

Effect of *Scrophularia buergeriana* Extract on the Degranulation of Mast Cells and Ear Swelling Induced by Dinitrofluorobenzene in Mice

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Abstract—*Scrophularia buergeriana* Miquel (Scrophulariaceae, SB) is a biennial plant native to Korea, northern China, and Japan that plays an important role in traditional medicine. The dried root of SB has long been used in oriental medicine for treatment of fever, swelling, constipation, pharyngitis, neuritis, and laryngitis. In the present study, we evaluated the ethanol extract of SB (SBE) to determine if it exerted any anti-allergic effects that had not previously been demonstrated. SBE markedly inhibited β -hexosaminidase and histamine release and suppressed the expression of tumor necrosis factor- α and interleukin-4 cytokines by RBL-2H3 mast cells. In addition, topical treatment with SBE effectively reduced allergic inflammation in a dinitrofluorobenzene-induced contact hypersensitivity mouse model. These results strongly suggest that SBE is a promising source of anti-allergic agents.

KEY WORDS: *Scrophularia buergeriana*; allergy; inflammation; RBL-2H3 cell.

INTRODUCTION

Scrophularia buergeriana Miquel (Scrophulariaceae, SB) is a strong-scented perennial or biennial plant native to Korea, northern China, and Japan that plays an important role in traditional medicine. The dried root of SB has long been used in oriental medicine for the treatment of fever, swelling, constipation, pharyngitis, neuritis, and laryngitis

[1, 2]. A number of iridoid glycosides, phenylpropanoids, terpenoids, and flavonoids have been isolated from *Scrophularia* spp. [3–5]. In addition, recent studies have shown that SB extracts and several compounds isolated from SB provide immune-enhancement effects [6], antioxidant effects [7], neuroprotective effects [7–9], as well as hepatoprotective effects [10]. However, the effect of SB on allergic reaction has not been investigated to date.

Allergic reactions can be classified into four types [11]. Among these, there has recently been an increased incidence of type I allergic disorders in response to food- and air-borne allergens worldwide [12]. This type of allergy is recognized as an IgE-mediated immediate hypersensitive reaction against environmental allergens and is believed to be associated with an elevated level of IgE antibodies produced against allergens [13]. Mast cells/basophils are commonly found at sites of contact with the outside environment and play pivotal roles in immediate-type allergic reactions and allergic inflammation. Degranulation of mast cells/basophils causes the secretion of biologically active substances including histamine, eicosanoids, proteolytic enzymes, cytokines, and chemokines. Cytokines released following degranu-

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lation, such as tumor necrosis factor- α (TNF- α), interleukin-4 (IL-4), IL-6, and IL-8, induce late-phase allergic reactions and allergic inflammation via the recruitment of immune cells to the inflamed site [14]. It is well known that the degranulation of mast cells/basophils is induced by multivalent antigen-IgE cross-linking and the aggregation of high affinity IgE receptor I (Fc ϵ RI) [15]. The aggregation of high affinity Fc ϵ RI triggers intracellular signaling pathways, such as the phosphorylation of various protein kinases including spleen tyrosine kinase (Syk), Grb2-associated binder-2 (Gab2), transmembrane adapter molecule linkers for activated T cells (LAT), as well as mitogen-activated protein kinase (MAPK) [16]. Based on this background, an assay for degranulation of mast cells/basophils has been used for screening of anti-allergic agents including phytochemicals.

In the present study, we evaluated the anti-allergic effects of ethanol extracts of SB (SBE) by using an *in vitro* culture of RBL-2H3 cells, a rat basophilic leukemia cell line. RBL-2H3 cells possess phenotypic characteristics of mucosal mast cells, are a tumor analog of mast cells, and are widely used in mast cell associated studies. Following stimulation with antigens, these cells release β -hexosaminidase and histamine, which are markers of mast cell degranulation [17]. To demonstrate the effects of SBE on antigen-induced activation of mast cells, the antigen-induced release of β -hexosaminidase, histamine, and cytokine production were examined. To further correlate these changes to the IgE receptor-induced signaling pathway, phosphorylation of regulatory proteins was also investigated. Furthermore, *in vivo* anti-allergic activity was evaluated based on the dinitrofluorobenzene (DNFB)-induced contact hypersensitivity reaction with mice.

