

Scrophularia buergeriana attenuates allergic inflammation by reducing NF- κ B activation



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ABSTRACT

Background: *Scrophularia buergeriana* Miq. (Scrophulariaceae) (SB) has been used as an oriental medicine for the treatment of inflammatory diseases, such as neuritis and pharyngolaryngitis.

Purpose: We explored the therapeutic effects of *S. buergeriana* ethanol extract (SBE) on airway inflammation in ovalbumin (OVA)-induced asthmatic mice and lipopolysaccharide (LPS)-stimulated RAW264.7 cells.

Methods: Mice were intraperitoneally injected with OVA on days 0 and 14 to elevate the immune response. On days 21 to 23, the mice were challenged with OVA solution and SBE (20 and 40 mg/kg) was administered daily by oral gavage from days 18 to 23. RAW264.7 cells were pretreated with SBE 1 h before LPS stimulation.

Results: SBE administration effectively suppressed inflammatory cell infiltration, the expression of interleukin (IL)-5, IL-13, and IL-17, immunoglobulin E, and airway hyperresponsiveness in an OVA-induced allergic asthma model. A reduction in histological alterations, including airway inflammation and mucus hypersecretion, was observed. These effects of SBE were accompanied by a decrease in matrix metalloproteinase-9 (MMP-9) expression and nuclear factor kappa B (NF- κ B) phosphorylation. These responses were observed in LPS-stimulated RAW264.7 cells. SBE treatment reduced the mRNA expression of tumor necrosis factor (TNF)- α , IL-6, and MMP-9, and NF- κ B phosphorylation, in LPS-stimulated RAW264.7 cells.

Conclusion: Our results indicated that SBE effectively attenuated airway inflammation in an OVA-induced allergic asthma model. These properties of SBE were thought to be involved in the suppression of NF- κ B phosphorylation, suggesting that the material has the potential to regulate the development of allergic asthma.

Introduction

Asthma is a representative chronic inflammatory pulmonary disease mediated by environmental allergens. A consistent increase in the morbidity and mortality associated with asthma has been observed over

the last few decades (Manka and Wechsler, 2018; Yang et al., 2014). The clinical symptoms of asthma involve cough, airway hyperresponsiveness (AHR), and mucus secretion; these are associated with airway inflammatory responses, particularly with the recruitment of eosinophils (Barnes, 2002; Manka and Wechsler, 2018). An increase in the

Abbreviations: BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPLC, High-performance liquid chromatography; IHC, immunohistochemistry; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase-9; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF- κ B, nuclear factor-kappa B; OVA, ovalbumin; PBS, phosphate-buffered saline; SB, *Scrophularia buergeriana* Miq.; SBE, *Scrophularia buergeriana* Miq. ethanol extract; TIC, total ion chromatogram; TNF- α , tumor necrosis factor- α ; XIC, extracted ion chromatogram

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eosinophil count in the bronchoalveolar lavage fluid (BALF) and bronchial mucosa of patients with asthma was reported in clinical trials, and was thought to be related to the severity of asthma (Barnes, 2002; Lloyd and Hessel, 2010). During the development of asthma, eosinophils release proinflammatory cytokines, which induce the conversion of immunoglobulin (Ig) E into B cells and the production of inflammatory mediators (Zhu, 2015; Sung et al., 2017). The proinflammatory cytokines are associated with asthma pathogenesis (Barnes, 2001). These factors are associated with the recruitment, maturation, and differentiation of eosinophils, as well as mucus production and IgE switching, eventually inducing airway inflammation through increase in the levels of inflammatory mediators (Richter et al., 2001; Wu et al., 2010; Liou et al., 2014).

Matrix metalloproteinases (MMPs), a family of zinc proteases, are involved in the destruction of the matrix components of cells and are regulated by tissue inhibitors of metalloproteinase (Visse and Nagase, 2003). MMP-9 is produced by bronchial epithelial cells, the alveolar epithelium, and many inflammatory cells (Cataldo et al., 2002), and plays a crucial role in the recruitment of inflammatory cells and the production of inflammatory mediators (McMillan et al., 2004). It is involved in the progression and aggravation of asthma (Chen et al., 2015) and is overexpressed in the sputum and alveolar macrophages in patients with asthma and in the BALF and lung tissue in an experimental animal asthma model (Cataldo et al., 2002; Ma et al., 2018; Hetzel et al., 2003; Shin et al., 2007). MMP-9 expression is modulated by signaling pathways, particularly the nuclear factor kappa B (NF- κ B) pathway (Bond et al., 2001; Teng et al., 2017). NF- κ B, a ubiquitous transcription factor, is activated in inflammatory diseases such as asthma, arthritis, and dermatitis (Tak and Firestein, 2001). Thus, the reduction in MMP-9 expression via NF- κ B suppression is thought to be a crucial strategy for asthma treatment.

Scrophularia buergeriana Miq. (Scrophulariaceae) (SB) is a perennial herb distributed in eastern Asia (i.e., Korea, Japan, Taiwan, Northeastern China) that grows to 1.8 m (Hong et al., 1998; Choi, 2007). The dried roots of SB have been used traditionally in the form of herbal medicine to control neuritis, sore throat, and laryngitis (Kim et al., 2012). The biological properties of SB have been evaluated in various studies. SB exhibited biological properties, such as neuroprotection and antioxidation activities, which were associated with the many active ingredients including phenylpropanoids, iridoids, and E-p-methoxycinnamic acid (Jeong et al., 2008; Kim et al., 2002, 2003a, 2003b). In addition, SB attenuated the inflammatory response in allergic disease models through the modulation of cytokine production (Kim et al., 2009, 2012). Based on these studies, we considered that SB may effectively suppress airway inflammation in an experimental asthma model.

Here, we explored the therapeutic properties of *S. buergeriana* Miq. ethanol extract (SBE) on airway inflammation using lipopolysaccharide (LPS)-stimulated RAW264.7 cells and an ovalbumin (OVA)-induced asthma mouse model. We evaluated the effect of SBE on inflammatory cell recruitment and proinflammatory cytokine and IgE production in this experimental model and then investigated the changes in MMP-9 expression and NF- κ B phosphorylation to elucidate the underlying mechanism of action.

Materials and methods

Plant materials

SB was collected from the experimental field of the National Institute of Horticultural and Herbal Science (NIHHS) of Rural Development Administration, Eumseong-gun, Chungbuk Province, Korea, and medicinal and plant voucher specimens (2-18-0144, KIOM-2019-53) were deposited in the Korean Herbarium of Standard Herbal Resources (Index Herbariorum code: KIOM) at the Korea Institute of Oriental Medicine, Naju, Korea. This species was identified and

confirmed based on key morphological characteristics (Choi, 2007; Jang and Oh, 2013). SB is distinguished from other related species of the genus *Scrophularia* by the leaf shape, such as the acute apex (Suppl. Fig. 1A), regular double serrated margins (Suppl. Fig. 1B), obtuse to truncated base (Suppl. Fig. 1C), and ovate calyx lobes (Suppl. Fig. 1D).

Instruments

We prepared 70% SB ethanol extract (SBE) by refluxing SB in 70% ethanol (v/v) for 2 h. The yield was 46.8% (w/w). Aucubin, harpagide, and harpagoside were obtained from Shanghai Sunny Biotech (Shanghai, China), whereas 8-acetyl harpagide and angoroside C were purchased from ChemFaces (Hebei, China). High-performance liquid chromatography (HPLC)-grade water, acetonitrile, and methanol were supplied by Merck (Darmstadt, Germany). The HPLC system (Waters Corporation, Milford, MA, USA) consisted of a Waters e2695 separation module, 2998 PDA detector, Acquity QDa detector, and micro-splitter (IDEX Health & Science LLC, Oak Harbor, WA, USA).

HPLC analysis

SBE (119.6 mg) was accurately weighed, dissolved in 10 ml of 70% ethanol, and filtered through 0.2 μ m syringe filters before HPLC analysis. A Kinetex Biphenyl 100A column (4.6 $\times$ 250 mm, 5 μ m, Phenomenex Inc., Torrance, CA, USA) was used with a mobile phase comprising a mixture of 0.05% aqueous formic acid (A), acetonitrile (B), and methanol (C), with the following gradient: 100% A (2 min) $\rightarrow$ 96% A (3% B and 1% C, 7 min) $\rightarrow$ isocratic 85% A (11% B and 4% C, from 15 min to 20 min) $\rightarrow$ 70% A (23% B and 7% C, 35 min) $\rightarrow$ 50% A (35% B and 15% C, 45 min) $\rightarrow$ 30% A (50% B and 20% C, 55 min). The flow rate was maintained at 0.8 ml/min, and the injection volume was 10 μ l. The UV wavelengths monitored were from 195 to 400 nm, and a QDa detector was used. The other parameters were set as follows: carrier gas, nitrogen; ESI capillary, 0.8 kV; probe temperature, 600 $^{\circ}$ C; con voltage, 15 V; source temperature, 120 $^{\circ}$ C; split ratio, 20:1. Aucubin, harpagide, 8-acetyl harpagide, and angoroside C were detected at 200 nm, whereas harpagoside was analyzed at 280 nm. Molecular weight detection proceeded in negative total ion chromatogram (TIC) mode and the mass range was detected from 300 to 800 m/z.

