



Prunus serrulata var. *spontanea* inhibits mast cell activation and mast cell-mediated anaphylaxis



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ARTICLE INFO

Keywords:

Prunus serrulata
Active systemic anaphylaxis
Immunoglobulin E
Passive cutaneous anaphylaxis
Histamine
Mast cells

Ethnopharmacological relevance: A promising approach to treat a variety of diseases are considered as complementary and alternative herbal medicines. *Prunus serrulata* var. *spontanea* L. (Rosaceae) is used as herbal medicine to treat allergic diseases according to the Donguibogam, a tradition medical book of the Joseon Dynasty in Korea.

Aim of the study: We prepared the aqueous extract of the bark of *P. serrulata* (AEBPS) and aimed to investigate the effects in mouse anaphylaxis models and various types of mast cells, including RBL-2H3, primary cultured peritoneal and bone marrow-derived mast cells.

Materials and methods: We used ovalbumin (OVA)-induced active systemic anaphylaxis (ASA) and immunoglobulin (Ig) E-mediated passive cutaneous anaphylaxis (PCA) models, *in vivo*. The control drug dexamethasone (10 mg/kg) was used to compare the effectiveness of AEBPS (1–100 mg/kg). *In vitro*, IgE-stimulated mast cells were used to confirm the role of AEBPS (1–100 µg/mL). For statistical analyses, *p* values less than 0.05 were considered to be significant.

Results: In ASA model, oral administration of AEBPS suppressed the hypothermia and increased level of serum histamine in a dose-dependent manner. AEBPS attenuated the serum IgE, OVA-specific IgE, and interleukin (IL)-4. Oral administration of AEBPS also blocked mast cell-dependent PCA. AEBPS suppressed degranulation of mast cells by reducing intracellular calcium level in mast cells. AEBPS inhibited tumor necrosis factor-α and IL-4 expression and secretion in a concentration-dependent manner through the reduction of nuclear factor-κB.

Conclusions: On the basis of these findings, AEBPS could serve as a potential therapeutic target for the management of mast cell-mediated allergic inflammation and as a regulator of mast cell activation.

1. Introduction

Oriental medicine has been a history of extending over 2000 years, since its origin in China. *Prunus serrulata* var. *spontanea* L. (Rosaceae) is a large sized tree widely distributed throughout Korea and Japan. The

fruits of this tree are edible and are used in folk medicine against heart failure, dropsy, and mastitis, and as an emmenagogue. The traditional Korean medicine Pruni cortex, which is a mixture of *P. serrulata*, *P. yedoensis*, and *P. sargentii*, have been used for detoxification and relaxation, and as an antitussive (Jung et al., 2004). The distinctive

Abbreviations: DNP, dinitrophenyl; HSA, human serum albumin; RPMCs, rat peritoneal mast cells; TNF, tumor necrosis factor; BMMCs, Bone marrow-derived mast cells

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<https://doi.org/10.1016/j.jep.2019.112484>

Received 26 August 2019; Received in revised form 19 November 2019; Accepted 12 December 2019

Available online 13 December 2019

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medical system of traditional Korean medicine was established after the publication of Donguibogam, by Dr. Heo Jun in 1613 (Song et al., 2016). According to donguibogam, bark and stems of *P. serrulata* are representative anti-allergic herbal medicines to treat red rash (urticaria or hives) caused by food allergy (Heo, 1613). Previous studies have reported that many species of *Prunus* have anti-allergic potentials (Han et al., 2015; Shin et al., 2010).

In developed countries, about 10–20% of the population are suffered from allergic diseases such as allergic asthma, allergic rhinitis, and atopic dermatitis (J.H. Kim et al., 2015). Mast cells are long-lived tissue-resident cells and are key effectors of allergic immune responses. Mast cells are activated through cross-linking of high affinity receptors to immunoglobulin (Ig) E, FcεRI, by the IgE/antigen complex. Activated mast cells release a number of pre-formed and newly synthesized inflammatory mediators including histamine, leukotrienes, prostaglandins, cytokines, and chemokines, which induces allergic diseases (Biethahn et al., 2014). Among them, inflammatory cytokines have a beneficial effect on host defense, whereas reduction of these cytokines from mast cells is one of the main indicators of allergic inflammatory symptoms, since overexpression may cause pathological problems (Kim et al., 2013; Lee et al., 2017). Inflammatory cytokine is regulated by the nuclear factor (NF)-κB, an important transcription factor possessing a pivotal role in the regulation of immune and inflammatory responses (Bhatt and Ghosh, 2014; Liu et al., 2017). Thus, the initiation and perpetuation of allergic inflammation is important for the expression of NF-κB activation and subsequent inflammatory cytokines.

Established mast cell-dependent experimental models were used by many investigators to concentrate on finding the effective therapeutics for allergic inflammation (Kim et al., 2010). The rat basophilic leukemia mast cells (RBL-2H3) are generally used as an *in vitro* model of IgE-mediated allergic responses. In addition, primary cultured mast cells are an attractive means of studying general point of view of mast cell biology and factors of mast cell functions relevant to human disease. Rat peritoneal mast cells (RPMCs) and bone marrow-derived mast cells (BMMCs) are used as primary mast cells (Im et al., 2011; Kim et al., 2018; Meurer et al., 2016). In particular, BMMCs can be derived from multipotent progenitor cells that are matured, and have been used in many studies (Mollerherm et al., 2017).

Although traditional medicines have been the subject of increased interest due to its potential in the treatment of various diseases, the pharmacological mechanism of most herbal medicines has not been elucidated (Oh et al., 2012). The present study was designed to investigate the anti-allergic effect of the aqueous extract of the bark of *P. serrulata* (AEBPS) in active systemic and passive anaphylaxis *in vivo*, and mast cell activation *in vitro*.