MATERIALS AND METHODS

Chemicals and Reagents

Anti-dinitrophenyl (DNP)-IgE, 1-fluoro-2,4-dinitrofluorobenzene, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St Louis, MO, USA). DNP-bovine serum albumin (DNP-BSA) was obtained from Calbiochem (San Diego, CA, USA). Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), and penicillin and streptomycin mixture solution were obtained from Hyclon (Logan, UT, USA). The mono-

clonal antibody and polyclonal antibody for ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-JNK (Thr183/Tyr185), p38, and phospho-p38 (Thr180/Tyr182) were obtained from Cell Signaling Technology (Beverly, MA, USA). The ECL advance western blotting system was obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). TNF- α and IL-4 ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA).

Preparation of SBE

The roots of SB were purchased from Kyungdong traditional herbal market (Seoul, Korea) and identified by Dr. Hyung Jun Ji. A voucher specimen (No. 3421) was deposited at RIC, Hallym University, Korea.

The roots of SB (800 g) were extracted with 70% ethanol for 2 h at 80°C in a Soxhlet extractor for three cycles. Each extract was filtered with Whatman filter paper and evaporated under reduced pressure. Overall, 153 g of the SBE were obtained and stored at -20°C until used.

Cell Culture

RBL-2H3 was obtained from the American Type Culture Collection (Bethesda, MD) and grown in MEM supplemented with 10% fetal FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified incubator under 5% CO₂.

Cell Proliferation and Cytotoxicity Assay

The effects of the SBE on cell viability and cytotoxicity were tested using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay and CytoTox 96® Non-Radioactive Cytotoxicity Assay, respectively (Promega). RBL-2H3 cells were plated at a density of 1×10^4 cells/well in a 96 well flat-bottomed plate, and SBE was added to each well at a concentration of 0–5,000 μ g/mL in complete MEM. After a 24 h incubation period, cell proliferation and cytotoxicity were measured according to the manufacturer's instructions.

β -Hexosaminidase Release Assay

Inhibitory effects on the release of β -hexosaminidase from RBL-2H3 cells were evaluated using a modification of the method described by Matsuda *et al.* [17]. Briefly, RBL-2H3 cells were plated at a density of 2×10^5 cells/well in a 24-well plate. The cells were then incubated overnight in complete medium containing

0.45 µg/mL anti-DNP IgE; after which, they were washed twice with 0.5 mL Siraganian buffer (119 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.4 mM MgCl₂, 5.6 mM glucose, 25 mM piperazine-*N*, *N'*-bis (2-ethanesulfonic acid)(PIPES), and 1 mg/mL BSA at pH 7.2) and then incubated in 0.16 mL of Siraganian buffer for additional 10 min at 37°C. Next, 20 µL of SBE (0, 10, 100, 1,000 µg/mL) were added to each well, and the samples were incubated for 45 min, followed by the addition of 20 µL of DNP-BSA (final concentration 10 µg/mL) at 37°C for 20 min to stimulate the cells to degranulate. The supernatant was then transferred into a 96-well plate and incubated with 50 µL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 h. The reaction was stopped by adding 100 µL of stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). Finally, the absorbance at 405 nm was measured using a SpectraMaxM2 Microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Histamine Release Assay

RBL-2H3 cells were plated at a density of 2×10^5 cells/well in a 24-well plate. The cells were then incubated overnight in a complete medium containing 0.45 µg/mL DNP-specific IgE. The cells were then washed twice with 500 µL of Siraganian buffer and incubated in 160 µL of Siraganian buffer for an additional 10 min at 37°C. Next, 20 µL of SBE (0, 10, 100, 1,000 µg/mL) were added to each well and incubated for 30 min, followed by the addition of 20 µL of DNP-BSA (final concentration 10 µg/mL) at 37°C for 10 min to stimulate the cells to degranulate. The histamine content was measured using a Histamine kit (Oxford Biomedical Research, MI, USA) according to the manufacturer's instructions.