Cell culture and viability

RAW264.7, a murine macrophage cell line, was maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum and antibiotics at 37 $^{\circ}$ C in a 5% CO₂ incubator. Cell viability after SBE treatment was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Shin et al., 2014). Nontoxic concentration of (SBE) is determined as the high concentration (Suppl. Fig. 2). SBE was added to each well at 10, 20, 40, or 80 μ g/ml. The optical density was measured at 570 nm by using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time polymerase chain reaction (PCR)

RAW264.7 cells were seeded in six-well plates at a density 1×10^5 cells/well and treated with SBE for 1 h. After incubation, the cells were stimulated with LPS, and total RNA was isolated using an RNA isolation kit (Biofact, Daejeon, Republic of Korea). cDNA was synthesized by PCR using Oligo DT and cDNA synthesis kit (Biofact) and specific forward and reverse primers (tumor necrosis factor (TNF)- α , forward, 5'-CAA AGTAGACCTGCCAGAC-3', reverse, 5'-GACCTCTCTCTAATCAG CCC-3'; interleukin (IL)-6, forward, 5'-ATGCAATAACCCACCCCT GAC-3', reverse, 5'-ATCTGAGGTGCCCATGCTAC-3'; MMP-9, forward, 5'-AAGGGCGTCGTGGTTCCAATC-3' and reverse, 5'-AGCATTGCCGT

CCTGGGTGTAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward, 5'-CAAAAG GGTCTCTCTG-3', reverse, 5'- CCTG CTTACACACCTTCTTG-3'). The expression of mRNA was normalized to that of the housekeeping gene *GAPDH*. In addition, to investigate the effect of SBE on NF- κ B signaling in LPS-stimulated RAW264.7 cells, the cells were pretreated with SBE (40 μ g/ml) and Bay11-7085 (NF- κ B inhibitor, 10 μ M, α Cruz Biotechnology Inc., Santa Cruz, CA, USA) and incubated in the presence of LPS. The mRNA expression of IL-6 and TNF- α was evaluated via real-time PCR.

Immunoblotting for RAW264.7 cells

The cells were treated as described above and then incubated in the presence of LPS (1 μ g/ml) for 1 h or 24 h. The cells were collected by centrifugation, washed twice with phosphate-buffer saline (PBS), and suspended in an extraction lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitors. The protein concentration was determined using a protein assay reagent (Bio-Rad Laboratories) in accordance with the manufacturer's instructions. Equal masses of total cellular protein (30 μ g) were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution (5% skim milk), followed by overnight incubation at 4 °C with the appropriate primary antibody. The primary antibody dilutions used were as follows: anti-MMP-9 (1:1000; Abcam, Cambridge, UK), anti-NF- κ B (1:1000; Cell Signaling Technologies, Denver, MA, USA), anti-pNF- κ B (1:2000; Cell Signaling Technologies), and anti- β -actin (1:2000; Cell Signaling Technologies). The blots were washed three times with Tris-buffered saline containing Tween 20 (TBST), and then incubated with a 1:10000 dilution of a horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immuno Research, PA, USA) for 30 min at room temperature. The blots were again washed three times with TBST, and then developed by the application of an enhanced chemiluminescence (ECL) kit (Thermo Scientific, CA, USA). The density of protein band was determined with ChemiDoc (Bio-Rad Laboratories).

Allergic asthma model

BALB/c mice (female, 6 weeks old) were purchased from Samtako Co. (Osan, Republic of Korea) and acclimated to the experimental environment for 1 week. All mice were maintained in a controlled environment (22 °C $\pm$ 2 °C and 55% $\pm$ 5% humidity). Ethical and scientific management procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of Chonnam National University.

To establish the asthma model, the mice were intraperitoneally administered with OVA (20 μ g/mouse, Sigma-Aldrich) emulsified with aluminum hydroxide (20 mg in 200 μ l of PBS) on days 1 and 14, and then exposed to 1% OVA aerosol using a nebulizer (Omron, Tokyo, Japan) twice daily for 30 min from days 21 to 23. The drug was administered via oral gavage 1 h before OVA inhalation from days 18 to 23. Mice were randomly divided into the following five groups: NC, (normal control group, PBS sensitization and inhalation + PBS administration); OVA (asthma group, OVA sensitization and inhalation + PBS administration); Mon (montelukast-treated group, OVA sensitization and inhalation + montelukast (10 mg/kg administration); SBE20 and SBE40 (SBE-treated groups, OVA sensitization and inhalation + SBE administration (20 and 40 mg/kg, respectively). On day 24, airway hyperresponsiveness (AHR) in conscious and unrestrained mice was determined by using whole-body plethysmography (OCP3000; Allmedicus, Seoul, South Korea). AHR was measured following methacholine aerosolization (doses of 0, 10, 20, and 30 mg/ml in PBS) for 3 min. The results were recorded as the dimensionless parameter, enhanced pause (Penh).

BALF and serum analysis

To obtain BALF, we performed tracheostomy in mice anesthetized with alfaxan (Alfaxalone®, Jurox Pty Ltd., Hunter Valley, Australia). The lungs were lavaged twice with cold PBS (total volume 1.4 ml) and the obtained BALF was centrifuged (5 min, 1,500 rpm). The supernatant was used for the analysis of inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA). The pellets were used to count inflammatory cells in the BALF. The total inflammatory cell count was determined by using a cell counting machine (Countess II, Thermo Fisher Scientific, San Diego, CA, USA). To estimate differential cell counts, whole cells from the BALF pellet were attached to a slide glass using Cytospin (5 min, 1000 rpm, Hanil Electric, Wonju, Republic of Korea), and the slides were then stained with Diff-Quik® reagent (Sysmax, Kobe, Japan). The production of IL-5, IL-13, and IL-17 was measured in BALF supernatant by using an ELISA kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's protocol. Blood was collected and then centrifuged to obtain serum for analysis of IgE. IgE level was measured by using an ELISA kit (BioLegend Inc., San Diego, CA, USA), and OVA-specific IgE was determined as previously described (Shin et al., 2017).

Immunoblotting for lung tissue samples

To extract proteins from the lung tissue, the samples were homogenized and the protein concentration was determined with a protein assay kit (Bio-Rad Laboratories). Immunoblotting was performed as described above. The primary antibodies dilutions were as follows: anti-MMP-9 (1:1000; Abcam), anti-NF- κ B (1:1000; Cell Signaling Technologies), anti-pNF- κ B (1:2000; Cell Signaling Technologies), and anti- β -actin (1:2000; Cell Signaling Technologies). The density of the protein band was determined by using ChemiDoc (Bio-Rad Laboratories).

Histopathological analysis

The right lung of each mouse was fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned into 4- μ m thick slices, and stained with hematoxylin and eosin (Sigma-Aldrich), to evaluate inflammatory responses in lung tissues, and periodic acid-Schiff (PAS, IMEB Inc., San Marcos, CA, USA), to determine mucus production. Quantitative analysis of inflammation and mucus production was performed by using an image analyzer (IMT i-Solution software, IMT i-Solution Inc., Vancouver, BC, Canada). To evaluate MMP-9 expression in the lung tissue, immunohistochemical analysis was conducted as previously described (Ko et al., 2018). Anti-mouse MMP-9 antibody (1:100 dilution, Abcam) was used as the primary antibody. The slides were counterstained, rinsed with PBS to terminate the reaction, and the tissues were protected with coverslips.

Statistical analysis

The data were expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA), followed by a multiple comparison test with Dunnett's adjustment. A value of $p < 0.05$ indicated significant difference.