2. Materials and Methods

2.1. Reagents

The reagents were obtained from the following suppliers: DMEM (12800–017), MEM-α (11900–016), and fetal bovine serum (FBS) (16000–044) (Gibco, Grand Island, NY, USA). Monoclonal anti-dinitrophenyl (DNP) IgE (D8406), DNP-human serum albumin (HSA) (A6661), ovalbumin (OVA) (A5503), dexamethasone (Dexa) (D4902), 4-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NAG) (N9376), Histodenz (D2158), calcium ionophore A23187 (C7522), and o-phthalaldehyde (P1378) (Sigma-Aldrich, St. Louis, MO, USA). Alum adjuvant (77161) (Thermo Scientific, Waltham, MA, USA). RPMI 1640 (SH30027.01) (Hyclone, Logan, UT, USA).

2.2. Cell culture

RBL-2H3, RPMCs and BMMCs were cultured in DMEM, α-MEN and RPMI 1640, respectively, which contained heat-inactivated 10% FBS with 100 units/mL antibiotics (Hyclone, SV30079.01) at 37 °C in a

humidified atmosphere of 5% CO₂. For BMMCs, complete RPMI 1640 media was supplemented by 4 mM L-glutamine (25030–081), 25 mM HEPES (15630–080), 50 μM 2-mercaptoethanol (21985–023), 1 mM sodium pyruvate (11360–070), MEM nonessential amino acid solution (11140–050) (Gibco), 10 ng/mL murine IL-3 (213-13-100), and 2 ng/mL murine stem cell factor (250-03-100) (PeproTech EC, London, UK).

2.3. Animals

Male Imprinting Control Region (ICR) mice (6-week-old) and male Sprague-Dawley (SD) rats (10-week-old) were obtained from the Dae-Han Experimental Animal Center (Daejeon, Korea). Animal rooms were maintained with temperature of 22 ± 1 °C, humidity of 55 ± 5%, a 12 h light/dark cycles and air exchanges at 15 times/h. Feed and water were supplied *ad libitum*. Animal experiments in our research were carried out in accordance with the guide for the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyungpook National University (IRB #2017-0002-9). All efforts were made during the animal experiments to minimize suffering.

2.4. Preparation of AEBPS and high performance liquid chromatography (HPLC) analysis

Bark of *P. serrulata* was purchased from the oriental drug store, Bohwa Dang, in Jeonju, Republic of Korea. A voucher specimen (number WSP-17-099) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The dried material (30 kg) was ground and extracted with purified water at 70 °C for 5 h (2 times) in water bath. Then, the extract was filtered, lyophilized and then kept at 4 °C. The yield of dried extract from starting crude materials was about 13.2%. Doses were determined based on previous study using *Prunus* species and maximum/minimum effects in our preliminary experiments (Kang et al., 2015).

To identify the individual components of the AEBPS, we used HPLC as previously reported (Kang et al., 2015; Yun et al., 2014). Analyses were performed using a reversed-phase HPLC system (Agilent model 1260 series) (Agilent Technologies, Waldbronn, Germany) with a Capcell Pak C18 column (5 μm × 4.6 mm × 250 mm; shiseido, Japan) and agilent 6120 single quadrupole. Chromatography was performed at room temperature at a flow rate of 1 mL/min, and 10 μL was analyzed for 120 min. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) in a ratio specified by the following binary gradient with linear interpolation: 0 min 20% B, 60 min 30% B, 70 min 60% B, 100 min 70% B, and 120 min 20% B.

2.5. Active systemic anaphylaxis (ASA)

On day 0 and 7, mice ($n = 5/\text{group}$, total $n = 30$) were sensitized with the OVA mixture (100 μg of OVA and 2 mg of alum adjuvant in 200 μL of phosphate-buffered saline [PBS]) by intraperitoneal injection (Kim et al., 2017). AEBPS (1–100 mg/kg) and Dexa (10 mg/kg) were administered orally on days 9, 11 and 13. On day 14, OVA (200 μg) was intraperitoneally injected and rectal temperature was measured every 10 min for up to 80 min. After 80 min, blood samples were obtained from the abdominal arteries of each mouse.

2.6. Passive cutaneous anaphylaxis (PCA)

An IgE-mediated PCA model was established as described in previous study (Kim et al., 2017). Five mice per each group were experimented in six groups (PBS only, DNP-HSA mixture only, DNP-HSA mixture and AEBPS at 1, 10, and 100 mg/kg, and Dexa at 10 mg/kg). For sensitization, anti-DNP IgE (0.5 μg) was injected into the mouse ear and incubated for 48 h. After 48 h, AEBPS was orally administered at doses of 1, 10 and 100 mg/kg. One hour later, intravenous injection of

1 µg of DNP-HSA containing 4% Evans blue (1:1) into the tail vein was administered to each mouse. After 30 min of challenge, the mice were euthanized and the ears removed for pigment area measurements. The amount of dye was measured with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 620 nm.

2.7. Preparation of RPMCs

Peritoneal mast cells were isolated from SD rats as previously described (Kim et al., 2018). First, rats were euthanized with CO₂ and 50 mL of Tyrode's buffer (137 mM NaCl, 5.6 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄ and 0.1% gelatin) was injected intraperitoneally. The center of the abdominal cavity was carefully opened and a liquid containing peritoneal cells were collected using a Pasteur pipette. The harvested cells were centrifuged at 150 g for 10 min at room temperature and resuspended in 1 mL of Tyrode's buffer. The suspension was placed on top of 2 mL of 0.235 g/mL Histodenz solution and centrifuged at 400 g for 15 min at room temperature to separate mast cells from other peritoneal cells, namely macrophages and small lymphocytes. The cells in the pellet were washed once. Toluidine blue staining determined approximately 95% purity for mast cell preparation, and trypan blue staining showed that more than 97% of cells were viable.