Measurement of Cytokine Production

RBL-2H3 cells were plated at a density of 1×10^6 cells/well in a 6-well plate. The cells were incubated overnight in a complete medium containing 50 ng/mL DNP-specific IgE. Next, the cells were washed and stimulated with 25 ng/mL DNP-BSA for 4 h with SBE (0, 10, 100, 1,000 µg/mL). The TNF- α and IL-4 concentration in the culture supernatant were determined using a Bio-Rad Multiplex bead array instrument and a cytokine kit (Bio-Rad, Irvine, CA, USA).

Western Blotting

RBL-2H3 cells were plated at a density of 2×10^6 cells/well in a 10-mm dish. The cells were then incubated overnight in complete MEM containing 100 ng/mL DNP-specific IgE. Next, the cells were washed twice with 500 µL of Siraganian buffer and then incubated with SBE (0, 10, 100, 1,000 µg/mL) for 30 min; after which, they were incubated with 50 ng/mL DNP-BSA for 10 min. Whole cell extracts (30 µg protein) were separated on 10% SDS-polyacrylamide gels; after which, the separated proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were then incubated with the indicated Abs, and specific bands were visualized using an ECL kit (Amersham Biosciences).

Measurement of DNFB-Induced Mouse Ear Swelling

Specific pathogen-free BALB/c mice (female, 6 weeks old) were purchased from Koatech Laboratory Animal Research Co. (Pyeongtaek, Korea). The mice were housed under normal laboratory conditions with a 12-h light/dark cycle and free access to standard rodent food and water. All animal experiments were approved by the Animal Care and Use Committee of Hallym University (Chuncheon, Korea) and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals.

The *in vivo* anti-allergic activity was tested by the DNFB-induced contact hypersensitivity reaction. Briefly, mice were sensitized by painting 25 µL of 0.5% (v/v) DNFB in acetone/olive oil (3:1) onto the shaved abdomen for two consecutive days. Four days after the last DNFB sensitization, the animals were challenged by application of 20 µL of 2% DNFB to their ears. The control animals were painted with acetone/olive oil without DNFB. To evaluate the effect of SBE on contact hypersensitivity reactions, SBE (0, 10, 100, 1,000 µg/ear) or dexamethasone (50 µg/ear) was administered topically 2 h before DNFB challenge. At 24, 48, and 72 h after DNFB challenge, the ear swelling was evaluated by measuring the ear thickness using a dial thickness gauge (Mitutoyo Co., Tokyo, Japan) and the weight (based on a small round piece cut-out using a sharp clamp).

Part of the ear was homogenized in 50 mM Tris-HCl buffer (pH 7.5) with 1 mM EDTA; after which, the homogenate was incubated on ice for 20 min in the presence of 0.1% Triton X-100. The homogenate was

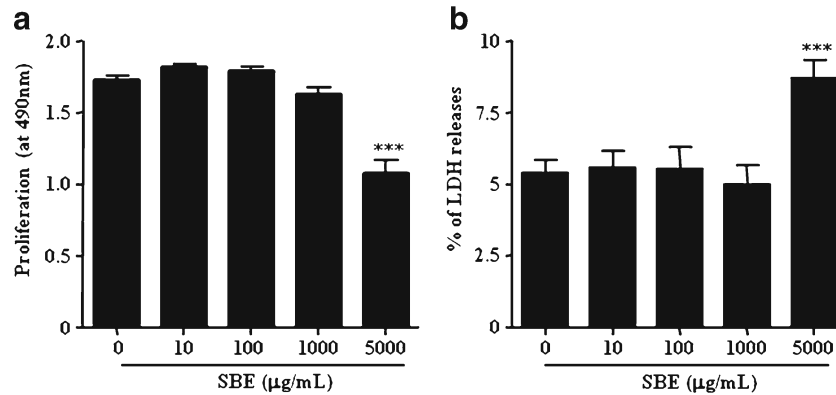


Fig. 1. Effect of SBE on the viability of RBL-2H3 cells. Cell proliferation (a) and cytotoxicity (b) were determined as described in the “Materials and Methods” section. The results are reported as the means±SEM of three independent experiments. Significant differences from cells treated with 0 µg/mL of SBE are indicated as follows *** $P<0.001$.

then centrifuged at $10,000\times g$ for 15 min; after which, the supernatant was collected for cytokine measurement.