Results

HPLC analysis of SBE

The HPLC chromatogram of SBE is shown in Fig. 1. Aucubin, harpagide, 8-acetyl harpagide, angoroside C, and harpagoside were detected at approximately 11.5, 13.4, 19.9, 37.2, and 42.2 min, respectively. The HPLC chromatogram of SBE at 200 nm is presented in Fig. 1A and shows aucubin, harpagide, 8-acetyl harpagide, and

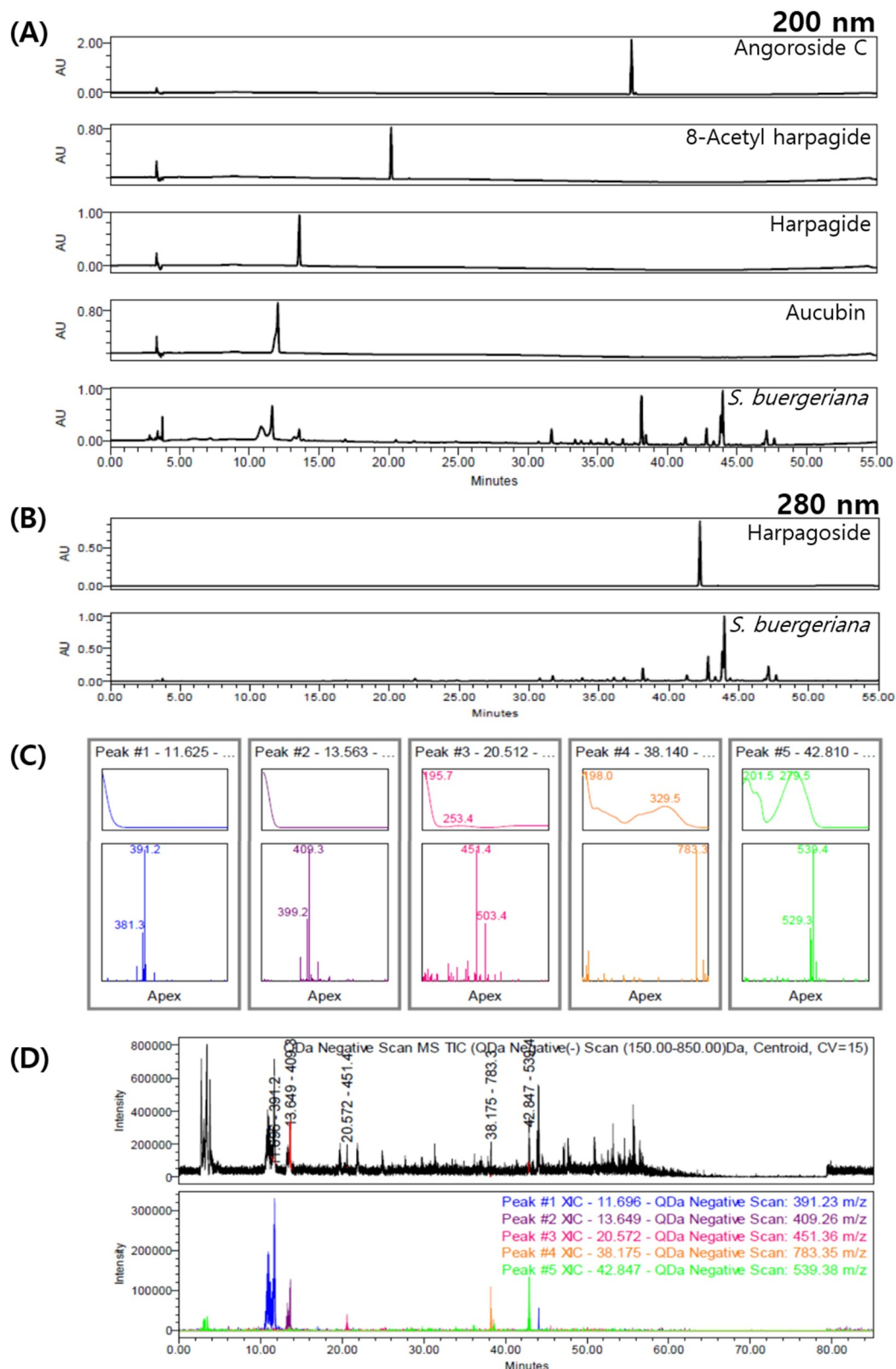


Fig. 1. Chromatogram of *S. buergeriana* at 200 nm (A) and 280 nm (B), UV spectra and MS spectra of peaks according to retention time (C), total ion chromatogram (TIC), and extracted ion chromatogram (D).

angoroside C. The chromatogram of SBE and harpagoside at 280 nm is shown in Fig. 1B. The content of each of the five compounds was 15.02 ± 0.375 , 4.29 ± 0.060 , 0.71 ± 0.024 , 4.85 ± 0.043 , and 2.16 ± 0.050 $\mu\text{g}/\text{mg}$, respectively. Aucubin, harpagide, 8-acetyl

harpagide, and harpagoside were classified as iridoids, whereas angoroside C was categorized as a phenylpropanoid. Aucubin was the main compound of SBE, and the sum of iridoids was $22.18 \mu\text{g}/\text{mg}$. The five peaks of SBE were compared by UV wavelength and molecular

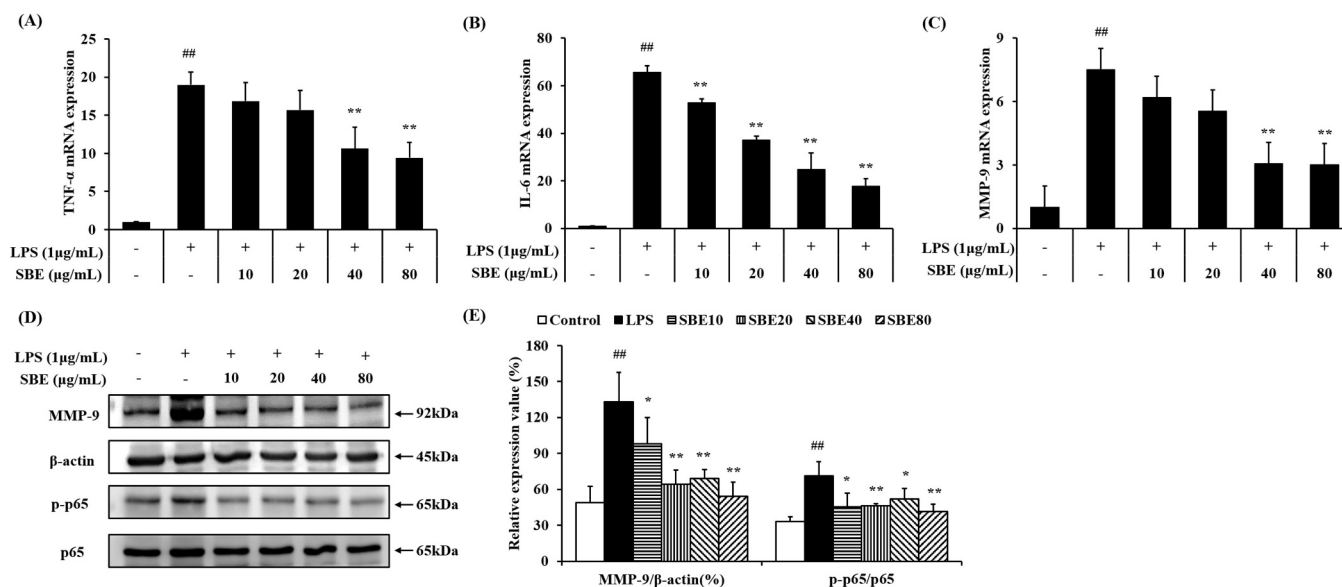


Fig. 2. SBE decreases the expression of inflammatory mediators in LPS-stimulated RAW264.7 cells. To evaluate the mRNA expression of proinflammatory mediators, we performed RT-PCR analysis. MMP-9 expression and NF- κ B phosphorylation were determined by western blotting. The band density was evaluated using ChemiDoc. (A) mRNA expression of TNF- α , (B) IL-6, (C) MMP-9. (D) Expression of MMP-9 and phosphorylation of NF- κ B, (E) Expression of NF- κ B. Values are shown as the mean $\pm$ SD ($n = 3$). ^{##} $p < 0.01$ versus non-stimulated cells; ^{*}, ^{**} $p < 0.05$ and 0.01 vs. LPS-stimulated cells, respectively.

weight (Fig. 1C). The UV spectra of these five compounds showed a $\lambda_{\text{max}} = 195.6, 195.3, 195.7, 198.0$, and 279.5 nm, respectively. In the negative mode, the masses of the compounds were confirmed as follows: aucubin $[M + \text{COOH}]^- = 391.2$ m/z , harpagide $[M + \text{HCOOH} + \text{OH}]^- = 409.2$ m/z , 8-acetyl harpagide $[M + \text{COOH}]^- = 451.2$ m/z , angoroside C $[M - \text{H}]^- = 783.4$ m/z , and harpagoside $[M + \text{COOH}]^- = 539.3$ m/z . The TIC and extracted ion chromatogram (XIC) were compared to verify the five compounds on the chromatogram. The TIC was a black line in the mass range from 300 to 800 m/z , and the XIC was shown as follows: blue, aucubin at 391.2 m/z ; purple, harpagide at 409.2 m/z ; pink, 8-acetyl harpagide at 451.2 m/z ; orange, angoroside C at 783.4 m/z ; green, harpagoside at 539.3 m/z (Fig. 1D).

SBE reduces the expression of inflammatory mediators in LPS-stimulated RAW264.7 cells

The mRNA expression of TNF- α and IL-6 was higher in LPS-stimulated cells than in non-stimulated cells (Fig. 2A and B). However, SBE treatment significantly decreased the mRNA expression of TNF- α and IL-6 in LPS-stimulated cells. LPS-stimulated cells showed elevated MMP-9 mRNA expression compared with the non-stimulated cells, whereas the expression of MMP-9 in SBE-treated cells was lower compared with that in LPS-stimulated cells (Fig. 2C).