2.8. Preparation of mouse BMMCs

BMMCs were isolated from male ICR mice as described in previous study (Kim et al., 2018). The femurs of the ICR mouse were obtained by removing the skin from the hips to the ankle and then carefully removing the muscles without scraping the bones with scissors. After that, the long bones of the second leg were prepared in the same way. To prevent contamination of the bone marrow, all further steps in the cell isolation protocol were performed under a sterile cell culture hood. The femur and tibia, from which the muscles were removed, were washed with PBS. Therefore, the end of each bone was successively removed with scissors to open the bone marrow cavity, followed by flushing the bone marrow with 1 mL culture medium and cells were collected in a Petri dish (Sarstedt, Nümbrecht, Germany). The cell suspension is transferred into T flask.

2.9. Cell viability

Cell viability was determined by colorimetric analysis using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Je et al., 2015a,b). RBL-2H3 (6×10^4 cells/well in a 96-well plate) were treated with AEBPS for 12 h, followed by incubation with MTT reagent for 2 h. The formed formazan crystals were dissolved in dimethyl sulfoxide and the absorbance was read at 570 nm using a spectrophotometer (Molecular Devices).

2.10. Histamine and β -hexosaminidase release assay

As a marker of degranulation, we measured the release of β -hexosaminidase and histamine (Dhakal et al., 2019). RBL-2H3 and BMMCs were inoculated into 12-well plates (5×10^5 cells/well, 500 µL/well) and cultured for 1 h. Then, monoclonal mouse anti-DNP-IgE was added to cultured medium at a concentration of 50 ng/mL and incubated for 12 h. The cells were washed three times with PBS, treated with AEBPS for 1 h, and then stimulated with DNP-HSA (100 ng/mL) for 4 h or 30 min in RBL-2H3 and BMMC, respectively. After incubation, the supernatant was separated by centrifugation at 150g for 5 min at 4 °C. The cells were disrupted with 0.5% Triton X-100. Serum was collected after centrifugation of blood samples at 400 g for 15 min at 4 °C.

To measure histamine release, 0.1 N HCl and 60% perchloric acid were added to the sample, followed by first centrifugation. The supernatant was transferred to a 1.5 mL tube, mixed with 5 M NaCl, 5 N

NaOH and *n*-butanol, mixed well and centrifuged for a second time. Again, the supernatant was mixed with 0.1 N HCl and *n*-heptane and then centrifuged a third time. Isolated histamine was measured using the *o*-phthalaldehyde spectrofluorometric procedure as previously described (Je et al., 2015a,b). Fluorescence intensity (excitation wavelength of 380 nm and emission wavelength of 440 nm) was detected using a fluorescent plate reader (Molecular Devices).

To measure β -hexosaminidase release, 40 µL of the supernatant was transferred to a 96-well plate and incubated at 37 °C for 1 h with 40 µL of substrate buffer (pH 4.5) containing 0.1 M sodium citrate and 1 mM 4-nitrophenyl-N-acetyl- β -D-glucosaminide. Cells attached to the bottom of the plate were dissolved in 0.5% Triton X-100. Total β -hexosaminidase activity was measured in the supernatants using a spectrophotometer (Molecular Devices, absorbance 405 nm). The percentage of β -hexosaminidase release was calculated from the equation: [β -hexosaminidase release (%) = (absorbance of supernatant) / (absorbance of supernatant + absorbance of pellet) $\times$ 100].

2.11. Intracellular calcium

Intracellular calcium was measured using the fluorescent indicator Fluo-3/AM (Invitrogen, Carlsbad, CA) (D.H. Kim et al., 2014). RBL-2H3 (6×10^4 cells/well in 96-well plates) were sensitized by overnight exposure to anti-DNP IgE (50 ng/mL). The cells were then incubated with Fluo-3/AM for 1 h at 37 °C and washed with Tyrode's buffer (137 mM NaCl, 5.5 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.2 mM NaH₂PO₄, 1 mM MgCl₂, and 1.8 mM CaCl₂) to remove the dye from the cell surface. RBL-2H3 were then pretreated with or without AEBPS for 1 h prior to antigen challenge with DNP-HSA (100 ng/mL). BAPTA-AM, a calcium chelator, was used as a positive control. Fluorescent intensity was detected using a fluorescent plate reader (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 510 nm. The intracellular calcium level in untreated control cells was assigned a relative absorbance value of 1.

2.12. Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were measured by ELISA. RBL-2H3 (5×10^5 cells/well in a 12-well plate) were sensitized with overnight exposure to anti-DNP IgE (50 ng/mL). The cells were pretreated with AEBPS for 1 h prior to a challenge with DNP-HSA (100 ng/mL) for 6 h. The ELISA was performed on a 96-well Nunc Immuno Plate using specific kits (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. After the substrate reaction was terminated, the absorbance was measured using a spectrophotometer (Molecular Devices) at a wavelength of 450 nm.