Histopathology

Formalin-fixed, paraffin-embedded ears were sectioned at a thickness of 7 µm, and the sections were then stained with hematoxylin and eosin (H&E). Next, the samples were then photographed using a light microscope equipped with a camera.

Statistical Analysis

All data are reported as the means±S.E.M. Statistical analyses were conducted by analysis of variance (ANOVA), followed by Duncan's multiple range tests. The GraphPad Prism 4.0 software (GraphPad Software

Inc., San Diego, CA, USA) was used for all calculations. A $P<0.05$ was considered statistically significant.

RESULTS

Effect of SBE on the Antigen-Induced Degranulation of RBL-2H3 Cells

To evaluate the SBE-induced anti-allergic effects, we used an *in vitro* model of RBL-2H3 cells. Because SBE showed no cytotoxicity at concentrations up to 1,000 µg/mL (Fig. 1) in RBL-2 H3 cells, we used up to 1,000 µg/mL SBE for the rest of the experiments.

The release of β -hexosaminidase and histamine from the cells was measured as an indicator of the degranulation of RBL-2H3 cells. Pretreatment with SBE (100–1,000 µg/

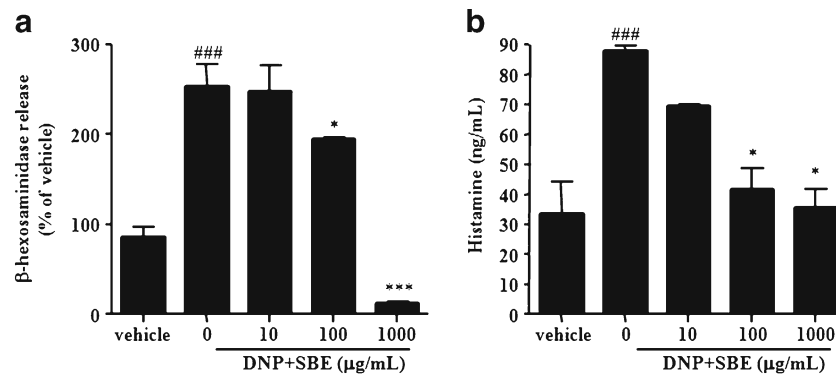


Fig. 2. Effect of SBE on antigen-induced degranulation of RBL-2H3 cells. The β -hexosaminidase (a) and histamine (b) release in RBL-2H3 cells was measured as described in the “Materials and Methods” section. The results are reported as means±SEM for three independent experiments. Significant differences from cells treated with DNP-BSA in the absence of SBE are indicated as follows: * $P<0.05$, *** $P<0.001$. ### $P<0.001$, a significant difference between vehicle and DNP-BSA treatment alone.

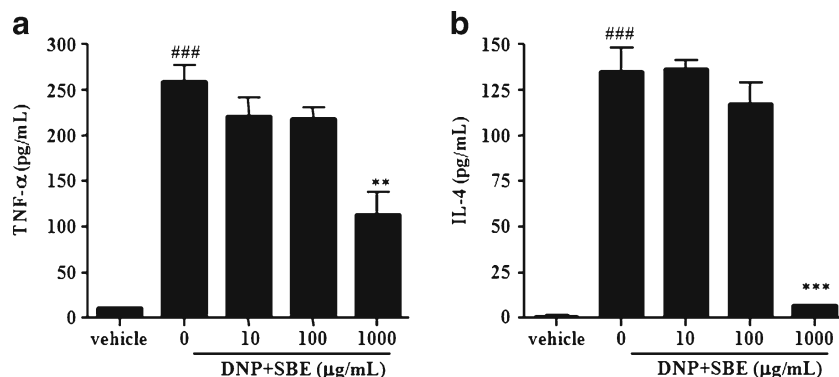


Fig. 3. Effect of SBE on the antigen-induced release of proinflammatory cytokines in RBL-2H3 cells. The TNF- α (a) and IL-4 (b) release in RBL-2H3 cells were measured as described in the “Materials and Methods” section. The results are reported as the means $\pm$ SEM for three independent experiments. Significant differences from cells treated with DNP-BSA in the absence of SBE are indicated as follows: ** P <0.01, *** P <0.001. ^{###} P <0.001, a significant difference between vehicle and DNP-BSA treatment alone.