MMP-9 expression was markedly higher in LPS-stimulated cells than in non-stimulated cells (Fig. 2D and E). However, SBE treatment significantly decreased MMP-9 expression in LPS-stimulated cells. The extent of NF- κ B phosphorylation was higher in LPS-stimulated cells than in the non-stimulated cells, but was significantly lower in SBE-treated cells than in LPS-stimulated cells.

The mRNA expression of IL-6 and TNF- α in LPS-stimulated cells was markedly decreased by treatment with an NF- κ B inhibitor (Bay11-7085) (Fig. 3A and B). Notably, cotreatment with an SBE and NF- κ B inhibitor also reduced the mRNA expression of IL-6 and TNF- α compared with that in LPS-stimulated cells; this effect was more than that on treatment with an NF- κ B inhibitor alone.

SBE reduces inflammatory cell recruitment and AHR in OVA-induced allergic asthma model

The inflammatory cell count was markedly higher in the BALF from OVA group than in the BALF from normal controls, whereas the SBE-treated group showed a significantly lower inflammatory cell count compared with the OVA group (Fig. 4A). In particular, SBE treatment effectively suppressed the elevation in eosinophil count induced by OVA inhalation. These effects were similar to those reported in the Mon group. The OVA group showed inflammatory cell infiltration of the airway, whereas the SBE-treated group showed a decrease in inflammatory cell recruitment compared with the OVA group, by histological examination (Fig. 5). AHR markedly increased in the OVA group with an increase in methacholine concentration compared with that in the normal controls. However, the SBE-treated group showed significant reduction in AHR compared with the OVA group. In particular, this reduction was clearly observed in the higher dose group (40 mg/kg, Fig. 4B).

SBE suppresses the physiological alteration in the BALF and serum of OVA-induced allergic asthma model

The expression of IL-5, IL-13, and IL-17 was higher in the OVA group than in normal controls (Fig. 6A, B, and C, respectively). However, the SBE-treated groups showed a significant decrease in these factors compared with the OVA group. These results were similar to those reported in the Mon group. In addition, the total and OVA-specific IgE levels in serum were significantly higher in OVA group than in the normal controls (Fig. 6D and E, respectively), but notably decreased in SBE-treated groups.

SBE decreases MMP-9 expression and NF- κ B phosphorylation in the OVA-induced allergic asthma model

The extent of NF- κ B phosphorylation was significantly increased in

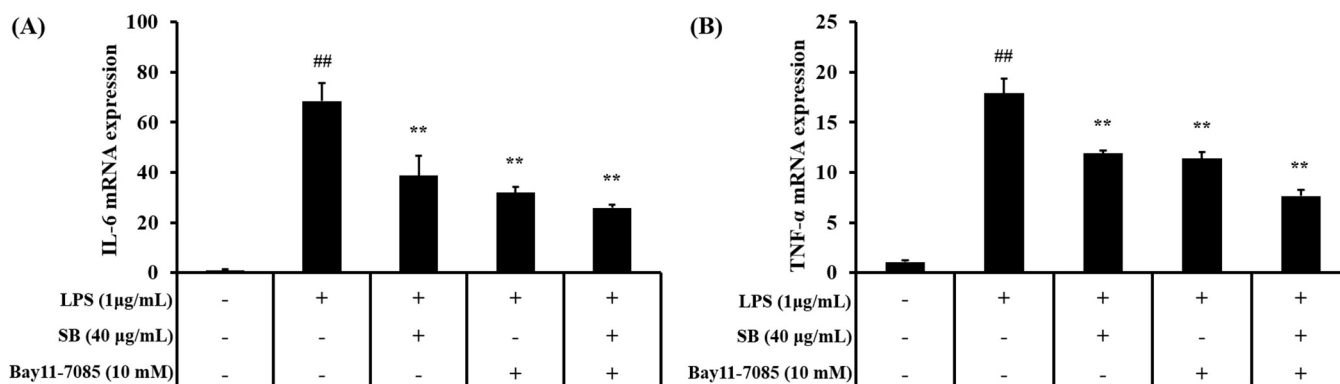


Fig. 3. SBE decreases the mRNA expression of IL-6 and TNF- α in LPS-stimulated RAW264.7 cells via suppression of NF- κ B. To investigate the effect of SBE on NF- κ B signaling in LPS-stimulated RAW264.7 cells, the cells were pretreated with SBE and Bay11-7085 (NF- κ B inhibitor, 10 μ M) and then stimulated with LPS. The mRNA expression of IL-6 and TNF- α was evaluated via real-time PCR analysis. (A) mRNA expression of IL-6 and (B) TNF- α . ^{##} p < 0.01 versus non-stimulated cells; ^{**} p < 0.01 vs. LPS-stimulated cell.

the OVA group compared with that in normal control group (Fig. 7A and B). In the Mon group, there was a reduction in NF- κ B phosphorylation compared with the OVA group. In addition, SBE treatment resulted in a significant decline in NF- κ B phosphorylation compared with the OVA treatment. Furthermore, the OVA group showed elevated MMP-9 expression compared with the normal control group, but SBE-treated group showed a significant decrease in the expression of MMP-9 compared with the OVA group. IHC results showed that SBE treatment effectively suppressed MMP-9 expression by OVA inhalation (Fig. 7C).

SBE suppresses mucus production in the OVA-induced allergic asthma model

The OVA groups showed higher mucus production than the normal control groups (Fig. 8). However, SBE treatment suppressed mucus production induced by OVA inhalation. These responses were similar to the results reported for the Mon group.

Discussion

The prevalence of asthma has gradually increased owing to the increase in exposure to allergens, airway pollutants, and chemicals (Manka and Wechsler, 2018). As a result, development of therapeutic agents for asthma treatment has garnered much attention from researchers. In the present study, we investigated the therapeutic properties of SBE against asthma by using an OVA-induced allergic asthma model and LPS-stimulated RAW264.7 cells. SBE treatment suppressed

pathophysiological alterations, including inflammatory cell infiltration, proinflammatory cytokine production, IgE, and mucus secretion in the OVA-induced allergic asthma mouse model. In addition, SBE treatment effectively decreased the mRNA expression of *TNF- α* , *IL-6*, and *MMP-9* in LPS-stimulated RAW264.7 cells. These responses were accompanied with a decrease in the MMP-9 expression and NF- κ B phosphorylation in *in vivo* and *in vitro* experiments. Aucubin, harpagide, angorside C, and harpagoside were quantitatively determined by using HPLC. A physicochemical analysis of five compounds following SBE administration was performed using HPLC-PDA-QDa with the addition 8-acetyl harpagide. The components identified in SBE were closely related to the reduction in pathological changes in respiratory disease. Aucubin was shown to cause a significant reduction the total number of BALF cells, neutrophils, and macrophages in LPS-induced acute lung injury mouse model (Pan et al., 2019). In addition, angorside C played a key role in the reduction of pulmonary edema (Zhang et al., 2017).

Airway inflammation and mucus overproduction are important features of asthma, attributed to the recruitment of inflammatory cells, in particular eosinophils (Murdoch and Lloyd, 2010; Shen et al., 2018). Proinflammatory cytokines, including IL-5 and IL-13, are closely involved in inflammatory responses of asthma. IL-5 and IL-13 accelerate eosinophil recruitment on airway, leading to the maturation and activation of eosinophils, and the production of other inflammatory cytokines and chemokines (Zhu et al., 2018). These events eventually contribute to eosinophil-rich inflammation and mucus hypersecretion in the airway (Deo et al., 2010). Therefore, the suppression of

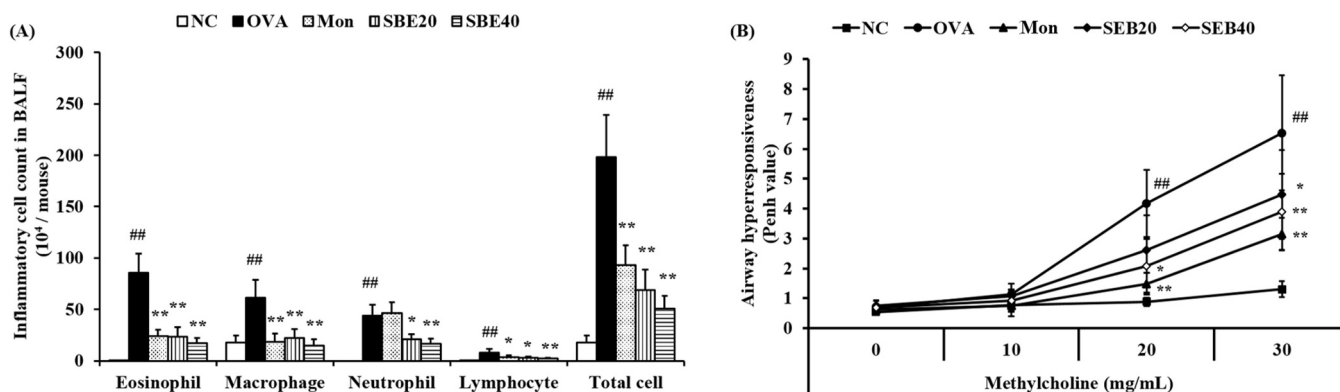


Fig. 4. SBE decreases the inflammatory cell count in BALF and airway hyperresponsiveness (AHR) of OVA-induced mice (an allergic asthma model). To count inflammatory cells in BALF, BALF was stained with Diff-Quik® reagent. AHR was determined by using whole-body plethysmography. (A) Inflammatory cell count, (B) AHR. NC: normal control; OVA: asthma group; Mon: asthma model with montelukast (10 mg/kg) administration; SBE20 and 40: asthma model with SBE administration (20 and 40 mg/kg, respectively). Values are shown as the mean $\pm$ SD (n = 5). ^{##} p < 0.01 vs. NC; ^{*}, ^{**} p < 0.05 and 0.01 vs. OVA, respectively.