2.13. qPCR

After stimulation with DNP-HSA with or without AEBPS, RBL-2H3 were seeded at a density of 5×10^5 cells/well in a 12-well plate. Total cellular RNA was isolated using a RNAiso Plus kit (TakaraBio, Shiga, Japan) according to the manufacturer's protocol. The first strand complementary DNA (cDNA) was synthesized using a PCR kit (Thermo Scientific). The primer sets were chosen by the Primer3 program (Whitehead Institute, Cambridge, MA, USA). The cycle number was optimized to ensure product accumulation was in the exponential range. qPCR was carried out using a Thermal Cycler Dice TP850 (TakaraBio) according to the manufacturer's protocol. Briefly, 2 µL of cDNA (100 ng), 1 µL of sense and antisense primer solution (0.4 µM), 12.5 µL of SYBR Premix Ex Taq (TakaraBio), and 9.5 µL of were mixed together to obtain a final volume of 25 µL in each reaction tube. Relative quantification of mRNA expression was performed using the TP850 software. The primer sequences used were as follows: TNF- α (F: 5'-TCC CAA ATG GGC TCC CTC TC-3', R: 5'-AAA TGG CAA ACC GGC TGA CG-3'), IL-4 (F: 5'-TGC ACC GAG ATG TTT GTA CCA GA-3', R: 5'-

TTG CGA AGC ACC CTG GAA G-3'), and β -actin (F: 5'-GAA GCT GTG CTA TGT TGC CCT AGA-3', R: 5'-GTA CTC CTG CTT GCT GAT CCA CAT-3'). The conditions used for PCR were previously described (Kim et al., 2017).

2.14. Western blot

Nuclear and cytosolic proteins were extracted as previously described (Choi et al., 2016). Anti-DNP IgE (50 ng/mL)-sensitized RBL-2H3 (1.5×10^6 cells/well in a 6-well plate) were washed three times with PBS, treated with AEBPS for 1 h, and then stimulated with DNP-HSA (100 ng/mL) for 1 h ($\text{IkB}\alpha$ and NF- κ B). The cells were washed with PBS, resuspended in 100 μ L of cell lysis buffer A (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA/ Na_3VO_4 , 0.5 mM PMSF/DTT, and 5 μ g/mL leupeptin/aprotinin), vortexed, incubated for 5 min on ice, and centrifuged at 400 g for 5 min at 4 °C. The supernatant was collected and used as the cytosolic protein extract. The pellets were washed three times with 1 mL of PBS, suspended in 25 μ L of cell lysis buffer B (25% glycerol, 420 mM NaCl, 20 mM HEPES, 1.2 mM MgCl_2 , 0.2 mM EDTA, 1 mM Na_3VO_4 , 0.5 mM PMSF/DTT, and 5 μ g/mL leupeptin/aprotinin), vortexed, sonicated for 30 s, incubated for 20 min on ice, and centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was collected and used as the nuclear protein extract. Equal amounts of cellular protein were electrophoresed using a 7.5–10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with a primary antibody against the target and then with anti-IgG horseradish peroxidase-conjugated secondary antibody. The following antibodies were purchased from Santa Cruz Biotechnology: NF- κ B (sc-109, rabbit polyclonal, 1:1000), $\text{IkB}\alpha$ (sc-371, rabbit polyclonal, 1:1000), β -actin (sc-8432, mouse monoclonal, 1:1000), and lamin B1 (sc-6217, goat polyclonal, 1:1000). Immunodetection was carried out using a chemiluminescent substrate (Thermo Scientific).

2.15. Statistical analysis

Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) using one-way analysis of variance with a Turkey's post-test, *p* values less than 0.05 were considered to be significant.

3. Results

3.1. Effects of AEBPS on systemic and local anaphylaxis

The systemic anaphylaxis model is widely used model to examine immediate-type hypersensitivity, which is strongly associated with mast cells (Reber et al., 2017). After intraperitoneal injection of OVA, mice were observed to have symptoms of decreased rectal temperature from 20 to 60 min. After 80 min, the mice were euthanized and their serum histamine levels were significantly increased. Oral administration of AEBPS reduced observed decrease in rectal temperature and increased serum histamine levels (Fig. 1A–C). Additionally, increased total serum IgE, OVA specific IgE and IL-4 levels after OVA inoculation was reduced by AEBPS (Fig. 1D–F). Dexa was used as a positive control drug.

To investigate the effect of AEBPS on IgE mediated allergic responses *in vivo*, we used the PCA model as an *in vivo* model of local allergic reaction (Kim et al., 2017). To sensitize the mouse ear, anti-DNP-IgE was injected intradermally. After 48 h, AEBPS (1, 10, and 100 mg/kg) or Dexa (100 mg/kg) was orally administrated. The oral administration of AEBPS significantly reduced the PCA reaction based on Evans blue extravasation (Fig. 2).

3.2. Effects of AEBPS on degranulation of mast cells

For *in vitro* assay, first, the cytotoxicity of AEBPS was tested by the MTT assay. RBL-2H3 were treated with various concentrations (0.1–1000 μ g/mL) of AEBPS for 12 h. As results, up to 1000 μ g/mL of AEBPS did not induce cytotoxicity (Fig. 3A). Within a concentration without cytotoxicity, we evaluated the release of histamine and β -hexosaminidase to confirm the effect of AEBPS on the degranulation of mast cells. DNP-HSA-stimulated mast cells (RBL-2H3, RPMCs and BMMCs) released high levels of histamine and β -hexosaminidase, which was reduced by AEBPS treatment in a concentration-dependent manner (Fig. 3B–F).

Calcium is an important secondary messenger in mast cell signaling (Alsaleh et al., 2016). To investigate the mechanism by which AEBPS causes a decrease in mast cell degranulation, we assayed the intracellular calcium. Fluorescence indicator Fluo-3/AM was used to examine the inhibition of calcium influx by AEBPS. The treatment of RBL-2H3 with AEBPS (1–100 μ g/mL) decreased intracellular calcium levels in a concentration-dependent manner (Fig. 3G).