mL) led to a significant reduction in the release of β -hexosaminidase as well as histamine from sensitized RBL-2H3 cells stimulated with DNP-BSA (Fig. 2). In contrast, 10 μ g/mL of SBE had no effect on the antigen-induced degranulation of mast cells (Fig. 2b).

Effect of SBE on the Expression of TNF- α and IL-4

Because there is increasing evidence that various cytokines, including TNF- α and IL-4, are critical for allergic inflammation [18], we examined SBE to determine if it could modulate the release of TNF- α and IL-4 induced by antigens in RBL-2H3 cells. SBE

(1,000 μ g/mL) was found to lead to a significant inhibition of the antigen-stimulated release of TNF- α and IL-4 in RBL-2H3 cells (Fig. 3).

Effect of SBE on the Activation of Intracellular Signaling Molecules

The aggregation of Fc ϵ RI induces various intracellular signaling pathways. Therefore, we investigated which signaling pathways leading to mast cell degranulation were inhibited by SBE. To accomplish this, RBL-2H3 cells were sensitized with anti-DNP IgE; after which, they were incubated with SBE before DNP-BSA stimulation.

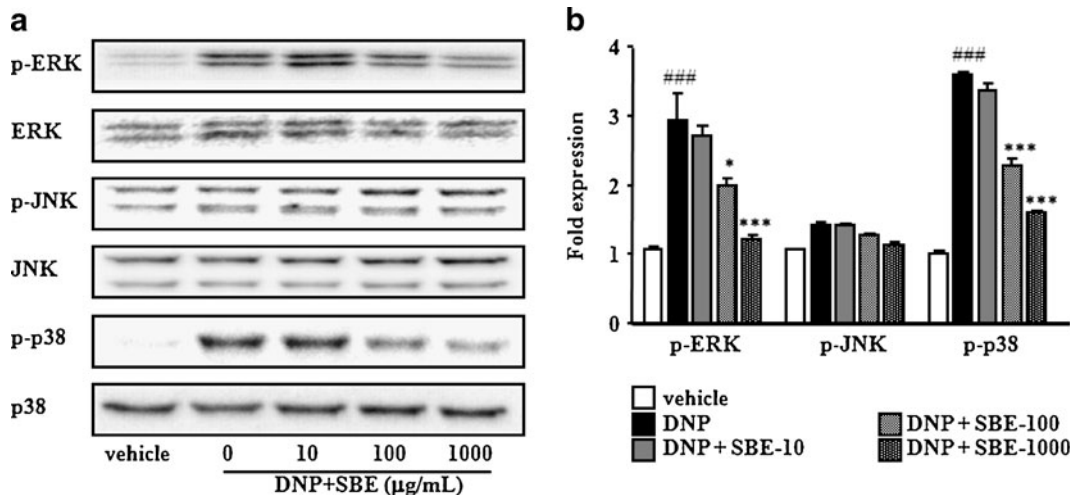


Fig. 4. Effect of SBE on antigen-induced activation of MAPK. **a** The RBL-2H3 cells were incubated overnight with 100 ng/mL DNP-specific IgE in a complete growth medium. The cells were treated with various concentrations of SBE and stimulated with 50 ng/mL DNP-BSA for 10 min. ERK, JNK, and p38 MAPK were subjected to immunoblot analysis to detect phosphorylated or total proteins. **b** Band densities are expressed as the mean $\pm$ SEM for the three independent experiments. Significant differences from cells treated with DNP-BSA in the absence of SBE are indicated as follows: * P <0.05, *** P <0.001. ^{###} P <0.001, a significant difference between vehicle and DNP-BSA treatment alone.

