelium, and the accumulation of mucus and debris in the lumen of bronchioles in OVA-sensitized/challenged mice. Spicatoside A (1mg/kg) markedly reduced these pathological lung changes in OVA-sensitized and challenged asthma mice. In addition, spicatoside A inhibited mucin accumulation as goblet cells hyperplasia in the lumen of bronchioles (fig. 5B) and reduced collagen accumulation around the bronchiole, peribronchial and vascular region (fig. 5C) in OVA-sensitized/challenged asthma mice. Ketotifen also reduced the inflammation in peribronchial and perivascular region.

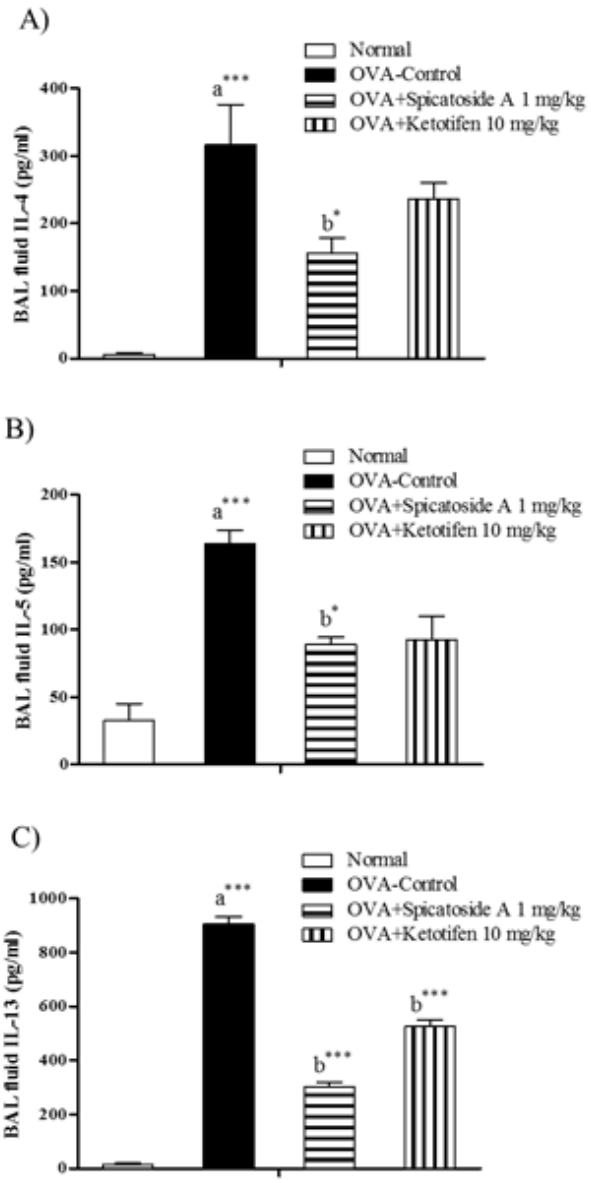

Fig 3: Effect of spicatoside A on the levels of Th2 cytokines in the BAL fluids of OVA-sensitized/challenged asthma mice. The levels of IL-4 (A), IL-5 (B), and IL-13 (C) were measured by ELISA. Data are expressed as means ± S.E.M. of 5 mice per group. *P<0.05 and ***P<0.001 vs. normal (a) or OVA-Control group (b).

DISCUSSION

The general principles for allergic asthma management are avoidance of allergens/triggering factors, and symptomatic treatment using a variety of drugs such as short acting beta-agonists, anti-cholinergics, corticosteroids and anti-inflammatory drugs or recently, therapies based on immunomodulation like allergen-specific immunotherapy (Shum *et al*., 2008). Although

pharmacotherapy is required, the current treatment of allergic asthma is not ideal and most modern drugs transiently improve clinical symptoms, but cannot cure the cause. Therefore, it is a challenge to choose the most appropriate treatment for patients from the wide range array of available agents.

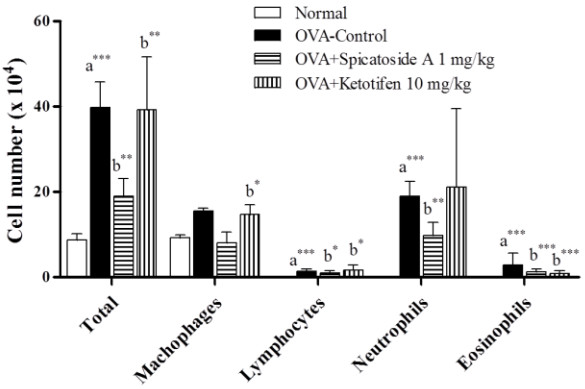

Fig. 4: Effect of spicatoside A on the numbers of inflammatory cells in the BAL fluids of OVA-sensitized/challenged asthma mice. The numbers of each cell type were counted in the BAL fluids by Giemsa staining. Data are expressed as means ± S.E.M. of 5 mice per group. *P<0.05, **P<0.01 and ***P<0.001 vs. normal (a) or OVA-Control group (b).