3.3. Effects of AEBPS on inflammatory cytokine expression and NF- κ B activation in mast cells

To test the effect of AEBPS on the expression of proinflammatory cytokines such as TNF- α and IL-4, we stimulated RBL-2H3 with DNP-HSA for 1 h resulted in a high level of all cytokines. The high levels of cytokine gene expression and secretion were inhibited by AEBPS in a concentration-dependent manner (Fig. 4A and B). In addition, NF- κ B was evaluated to determine the inhibitory mechanism of proinflammatory cytokines. In our previous experiment, after 1 h of DNP-HSA challenge, degradation of $\text{IkB}\alpha$ and nuclear translocation of NF- κ B were observed. In our results, these effects were significantly inhibited by AEBPS (Fig. 4C).

4. Discussion

Mast cells are multifunctional immune cells implicated in the pathogenesis of inflammatory diseases, specifically allergy, because a prominent contributor to allergic disease, histamine, is stored mainly in mast cells (Frydas et al., 2013; White, 1990). In general, histamine-induced wheal and flare response in tissue is treated by antihistamines. Mechanism of antihistamines is blocking the histamine binding to receptors or reducing the histamine receptor activity on neurons, vascular smooth muscle, smooth muscle cells, endothelial cells and mast cells. Thus, antihistamine that act on histamine receptors inhibits itching, sneezing, and inflammatory responses (Canonica and Blaiss, 2011; Monroe et al., 1997). Although antihistamines have been used to treat allergic diseases, none of these drugs is completely free of side effect (Aaronson, 1998; Kuna et al., 2016). Therefore, development of new therapeutic drug candidates is important for human health. For centuries, traditional herbal medicine has been used effectively based on traditional knowledge. However, pharmacological mechanisms for these herbal have yet to be elucidated (S.J. Kim et al., 2015).

Anaphylaxis, which can cause allergic reactions to asphyxiation, is dangerous and acute. When external allergens such as insect poison, food and pollen infiltrate the body of an allergic patient, systemic or local anaphylaxis may occur (Simons and Organization, 2010). Mast cells contribute to anaphylaxis by the release of inflammatory mediators and cytokines (Theoharides et al., 2012). To confirm the anti-allergic effect of AEBPS in animal models, we utilized OVA-induced ASA and IgE-mediated PCA model. Both ASA and PCA models are well-known models for evaluating the antiallergic effects of drug candidates based on systemic and local anaphylaxis (Kim et al., 2018). OVA challenges induce hypothermia and vasodilation by the increase of serum histamine levels, which is associated with the severity of the anaphylaxis (Hogan et al., 2012; Ishikawa et al., 2010). In PCA, IgE