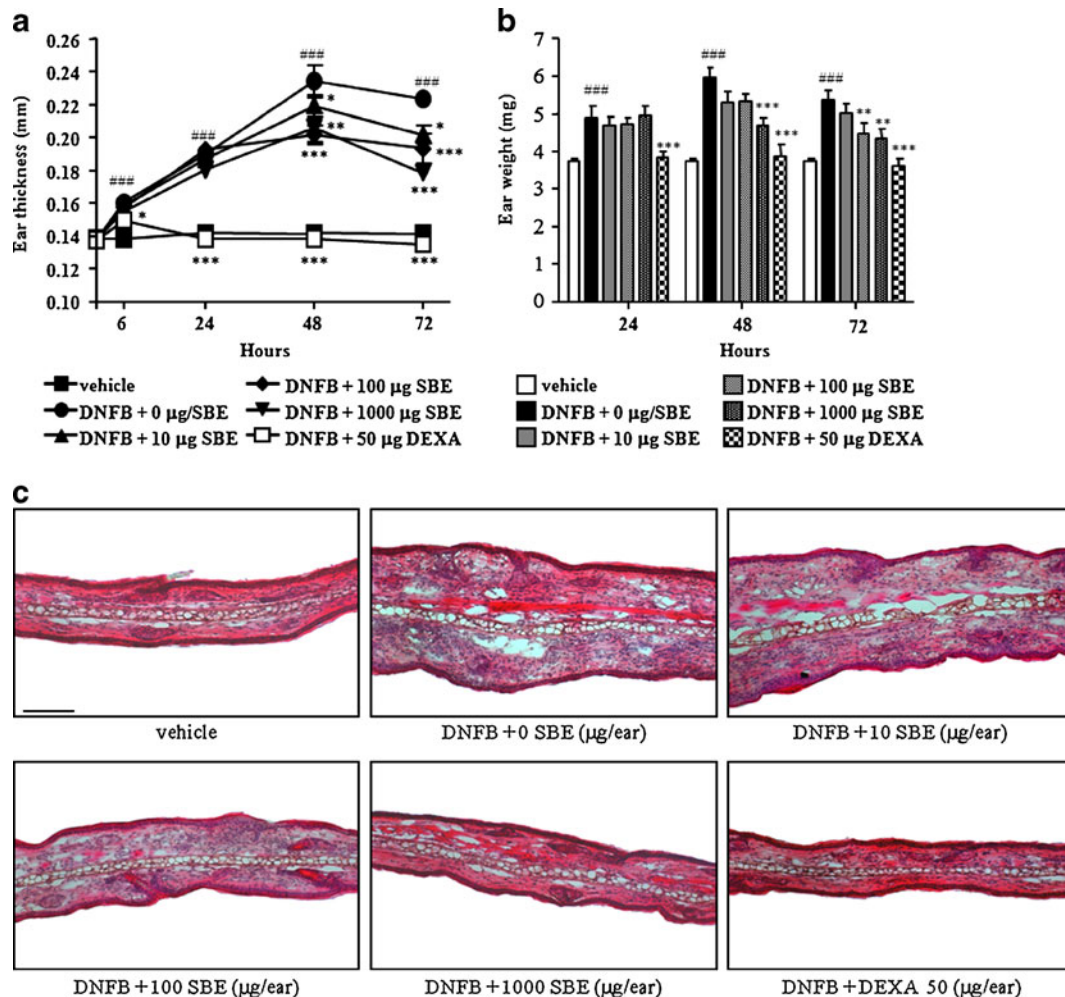


Fig. 5. Effect of SBE on DNFB-induced allergic inflammation. Inhibition of DNFB-induced ear edema by topical application of SBE was analyzed by measuring changes in ear thickness (a) and ear weight (b). The data are the means $\pm$ S.E.M of five mice per group. Significant differences from mice treated with DNFB in the absence of SBE are indicated as follows: * P <0.05, ** P <0.01, *** P <0.001. #### P <0.001, a significant difference between vehicle and DNFB challenge alone. **c** For histological analysis, ear skin sections were prepared as described in the “Materials and Methods” section and stained with hematoxylin and eosin (original magnification, $\times 100$, scale bar, 200 μ m).