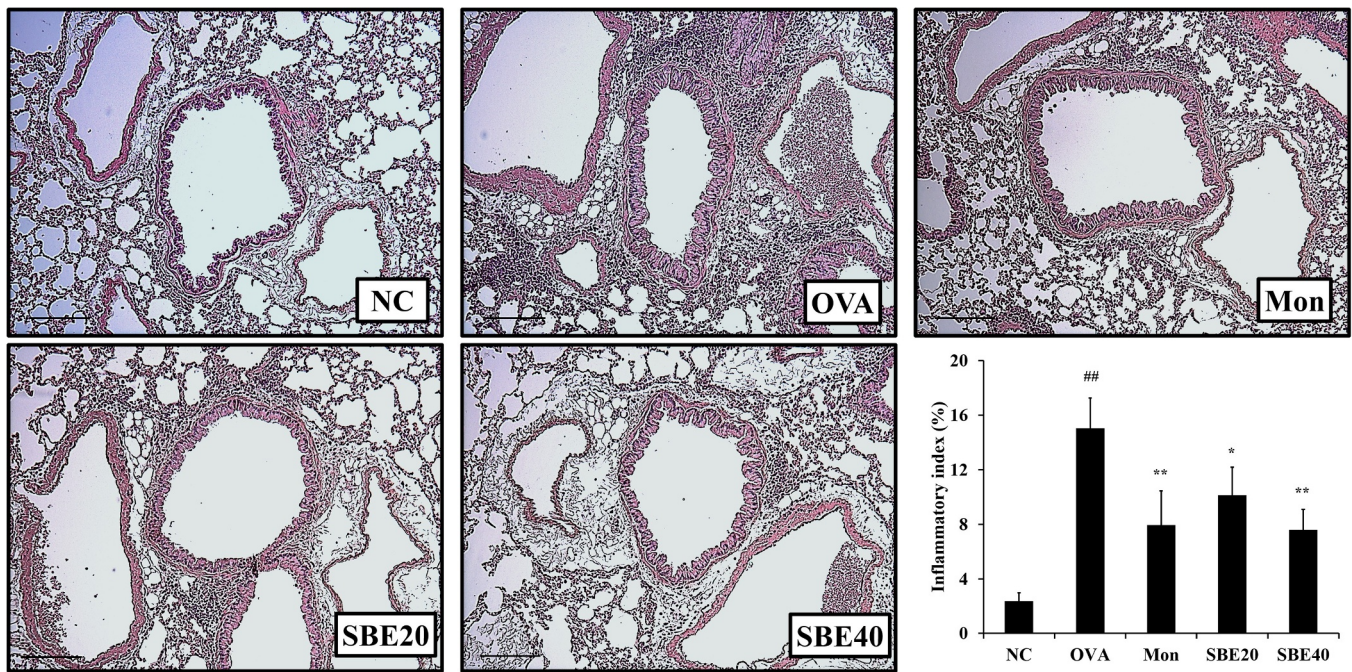


Fig. 5. SBE suppresses inflammatory cell recruitment in the lung of OVA-induced mice (an allergic asthma model). To evaluate the airway inflammation, lung tissue samples were stained with H & E solution. Quantitative analysis of inflammation was performed by using an image analyzer. NC: normal controls; OVA: asthma group; Mon: asthma model with montelukast (10 mg/kg) administration; SBE20 and 40: asthma model with SBE administration (20 and 40 mg/kg, respectively). Scale bars = 50 μ m. The values shown are the mean $\pm$ SD ($n = 5$). ^{##} $p < 0.01$ vs. NC; ^{*}, ^{**} $p < 0.05$ and 0.01 vs. OVA.

proinflammatory cytokines may be an important step in the attenuation of asthma severity or in the evaluation of efficacy of test agents in asthma. Here, SBE treatment effectively suppressed the elevated expression of proinflammatory cytokines, including IL-5 and IL-13, in the OVA-induced allergic asthma model that was accompanied by a reduction in inflammatory cell count, OVA-specific IgE, pulmonary inflammation, and mucus secretion. These results indicated that SBE treatment effectively inhibited the development of asthma through

suppression of the production of proinflammatory cytokines.

MMPs are proteinases expressed during acute and chronic asthma (McMillan et al., 2004) and regulated by several mediators, such as cytokines, growth factors, and substrate components (Sternlicht and Werb, 2001). MMP-9 expression is known to be increased by inflammatory cells or pulmonary resident cells in patients with asthma and experimental animals with asthma (Ingram and Kraft, 2015; Schwingshackl et al., 1999). MMP-9 is involved in the production of

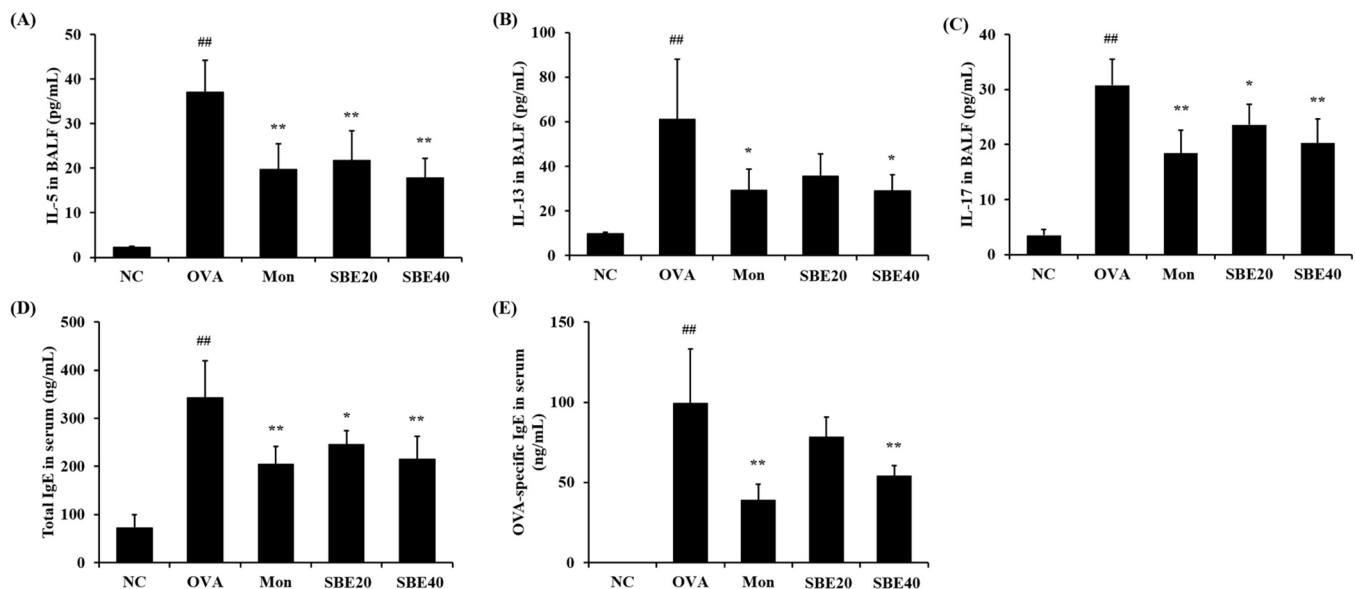


Fig. 6. SBE decreases the production of proinflammatory cytokines and IgE in OVA-induced mice (an allergic asthma model). IL-5, IL-13, IL-17, total IgE, and OVA-specific IgE were determined by using commercial ELISA kits. (A) IL-5, (B) IL-13, (C) IL-17, (D) total IgE, and (E) OVA-specific IgE. NC: normal controls; OVA: asthma group; Mon: asthma model with montelukast (10 mg/kg) administration; SBE20 and 40: asthma model with SBE administration (20 and 40 mg/kg, respectively). The values shown are the mean $\pm$ SD ($n = 5$). ^{##} $p < 0.01$ versus NC; ^{*}, ^{**} $p < 0.05$ and 0.01 vs. OVA, respectively.