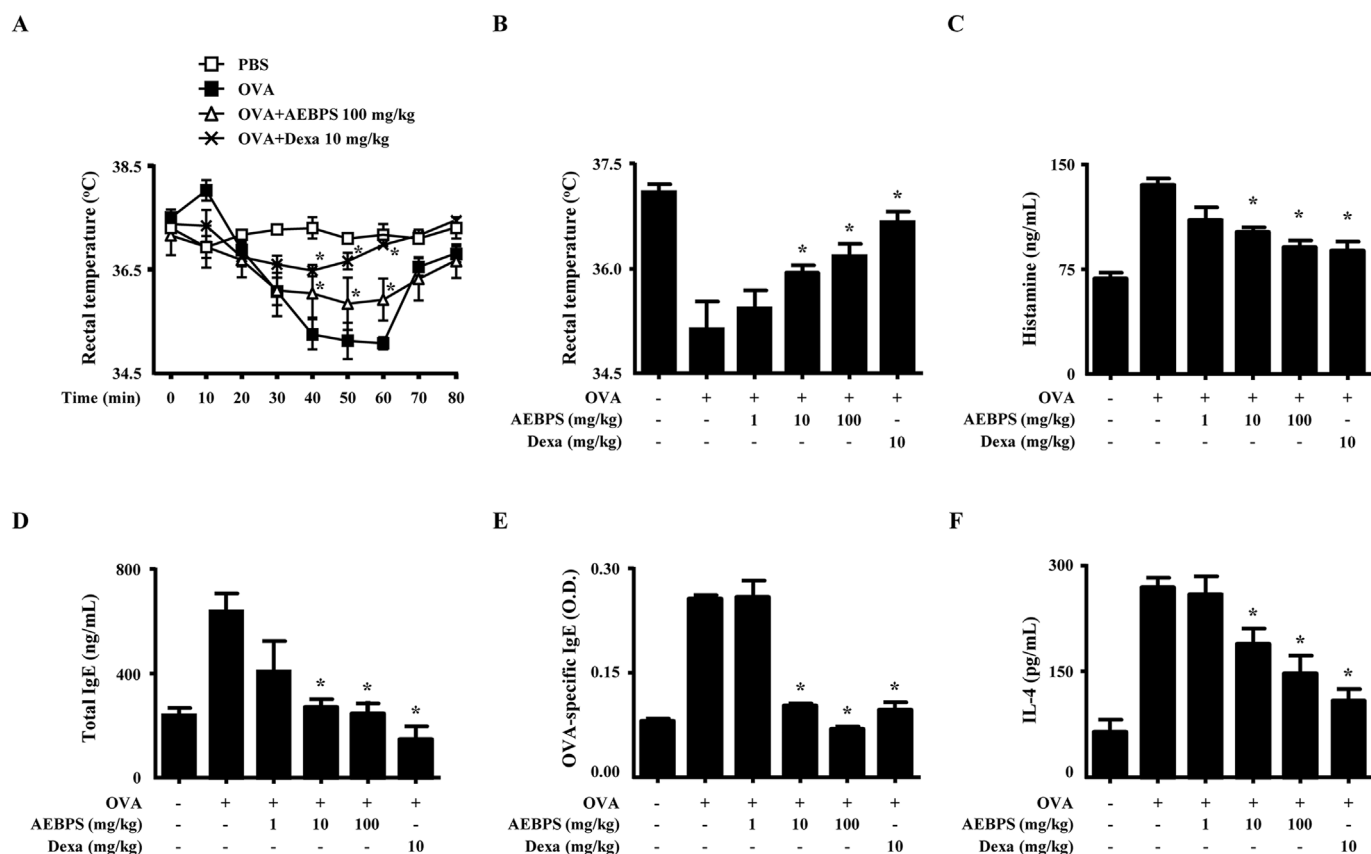


Fig. 1. Effects of AEBPS on OVA-induced active systemic anaphylaxis. (A) Rectal temperature was measured every 10 min for 80 min. (B) Rectal temperature of the mice at 50 min. (C–F) Serum levels of histamine, total IgE, OVA-specific IgE, and IL-4 represent the mean $\pm$ SEM ($n = 5$ /group) of two independent experiments. * $p < 0.05$ compared with the OVA-challenged group. Dexa: dexamethasone.

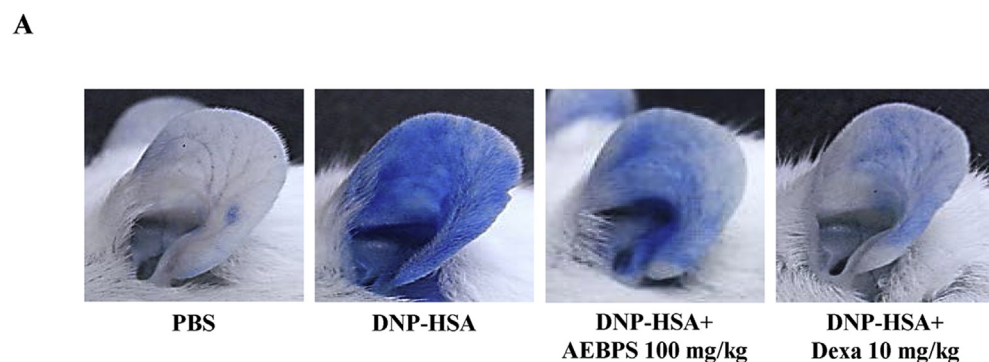
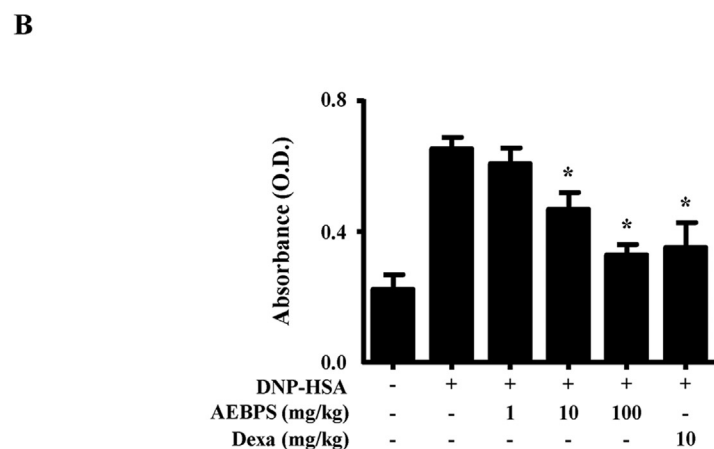


Fig. 2. Effects of AEBPS on IgE-mediated passive cutaneous anaphylaxis. (A) Representative photographic images of ears. (B) Each amount of the dye was extracted as described in the Materials and methods section and detected using a spectrophotometer. Graph data represent the mean $\pm$ SEM ($n = 5$ /group) of two independent experiments. * $p < 0.05$ compared with the OVA-challenged group. Dexa: dexamethasone.