Western blot analysis indicated that the phosphorylation of ERK and p38 was reduced by SBE pretreatment in a dose-dependent manner, whereas it had no effect on the phosphorylation of JNK (Fig. 4). These data suggest that SBE inhibits degranulation in RBL-2H3 cells via inhibition of the ERK and p38 pathway activation.

Effect of SBE on DNFB-Induced Ear Swelling in Mice

Evaluation of the *in vivo* anti-allergic activity of SBE was performed using the DNFB-induced mouse ear swelling model of contact hypersensitivity. The efficacy

of SBE against ear swelling was evaluated by measuring the ear thickness at 6–72 h after DNFB challenge. As shown in Fig. 5, exposure to DNFB resulted in marked increases in skin thickness and weight. Topical application of SBE dose-dependently inhibited ear swelling in DNFB sensitized mice. The inflamed auricles of the mice were examined histologically by hematoxylin and eosin staining (Fig. 5c). The epidermises of the DNFB-challenged mice showed hyperplasia. In addition, the dermis showed significant edema and infiltration of mononuclear cells. Furthermore, there was less hyperplasia of the epidermis of SBE-treated mice than for DNFB-applied mice that were not treated with SBE.

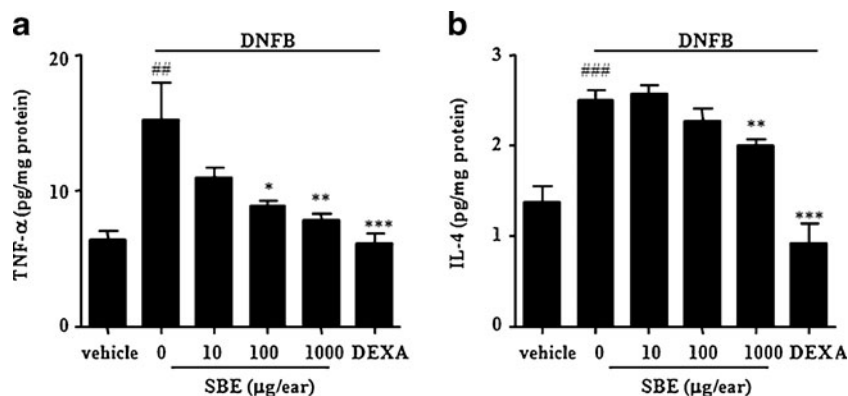


Fig. 6. Effect of SBE on TNF- α and IL-4 production in CHS induced by DNFB. Allergic inflammation was induced by topical application of DNFB to ears as described in the “Materials and Methods” section. TNF- α (a) and IL-4 (b) content in ear homogenates was determined at 24 h after challenge by ELISA. The data are the means $\pm$ S.E.M of five mice per group. Significant differences from mice treated with DNFB in the absence of SBE are indicated as follows: * P <0.05, ** P <0.01, *** P <0.001. ### P <0.001, a significant difference between vehicle and DNFB challenge alone.

Because contact hypersensitivity is caused by cytokines and infiltrating neutrophils, the topical anti-allergic activity of SBE was confirmed by measuring the TNF- α and IL-4 levels of samples collected during inner ear biopsies (Fig. 6). Topical application of DNFB resulted in increased TNF- α and IL-4 levels in ear homogenates. In contrast, these increased levels were suppressed in mice treated with DNFB plus SBE (100–1,000 μ g/ear) or dexamethasone.

DISCUSSION

Allergic diseases are currently a major health problem in many industrialized countries [19]. Since the current therapeutic approaches for the treatment of allergic disorders are largely ameliorative rather than curative and can cause unexpected side effects, novel approaches are being explored to develop inhibitors of allergic diseases [20]. Among these, natural remedies have been used for centuries for the treatment of skin conditions and a wide variety of dermatological disorders, including atopic dermatitis, inflammation, phototoxicity, psoriasis, and alopecia areata. Indeed, such remedies are considered to be an ideal approach to cure various allergic diseases. Although such remedies are currently widely accepted by patients, the respect they receive from the scientific community is limited. Therefore, we screened over 350 plant extracts for their ability to inhibit allergic reactions in RBL-2H3 cells and found that SBE has anti-allergic activity both *in vitro* and *in vivo*.