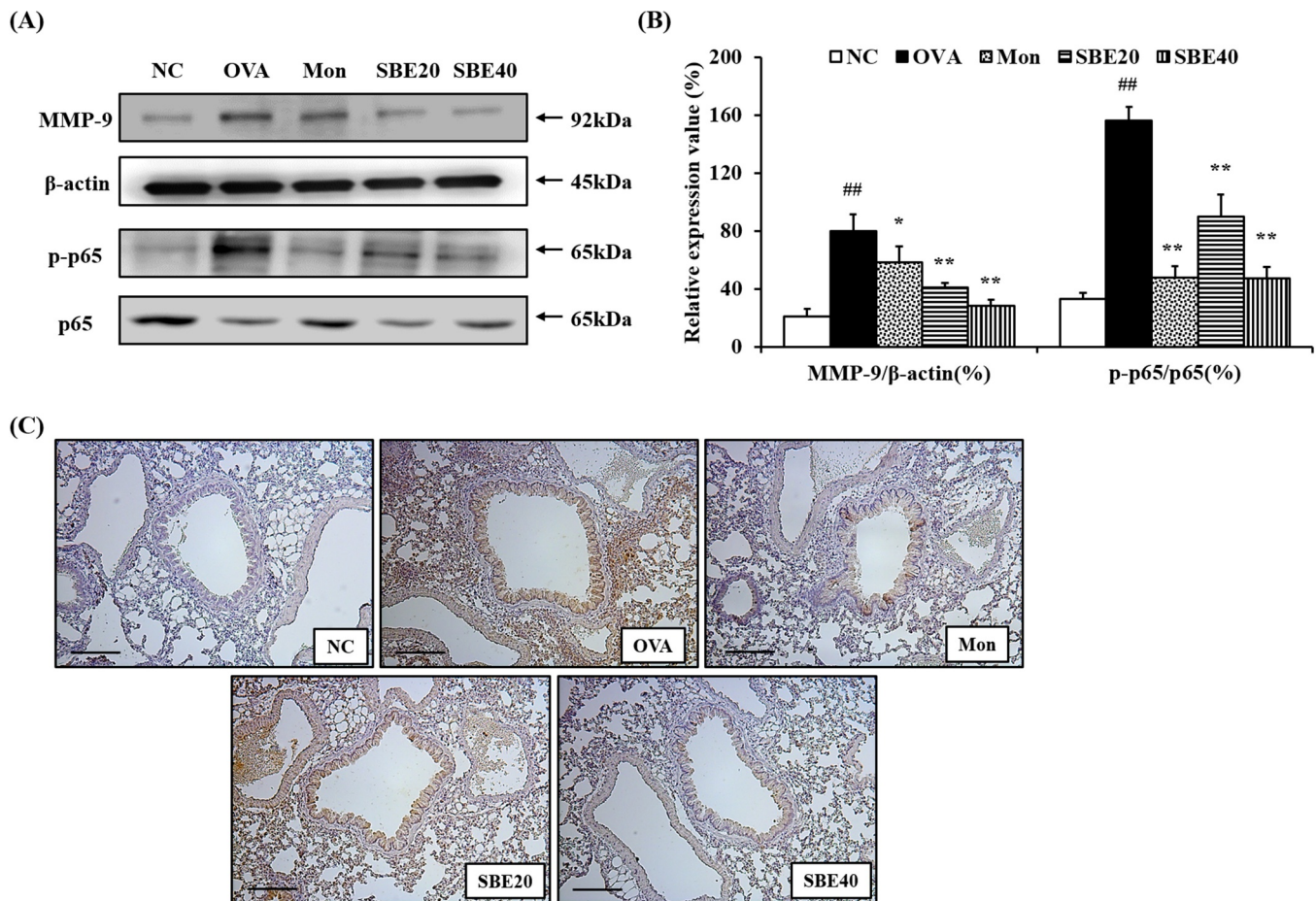


Fig. 7. SBE inhibits the expression and activity of MMP-9 and phosphorylation of NF-κB in OVA-induced mice (an allergic asthma model). MMP-9 expression and NF-κB phosphorylation were determined by using western blotting and IHC. (A) Expression of MMP-9 and phosphorylation of NF-κB, (B) density of MMP-9 and phosphorylation of NF-κB, and (C) MMP-9 expression in IHC. NC: normal control; OVA: asthma group; Mon: asthma model with montelukast (10 mg/kg) administration; SBE20 and 40: asthma model with SBE administration (20 and 40 mg/kg, respectively). The values shown are the mean $\pm$ SD ($n = 5$). ^{##} $p < 0.01$ vs. NC; ^{*}, ^{**} $p < 0.05$ and 0.01 vs. OVA, respectively. Scale bars = 50 μ m.

proinflammatory mediators and induces airway remodeling. The role of MMP-9 in the pathogenesis of asthma has been demonstrated in various studies, and MMP-9-deficient mice were shown to exhibit a decline in airway inflammation and mucus secretion following OVA stimulation (Cataldo et al., 2002; McMillan et al., 2004). In the present study, SBE treatment reduced the expression and activity of MMP-9 in the lung in an OVA-induced allergic asthma model. These results showed that the therapeutic effects of SBE on asthma were related to the suppression of MMP-9. MMP-9 expression is associated with NF-κB phosphorylation (Rhee et al., 2007). Many *in vitro* and *in vivo* studies have reported that the expression and activity of MMP-9 are increased by an increase in NF-κB phosphorylation (Bond et al., 2001; Rhee et al., 2007; Yan and Choi, 2014). NF-κB is a crucial transcription factor involved in inflammatory processes and serves as a key mediator in the development of asthma because it regulates not only MMP-9 expression, but also Th2 cell differentiation and Th2 cytokine production (Yan and Choi, 2014). Thus, the suppression of NF-κB phosphorylation is considered to be a crucial therapeutic target for asthma treatment. In the present study, SBE markedly decreased the expression of TNF-α, IL-6, and MMP-9 in LPS-stimulated RAW264.7 cells, and this effect was accompanied by a

reduction in NF-κB phosphorylation. SBE treatment also attenuated airway inflammation in an OVA-induced allergic asthma model along with a decrease in the extent of NF-κB phosphorylation. From these results, we concluded that the therapeutic effects of SBE were involved in the suppression of NF-κB phosphorylation.

In summary, we have demonstrated that SBE effectively attenuated inflammatory responses in an OVA-induced mouse model of allergic asthma and LPS-stimulated cells. These effects of SBE were closely related to the decrease in MMP-9 expression following suppression of NF-κB phosphorylation. Therefore, SBE could be effective in regulating the development of allergic asthma.

Author contributions

N.R.S., I.S.S. and J.S.K. designed carried out the experiments. A.Y.L., J.H.S., S.Y., I.P., J.O.L., T.Y.J., J.W.K., K.S.L., and M.Y.L. performed extraction, HPLC analysis and biochemical experiments. N.R.S. and A.Y.L. prepared manuscript. J.C.K., I.S.S. and J.S.K. revised the manuscript. I.S.S. and J.S.K. supervised all research. All authors reviewed the manuscript.

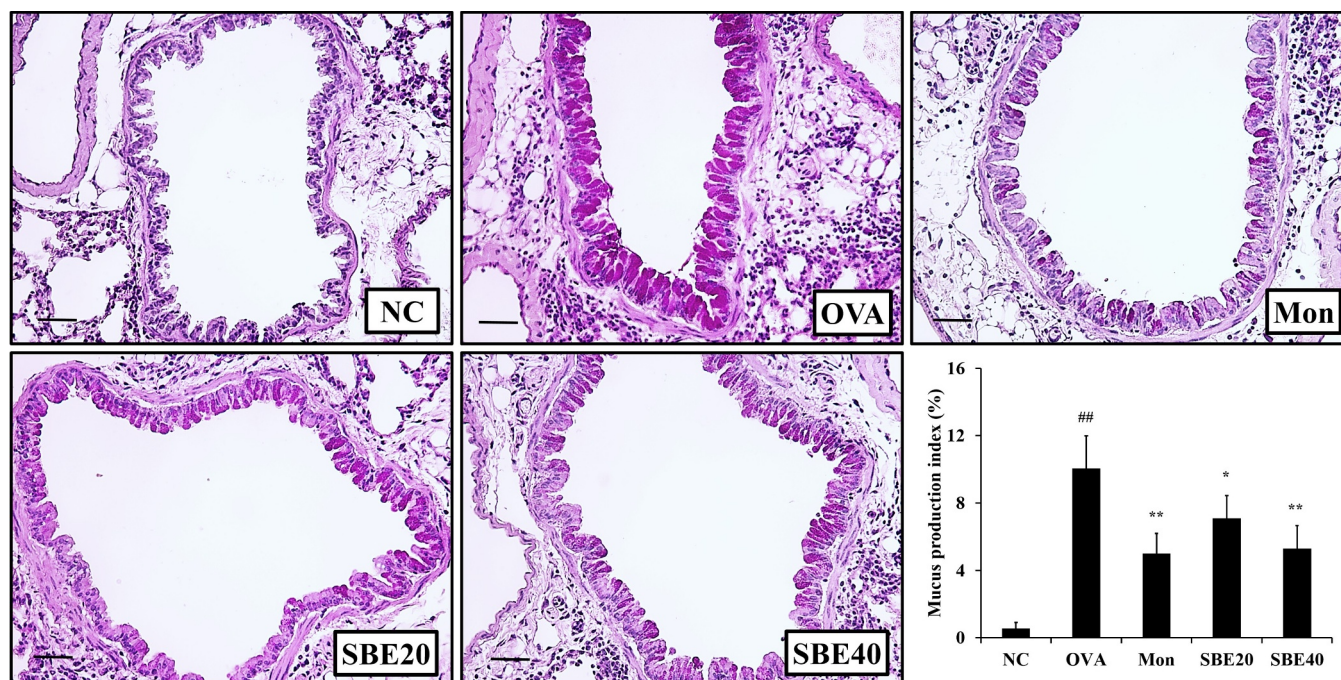


Fig. 8. SBE attenuates mucus production in the lungs of OVA-induced mice (an allergic asthma model). To evaluate mucus production, lung tissue samples were stained with PAS solution. Quantitative analysis of mucus production was performed by using an image analyzer. NC: normal controls; OVA: asthma group; Mon: asthma model with montelukast (10 mg/kg) administration; SBE20 and 40: asthma model with SBE administration (20 and 40 mg/kg, respectively). Scale bars = 50 μ m. The values are shown are the mean $\pm$ SD ($n = 5$). ## $p < 0.01$ vs. NC; *, ** $p < 0.05$ and 0.01 vs. OVA.