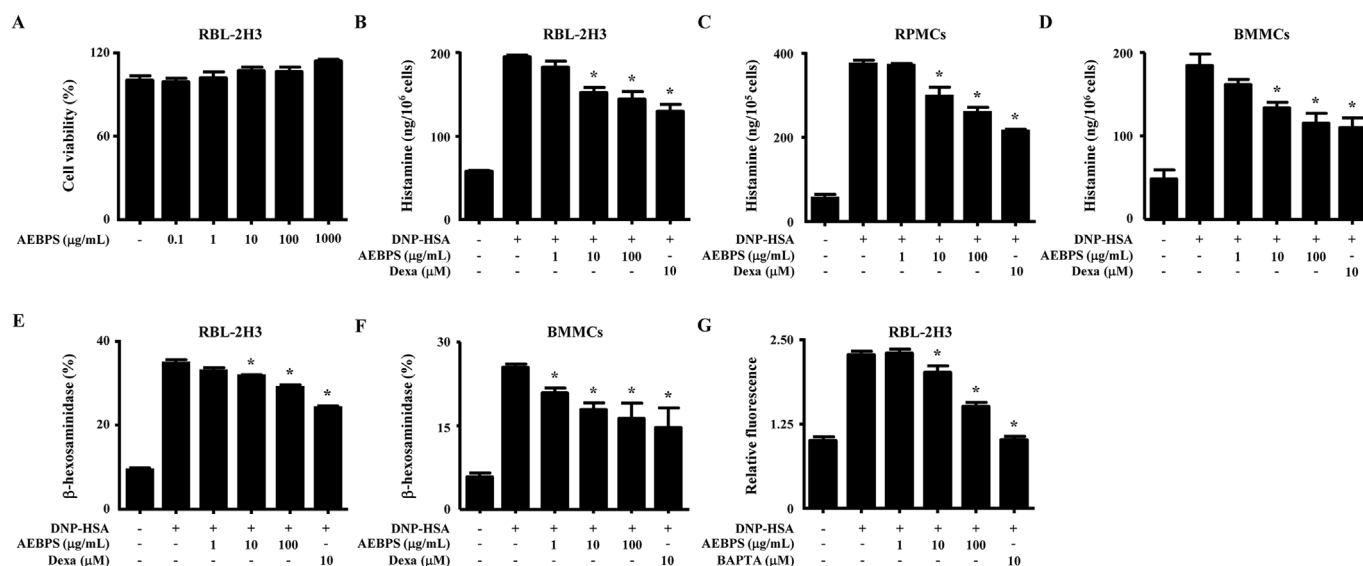


Fig. 3. Effects of AEBPS on mast cell degranulation and intracellular calcium. (A) RBL-2H3 (6×10^4 /well) were pretreated with or without AEBPS for 12 h and then incubated with 1 mg/mL MTT for 2 h. The absorbance intensity was measured using a spectrophotometer. (B–F) For mast cell degranulation, anti-DNP IgE (50 ng/mL)-sensitized RBL-2H3 (6×10^4 /well), RPMCs (2×10^4 /well), and BMMCs (5×10^5 /well) were pretreated with or without drugs, including AEBPS and Dexa, for 1 h or 30 min and then challenged with DNP-HSA (100 ng/mL). Histamine and β -hexosaminidase levels were detected using a fluorescent plate reader or a spectrophotometer, respectively. (G) After overnight anti-DNP IgE incubation, RBL-2H3 (5×10^5 /well) were incubated with Fluo-3/AM for 1 h, treated with or without AEBPS for 1 h, and then challenged with DNP-HSA. Intracellular calcium was detected using a fluorescent plate reader. BAPTA-AM, a calcium chelator, was used as a positive control. Graph data represent the mean $\pm$ SEM of three independent experiments. * $p < 0.05$ compared with the DNP-HSA-challenged group. Dexa: dexamethasone.

sensitization and antigen challenge lead to histamine release from mast cells and consequently produce local effects, such as increased vascular permeability, which results in tissue swelling and plasma extravasation. Evans blue dye binds to the extravasated plasma albumin and quantify the increased permeability (Galli and Tsai, 2012). Results in the both *in vivo* models demonstrated that the mast cell-mediated anaphylactic responses were inhibited by AEBPS treatment. In addition, these inhibitory effects of AEBPS on mast cell-involved anaphylaxis were supported by the reduction of mast cell degranulation in RBL-2H3 and primary cultured mast cells (RPMCs and BMMCs).

Histamine and β -hexosaminidase are widely known as markers for mast cell degranulation. Histamine is a biogenic amine synthesized from L-histidine by histidine decarboxylase and stored mainly within granules in mast cells (Carlos et al., 2006). Most of β -hexosaminidase is localized in the granules rather than in the lysosome of mast cells (Fukuishi et al., 2014). Intravenous administration of histamine can reproduce symptoms of anaphylaxis, including temporary hemodynamic changes, such as skin flushing, headache, airway obstruction, and transient hypotension. Thus, histamine has long been regarded as an important mediator of anaphylaxis (Kalinin et al., 1981; Reber et al., 2017; Vigorito et al., 1983). In this study, we focused on histamine release from mast cells. AEBPS showed the ability to reduce both histamine and β -hexosaminidase release in RBL-2H3 and primary cultured mast cells. Inhibition of histamine release and the association of intracellular calcium levels have been known in previous study (Borriello et al., 2017). After antigen-IgE binds to Fc ϵ RI, the calcium stored in the ER is released into the cytoplasm through binding of the inositol 1,4,5-trisphosphate (IP₃) receptor on the ER surface with IP₃. When calcium is depleted in the ER, extracellular calcium is incoming and the intracellular calcium levels are risen sharply (Kalesnikoff and Galli, 2008). As mentioned above, increasing intracellular calcium level is responsible for the mast cell degranulation. In our experiment, increased intracellular calcium level was attenuated by AEBPS. Therefore, the suppressive effect of AEBPS on mast cell degranulation might be due to reduction of intracellular calcium level.

The activation of mast cells also leads to generation of inflammatory

cytokines such as TNF- α and IL-4 (Galli et al., 2008; Vo et al., 2014). Mast cells store TNF- α and release rapidly when activated (Benyon et al., 1991; Walsh et al., 1991). In models of immune complex-induced and cutaneous inflammation, mast cell-derived TNF- α is important for the induction and promotion of initial inflammatory events (von Stebut et al., 2003; Zhang et al., 1992). Since IL-4 is well known to play an important role in the pathogenesis of allergic diseases such as induction of IgE synthesis and development of mast cells, regulation of IL-4 expression in mast cells may be a useful therapeutic strategy for allergic inflammatory diseases (Nabeshima et al., 2005). Likewise, the treatment of AEBPS suppressed TNF- α and IL-4 generation in antigen-activated mast cells. The expression of cytokines is regulated by several intracellular signaling pathways, especially NF- κ B, transcription factor that plays a central role in the induction of gene expression, and is considered as a promising target for the treatment of diseases (Hayden and Ghosh, 2004). After stimulation, the I κ B protein is phosphorylated and degraded, allowing NF- κ B nuclear translocation. Since NF- κ B in the nucleus can bind specific DNA sequences located in the promoter region of the target gene and activate gene transcription, it can play a pivotal role in regulating inflammatory responses and control the transcription of inflammatory cytokine genes. With this mechanism, inhibition of NF- κ B activation has been proposed as an anti-inflammatory strategy in allergy (Chen et al., 2001). Therefore, we attempted to determine whether the anti-inflammatory effect of AEBPS is through the regulation of NF- κ B activation. The results demonstrated that AEBPS inhibited the NF- κ B translocation into nucleus in stimulated mast cells. These findings suggest that AEBPS attenuates NF- κ B dependent gene expression through the inhibition of NF- κ B nuclear translocation.