Mast cells and basophils are secretory cells that play important roles in both immediate allergic reactions and inflammatory responses [13]. These cells have receptors with high affinity for IgE, Fc ϵ RI [21]. Recently, activated mast cells were shown to release a variety of cytokines and chemokines. Of these cytokines, mast cell-derived TNF- α and IL-4 are of particular importance in terms of causing allergic inflammation [17, 22]. The immediate inflammatory responses are primarily due to small-molecule chemical mediators (e.g., histamine and β -hexosaminidase) from mast cells. SBE was found to significantly inhibit the β -hexosaminidase and histamine release from RBL-2H3 cells stimulated with antigen. These data suggest that SBE can potentially protect against immediate immune response by suppressing the rate of mast cell degranulation. Mast cells also produce cytokines such as TNF- α , IL-4, and IL-5, and these cytokines are known to play an important role in late-phase reactions. Several flavones (e.g., luteolin, apigenin, diosmetin, and quercetin) obtained from nature have been reported to inhibit the release of TNF- α and IL-4 [17, 23]. Similarly, we found that SBE suppressed TNF- α and IL-4 secretion in RBL-2H3 cells sensitized with anti-DNP IgE.

It is well known that crosslinking of Fc ϵ RI by antigen leads to a cascade of events, which include activation of the Src family tyrosine kinase Lyn, the interaction of lipid rafts with IgE receptors, and the tyrosine phosphorylation of IgE receptors [15, 16]. These molecular events propagate a series of intracellular signals that result in elevation of the intracellular Ca $_2^{+}$ concentration, phosphorylation, as well as translocation of MAPK from the cytoplasm to the

nucleus and exocytotic release of inflammatory mediators such as histamine [24]. In the present study, we showed that SBE significantly inhibits the antigen-induced phosphorylation of ERK and p38 MAPK in RBL-2H3 cells. Our findings suggest that suppressed activation of ERK and p38 by SEB might lead to inhibition of both degranulation and the upregulation of cytokine.

Consistent with these *in vitro* observations, topical application of SBE was found to suppress allergic inflammatory reactions in DNFB-induced contact allergy model mice (Fig. 5). DNFB is used as a contact allergen to induce ear inflammatory reactions including the degranulation of mast cells [15]. SBE inhibited the production of proinflammatory cytokines such as IL-4 and TNF- α . During contact hypersensitivity reactions, nitric oxide is also produced by other inflammatory cells such as Langerhans cells and keratinocytes and contributes to swelling and infiltration of effector cells [25]. Furthermore, TNF- α exerts a stimulatory effect on local endothelium on the attraction of leukocytes and the upregulation of surface molecules on resident cells during the elicitation of contact hypersensitivity responses, whereas interleukin-4 is the predominant cytokine in contact hypersensitivity [26]. Indeed, the suppression of these cytokines may be directly related to the observed pharmacological effects of SBE.

TLC profile of SBE suggested that angoroside C is a major compound of SBE (Supplementary Fig. 1). Previous studies demonstrated that angoroside C isolated from various Scrophulariaceae, *Scrophularia ningpoensis* [27, 28], *Scrophularia lepidota* [29], and *Scrophularia scorodonia* [30]. Díaz *et al.* reported that angoroside C inhibited the release of prostaglandin E₂ by mouse peritoneal macrophages stimulated with calcium ionophore [30]. In addition, angoroside C had inhibitory activity on nitric oxide production in lipopolysaccharide-stimulated macrophages. These results including the present study indicate that pharmacology of angoroside C may participate in the anti-inflammatory effect of SB.

To date, there have been no reports of the activity of SB on allergic and allergic-induced inflammations. Based on the present study, we believe that SBE can be used to treat allergic diseases such as rhinitis and asthma and may be effective for patients who exhibit side effects in response to steroids.

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