Conflict of interest

No competing financial interests exist.

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Supplementary materials

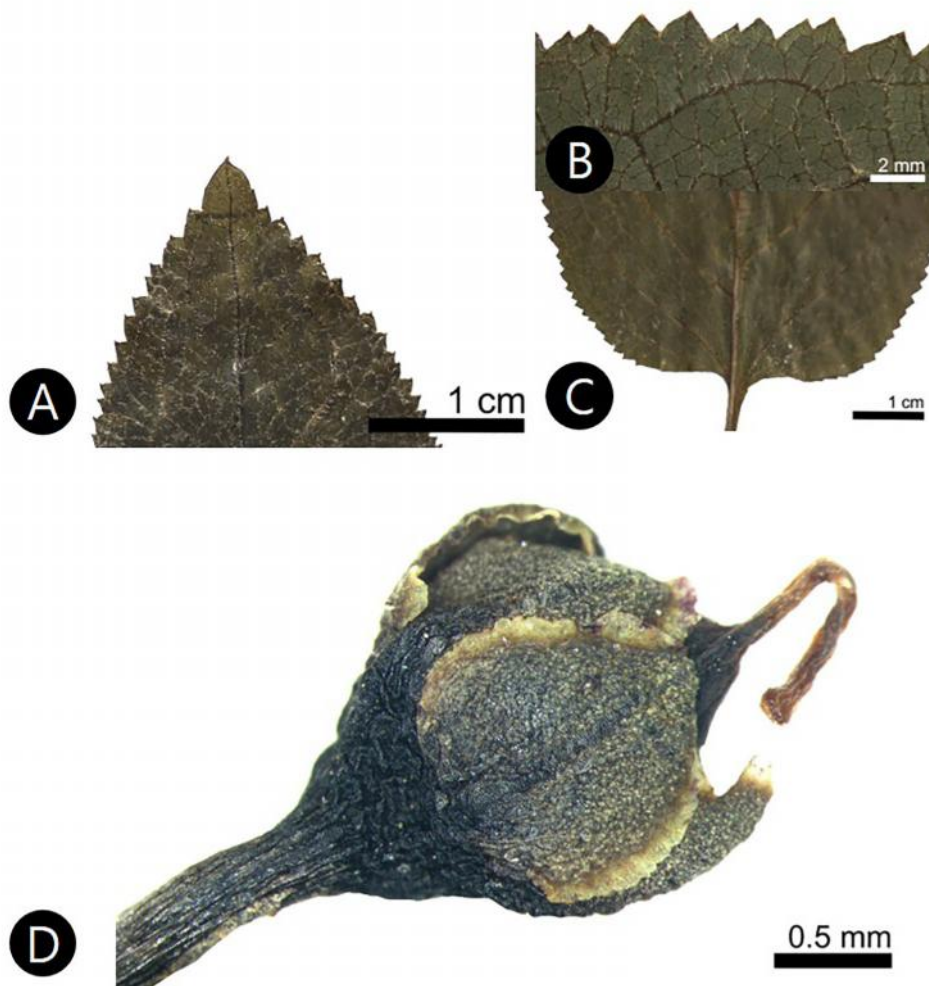
Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.phymed.2019.153159](https://doi.org/10.1016/j.phymed.2019.153159).

References

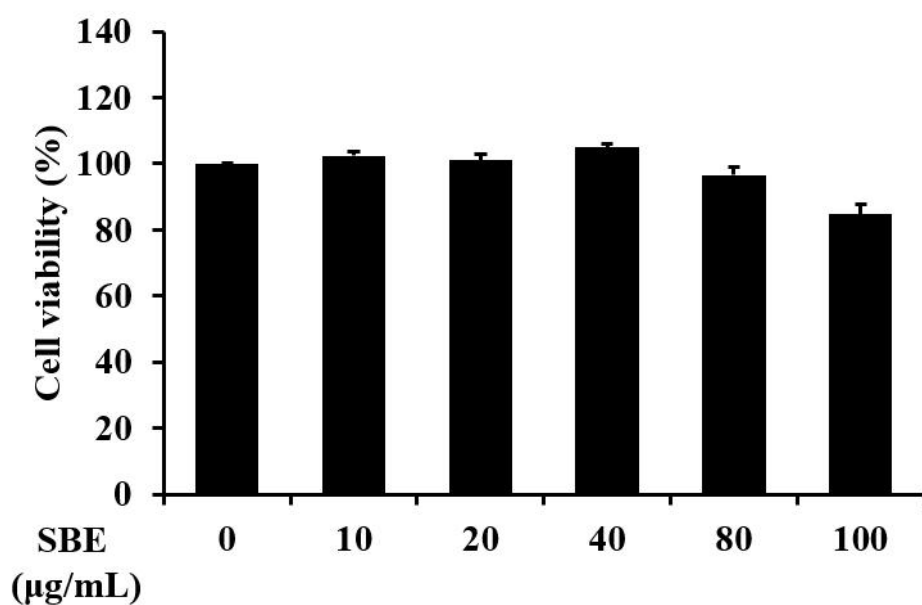
- Barnes, P.J., 2001. Th2 cytokines and asthma: an introduction. *Respir. Res.* 2, 64–65.
- Barnes, P.J., 2002. The role of inflammation and anti-inflammatory medication in asthma. *Respir. Med.* 96 (Suppl A), S9–15.
- Bond, M., Chase, A.J., Baker, A.H., Newby, A.C., 2001. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. *Cardiovasc. Res.* 50, 556–565.
- Cataldo, D.D., Tournoy, K.G., Vermaelen, K., Munaut, C., Foidart, J.M., Louis, R., Noël, A., Pauwels, R.A., 2002. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am. J. Pathol.* 161, 491–498.
- Chen, H., Xu, X., Teng, J., Cheng, S., Bunjhoo, H., Cao, Y., Liu, J., Xie, J., Wang, C., Xu, Y., Xiong, W., 2015. CXCR4 inhibitor attenuates allergen-induced lung inflammation by down-regulating MMP-9 and ERK1/2. *Int. J. Clin. Exp. Pathol.* 8, 6700–6707.
- Choi, H.K., 2007. *Scrophularia*. In: Park, C.W. (Ed.), *The Genera of Vascular Plants of Korea*. Academy Publishing Co., Seoul, Republic of Korea, pp. 864–865.
- Deo, S.S., Mistry, K.J., Kakade, A.M., Niphadkar, P.V., 2010. Role played by Th2 type cytokines in IgE mediated allergy and asthma. *Lung India* 27, 66–71.
- Hetzel, M., Walcher, D., Grüb, M., Bach, H., Hombach, V., Marx, N., 2003. Inhibition of MMP-9 expression by PPARgamma activators in human bronchial epithelial cells. *Thorax* 58, 778–783.
- Hong, D.Y., Yang, H.B., Jin, C.L., Fischer, M.A., Holmgren, N.H., Mill, R.R., 1998. *Scrophularia*. In: Wu, Z.Y., Raven, H. (Eds.), *Flora of China* 18. Science Press and Missouri Botanical Garden Press, Beijing and St. Louis, pp. 11–20.
- Ingram, J., Kraft, M., 2015. Metalloproteinases as modulators of allergic asthma: therapeutic perspectives. *Metalloproteinases Med.* 2015, 61–74.
- Jang, H.D., Oh, B.U., 2013. A taxonomic study of Korean *Scrophularia* L. (*Scrophulariaceae*) based on morphological characters. *Kor. J. Plant Res.* 26, 271–283.
- Jeong, E.J., Lee, K.Y., Kim, S.H., Sung, S.H., Kim, Y.C., 2008. Cognitive-enhancing and antioxidant activities of iridoid glycosides from *Scrophularia buergeriana* in scopamine-treated mice. *Eur. J. Pharmacol.* 588, 78–84.
- Kim, J.K., Kim, Y.H., Lee, H.H., Lim, S.S., Park, K.W., 2012. Effect of *Scrophularia buergeriana* extract on the degranulation of mast cells and ear swelling induced by dinitrofluorobenzene in mice. *Inflammation* 35, 183–191.
- Kim, S.R., Kang, S.Y., Lee, K.Y., Kim, S.H., Markelonis, G.J., Oh, T.H., Kim, Y.C., 2003a. Anti-amnesic activity of E-p-methoxycinnamic acid from *Scrophularia buergeriana*. *Brain Res. Cogn. Brain Res.* 17, 454–461.
- Kim, S.R., Koo, K.A., Sung, S.H., Ma, C.J., Yoon, J.S., Kim, Y.C., 2003b. Iridoids from *Scrophularia buergeriana* attenuate glutamate-induced neurotoxicity in rat cortical cultures. *J. Neurosci. Res.* 74, 948–955.
- Kim, S.R., Lee, K.Y., Koo, K.A., Sung, S.H., Lee, N.G., Kim, J., Kim, Y.C., 2002. Four new neuroprotective iridoid glycosides from *Scrophularia buergeriana* roots. *J. Nat. Prod.* 65, 1696–1699.
- Kim, S.J., Park, J.S., Myung, N.Y., Moon, P.D., Choi, I.Y., An, H.J., Kim, N.H., Na, H.J., Kim, D.H., Kim, M.C., An, N.H., Kim, I.K., Lee, J.Y., Jeong, H.J., Um, J.Y., Kim, H.M., Hong, S.H., 2009. *Scrophularia buergeriana* regulates cytokine production *in vitro*. *Immunopharmacol. Immunotoxicol.* 31, 246–252.
- Ko, J.W., Jeong, S.H., Kwon, H.J., Shin, N.R., Seo, Y.S., Kim, J.C., Shin, I.S., Kim, J.S., 2018. Preventive effect of garlic oil and its organosulfur component diallyl-disulfide on cigarette smoke-induced airway inflammation in mice. *Nutrients* 10, 1659.
- Liou, C.J., Cheng, P.Y., Huang, W.C., Chan, C.C., Chen, M.C., Kuo, M.L., Shen, J.J., 2014. Oral lovastatin attenuates airway inflammation and mucus secretion in ovalbumin-induced murine model of asthma. *Allergy Asthma Immunol. Res.* 6, 548–557.
- Lloyd, C.M., Hessel, E.M., 2010. Functions of T cells in asthma: more than just T(H)2 cells. *Nat. Rev. Immunol.* 10, 838–848.
- Ma, C., Zou, L., Xia, Y., Tu, Y., Xue, D., Yang, Y., Liu, D., Liu, Y., Wu, H., Dan, H., You, P., 2018. Extracts of *Coleus forskohlii* relieves cough and asthma symptoms via modulating inflammation and the extracellular matrix. *J. Cell. Biochem.* 120, 9648–9655.
- Manka, L.A., Wechsler, M.E., 2018. Selecting the right biologic for your patients with severe asthma. *Ann. Allergy Asthma Immunol.* 121, 406–413.
- McMillan, S.J., Kearley, J., Campbell, J.D., Zhu, X.W., Larbi, K.Y., Shipley, J.M., Senior, R.M., Nourshargh, S., Lloyd, C.M., 2004. Matrix metalloproteinase-9 deficiency results in enhanced allergen-induced airway inflammation. *J. Immunol.* 172, 2586–2594.
- Murdoch, J.R., Lloyd, C.M., 2010. Chronic inflammation and asthma. *Mutat. Res.* 690, 24–39.