Herbal medicines have long been used to treat human diseases including respiratory ailments, and to maintain good health. The search for appropriate therapeutic agents in immune imbalance-associated diseases such as allergic asthma has focused on medicinal plants because natural products may provide better safety and efficacy than currently used modern drugs. Working from this perspective, we confirmed that spicatoside A isolated from LT extract has an immune-modulatory activity on OVA sensitization/challenge-induced allergic asthma in mice. This mouse model is a standard experimental animal model of allergic asthma with clinical and pathological features similar to those of human allergic asthma that are dependent on both humoral and cellular immunity (Epstein 2006).

LT is traditionally used to relieve the symptoms with dry cough with sticky sputum or phthisic cough with hemoptysis by moistening the lung. LT promotes the production of body fluid, clearing away heart-fire to relieve vexation, and moistening the bowels to relieve constipation. In addition, its biologic activity including anti-asthma (Lee *et al*., 2005; Kim *et al*., 2015), anti-diabetes (Kim *et al*., 2012), anti-inflammation (Kim *et al*., 2012), and anti-atopic dermatitis (Kwak *et al*., 2013) were well-described in modern pharmacological studies. In the present study, we first identified that spicatoside A efficiently prevented the disease progression in OVA-sensitization/challenge-induced asthma of mice by reducing the levels of OVA-specific IgE and Th2 cytokines, IL-3, IL-5 and IL-13, but also by blocking the infiltration of inflammatory cells in lungs, to a degree similar to the reference drug, ketotifen, or better. Ketotifen is an anti-allergic drug with antihistaminic activity used for asthma prophylaxis that protects against bronchoconstriction, both immediate and late reactions (Yoshihara *et al*., 2009).

In animal models, OVA sensitization/challenge produces a significant increase in the serum IgE and BAL fluid IgE (Hamelmann *et al*., 1999). IgE is a type of antibody that is present in minute amounts in the body, plays a major role in allergic diseases, binds to allergens and triggers the release of substances from mast cells as part of the inflammatory process (Martinez and Vercelli 2013). Our results showed that the concentrations of OVA-specific IgE were significantly reduced in the serum and also BAL fluid of asthma mice after spicatoside A administration. This suggests that spicatoside A has an effect on allergic asthma dependent on IgE.

Asthma is classically recognized as the typical Th2 disease, with increased IgE levels and eosinophilic inflammation in the airway. Rising Th2 cytokines, particularly IL-4, IL-5 and IL-13 modulate the asthmatic inflammation, by triggering the activation/recruitment of IgE-producing B cells, mast cells and eosinophils (Kudo *et al*., 2013). IL-4 is the major factor regulating IgE production by B cells, and is required for optimal Th2 differentiation (Deo *et al*., 2010). IL-5 is a cytokine that is highly specific for eosinophilic inflammation in allergic disease, so antibodies that block IL-5 actions are efficacious in reducing eosinophilic inflammation and the airway hyper-responsiveness of severe asthma (Corren 2011). IL-13, independent of other Th2 cytokines, is both necessary and sufficient to produce all features of allergic asthma (Wills-Karp 2004), increases goblet cell differentiation, activation of fibroblasts, elevation of bronchial hyper-responsiveness, and switching of B cell antibody production from IgM to IgE. IL-13 antagonists are considered an important biological agent for asthma therapy in patients with poorly controlled asthma. In this study, the serum levels of IL-4, IL-5 and IL-13 decreased significantly in asthma mice after spicatoside A administration. This suggests that spicatoside A has the potential to modulate the Th2 cytokines and could be used as immunomodulatory agent in the treatment of allergic asthma.