The active component of AEBPS responsible for the suppression of allergic symptoms and the molecular targets of this effect are not elucidated yet. Species of *Prunus* been investigated to have compounds such as, tectochrysin, genistein, leucocyanadin, genkwanin, prunetin, and sakuranetin (Jangwan and Kumar, 2015; Wang et al., 1999; Yun et al., 2014). In this study, the HPLC profile of AEBPS indicated that genistein, prunetin, and sakuranetin are the components of AEBPS (Fig. S1). Previous studies showed that these components possess anti-

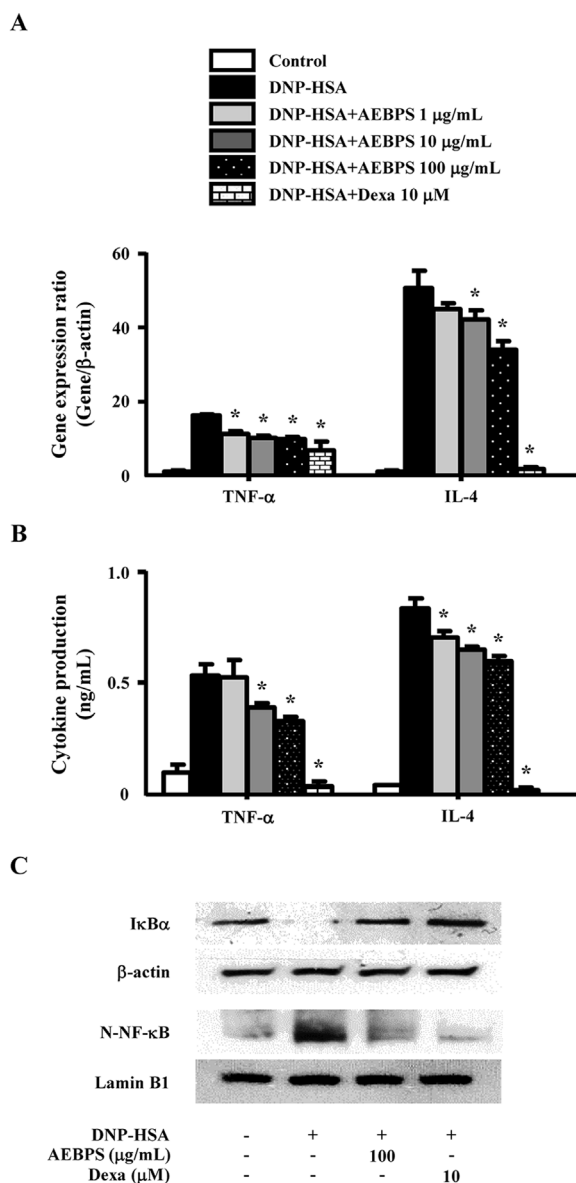


Fig. 4. Effects of AEBPS on expression and secretion of inflammatory cytokines and the activation of NF-κB. Anti-DNP IgE-sensitized RBL-2H3 (5×10^5 /well) were pretreated with or without drugs, including AEBPS and Dexa, for 1 h, and then challenged with DNP-HSA (100 ng/mL). (A) The gene expression of inflammatory cytokines was determined by qPCR. (B) The secretion of inflammatory cytokines was measured by ELISA. Graph data represent the mean $\pm$ SEM of three independent experiments. (C) NF-κB activation was assayed by Western blot (N: nuclear). β-actin and lamin B1 were used as loading controls. The band is a representative of three independent experiments. * $p < 0.05$ compared with the DNP-HSA-challenged group. Dexa: dexamethasone.

allergic properties (H.H. Kim et al., 2014; Ogawa et al., 2007; Ryu et al., 2013). Therefore, we assumed that these components might be responsible for the inhibitory effects of AEBPS on allergic responses. However, a more detailed study should be implemented.

5. Conclusion

The inhibitory actions of AEBPS on mast cell-mediated allergic responses via suppression of degranulation and cytokine generation might explain, at least in medicinal part, the traditional use of species of *Prunus*, as an anti-allergic agent. Therefore, we propose that AEBPS may

be a therapeutic candidate for mast cell activation and mast cell-mediated anaphylaxis.

Contributions

Kim M.J. and Choi Y.A. performed the experiments. Lee S.Y., Choi J.K., Kim Y.Y. performed the statistical analysis. Kim E.N. and Jeong G.S. performed HPLC and additional experiments. Shin T.Y. designed the study. Jang Y.H. and Kim S.H. participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgment

This work was supported by a National Research Foundation of Korea Grant funded by the Korean Government (2018R1D1A3A03000686, 2019M3A9H1103690, 2019R1C1C1005172 and 2017R1D1A1B03031032) and by a grant from the Korea Health Technology R&D Project of the Korea Health Industry Development Institute (KHIDI) and by the Ministry of Health & Welfare, Republic of Korea (grant number: HI18C0308).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2019.112484>.

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***Prunus serrulata* var. *spontanea* inhibits mast cell activation and mast cell-mediated anaphylaxis**

Supplementary Figures

Figure S1. HPLC profiling of aqueous extract of the bark of *P. serrulata*. HPLC analysis was performed on Capcell Pak C18 column (5 μ m $\times$ 4.6 mm $\times$ 250 mm). The peaks were confirmed by the addition of standard for naringenin, genistein, and sakuranetin.