- Pan, B., Zhou, Y., Kong, G., Li, P., Guo, Q., Guan, C., 2019. Aucubin alleviates lipopolysaccharide-induced acute lung injury in mice. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 44, 128–133.
- Rhee, J.W., Lee, K.W., Kim, D., Lee, Y., Jeon, O.H., Kwon, H.J., Kim, D.S., 2007. NF-kappaB-dependent regulation of matrix metalloproteinase-9 gene expression by lipopolysaccharide in a macrophage cell line RAW 264.7. *J. Biochem. Mol. Biol.* 40, 88–94.
- Richter, A., Puddicombe, S.M., Lordan, J.L., Bucchieri, F., Wilson, S.J., Djukanovic, R., Dent, G., Holgate, S.T., Davies, D.E., 2001. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am. J. Respir. Cell. Mol. Biol.* 25, 385–391.
- Schwingshackl, A., Duszyn, M., Brown, N., Moqbel, R., 1999. Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF-alpha. *J. Allergy Clin. Immunol.* 104, 983–989.
- Shen, Y., Huang, S., Kang, J., Lin, J., Lai, K., Sun, Y., Xiao, W., Yang, L., Yao, W., Cai, S., Huang, K., Wen, F., 2018. Management of airway mucus hypersecretion in chronic airway inflammatory disease: Chinese expert consensus (English edition). *Int. J. Chron. Obstruct. Pulmon. Dis.* 13, 399–407.
- Shin, Y., Yoon, S.H., Choe, E.Y., Cho, S.H., Woo, C.H., Rho, J.Y., Kim, J.H., 2007. PMA-induced up-regulation of MMP-9 is regulated by a PKCalpha-NF-kappaB cascade in human lung epithelial cells. *Exp. Mol. Med.* 39, 97–105.
- Shin, N.R., Ryu, H.W., Ko, J.W., Park, S.H., Yuk, H.J., Kim, H.J., Kim, J.C., Jeong, S.H., Shin, I.S., 2017. *Artemisia argyi* attenuates airway inflammation in ovalbumin-induced asthmatic animals. *J. Ethnopharmacol.* 209, 108–115.
- Shin, N.R., Shin, I.S., Jeon, C.M., Hong, J.M., Oh, S.R., Hahn, K.W., Ahn, K.S., 2014. Inhibitory effects of *Picrasma quassioides* (D.Don) Benn. on airway inflammation in a murine model of allergic asthma. *Mol. Med. Rep.* 10, 1495–1500.
- Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* 17, 463–516.
- Sung, Y.Y., Kim, S.H., Kim, D.S., Lee, J.E., Kim, H.K., 2017. *Illicium verum* extract and *trans*-Anethole attenuate ovalbumin-induced airway inflammation via enhancement of Foxp3⁺ regulatory T cells and inhibition of Th2 cytokines in mice. *Mediators Inflamm.* 2017, 7506808.
- Tak, P.P., Firestein, G.S., 2001. NF-kappaB: a key role in inflammatory diseases. *J. Clin. Invest.* 107, 7–11.
- Teng, Z., Guo, Z., Zhong, J., Cheng, C., Huang, Z., Wu, Y., Tang, S., Luo, C., Peng, X., Wu, H., Sun, X., Jiang, L., 2017. ApoE influences the blood-brain barrier through the NF-kB/MMP-9 pathway after traumatic brain injury. *Sci. Rep.* 7, 6649.
- Visse, R., Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* 92, 827–839.
- Wu, C.A., Peluso, J.J., Zhu, L., Lingenheld, E.G., Walker, S.T., Puddington, L., 2010. Bronchial epithelial cells produce IL-5: implications for local immune responses in the airways. *Cell. Immunol.* 264, 32–41.
- Yan, G.H., Choi, Y.H., 2014. *Phellinus linteus* extract exerts anti-asthmatic effects by suppressing NF-kB and p38 MAPK activity in an OVA-induced mouse model of asthma. *Immune Netw.* 14, 107–115.
- Yang, S.N., Hsieh, C.C., Kuo, H.F., Lee, M.S., Huang, M.Y., Kuo, C.H., Hung, C.H., 2014. The effects of environmental toxins on allergic inflammation. *Allergy Asthma Immunol. Res.* 6, 478–484.
- Zhang, Q., Lei, H.M., Wang, P.L., Ma, Z.Q., Zhang, Y., Wu, J.J., Nie, J., Chen, S.J., Han, W.J., Wang, Q., Chen, D.Y., Cai, C.K., Li, Q., 2017. Bioactive components from Qingwen Baidu Decoction against LPS-induced acute lung injury in rats. *Molecules* 22, 692.
- Zhu, J., 2015. T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. *Cytokine* 75, 14–24.
- Zhu, J., Dong, J., Ji, L., Jiang, P., Leung, T.F., Liu, D., Ng, L.G., Tsang, M.S., Jiao, D., Lam, C.W., Wong, C.K., 2018. Anti-allergic inflammatory activity of interleukin-37 is mediated by novel signaling cascades in human eosinophils. *Front. Immunol.* 9, 1445.

1 **Supplementary Figure**



2
3 **Supplementary Figure 1. The morphological characters of *Scrophularia buergeriana***
4 **Miq.** (A) leaf shape, (B) regularly double serrate margins, (C) obtuse to truncate base, (D)
5 ovate calyx lobes.



Supplementary Figure 2. Cell viability of SBE in RAW264.7 cells. Based on results of cell viability, 80 µg/mL of SBE was determined as a high concentration in *in vitro* experiment.