Research suggests that airway inflammation is central to asthma pathophysiology. As a result of airway inflammation, airway structural remodeling occurs, characterized by epithelial damage, goblet cell hyperplasia and airway smooth muscle hypertrophy, with profound consequences on the mechanics of airway narrowing in asthma, contributing to the chronicity and progression of the disease (Shikotra and Siddiqui 2013). Eosinophils are the predominant inflammatory cells in

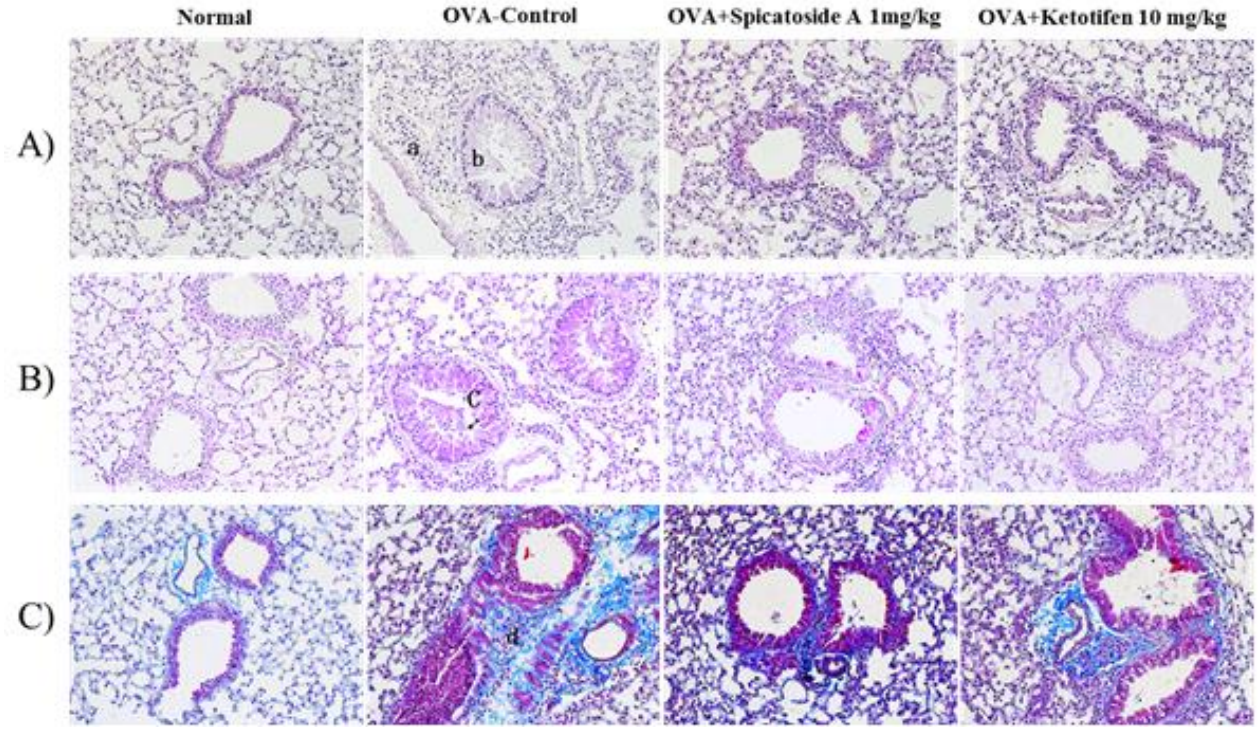

Fig. 5: Effect of spicatoside A on the pathological changes of lung tissues in OVA-sensitized/challenged asthma mice. The lung tissues were stained with H & E (A) for histopathological examination, PAS (B) for mucin-released goblet cells, and Masson's trichrome (C) for collagen-release lung fibrotic cells (×200 original magnification). A representative result of at least three independent experiments is shown. a, the infiltration of inflammatory cells; b, thickened epithelium of bronchiole; c, PAS-stained mucin releasing (violet) and d, trichrome-stained collagen accumulation (blue).

asthmatic lung tissues and contribute to the clinical features of allergic asthma and airway hyper-responsiveness. As expected, in this study, OVA-sensitization/challenge in mice engendered the structural remodeling of lungs with inflammatory alternations characterized by inflammatory cell infiltration in the peribronchial and perivascular areas, mucus overproduction and goblet cell hyperplasia in the bronchial airways. In contrast, the administration of spicatoside A inhibited increases in total number of cells and eosinophils in BAL fluids and lung tissues and prevented the development of pathological features including mucus hyper secretion and goblet cell hyperplasia in allergic asthma. These findings indicate that the protective effect of spicatoside A on allergic asthma induced by OVA is related to an attenuation of inflammatory cells in the lung tissues and goblet cell hyperplasia in the airways.

In summary, our data revealed that the anti-allergic effect with immune modulation by spicatoside A in OVA-sensitized/challenged asthma mice may be associated with its ability to prevent lung inflammation by decreasing the levels of OVA-specific IgE and Th2 cytokines, IL-3, IL-5 and IL-13 in the sera and BAL fluids. Our findings suggest that spicatoside A is useful in the treatment of respiratory disease such as asthma.

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