



Inhibitory effects of cyanidin-3-O-glucoside in black soybean hull extract on RBL-2H3 cells degranulation and passive cutaneous anaphylaxis reaction in mice

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ABSTRACT

Black soybean hull extract (BSHE) exhibits a variety of biological activities. However, little is known about the effects of BSHE on immunoglobulin E (IgE)-mediated type I allergic reactions. The anti-allergic effect of BSHE was assessed with the degranulation assay using rat basophilic leukemia RBL-2H3 cells and the passive cutaneous anaphylaxis (PCA) reaction in mice. An active compound in BSHE was identified by ultra-performance liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry analysis. BSHE inhibited the release of β -hexosaminidase and histamine in RBL-2H3 cells, and cyanidin-3-O-glucoside (C3G) was identified as one of its active compounds. Oral administering of 200 μ mol/kg of C3G to IgE-sensitized mice prior to antigen injection suppressed the PCA reaction, as compared with control ($p < 0.01$). Intravenous administration of BSHE (C3G content, 5.4%) more strongly inhibited PCA responses at lower doses (100 mg/kg, $p < 0.01$) than oral administration (1,000 mg/kg, $p = 0.059$). Intravenous C3G also suppressed PCA response at a low dose (40 mg/kg, $p < 0.05$), showing the same trend as BSHE. This information can be useful to design appropriate formulations of anthocyanin-based drug products to suppress allergic reactions. This study provides evidence for the potential use of BSHE and C3G for the prevention or the treatment of type I allergies.

1. Introduction

Food allergy and pollinosis are immunoglobulin E (IgE)-mediated type I allergic reactions, which sometimes cause serious symptoms, such as anaphylaxis. The prevalence of food allergy is reportedly increasing worldwide, likely affecting nearly 5% of adults and 8% of children [1]. In addition, allergic rhinitis (or hay fever) affects an estimated 10%–30% of the global population and sensitization to IgE antibodies against foreign proteins in the environment is present in up to 40% of the population [2]. Therefore, allergy is a global problem that needs to be addressed and controlled. Multiple steps have been proposed in the onset of type I allergic reactions. First, the IgE antibody binds to the high-affinity IgE receptor (FcεRI) on the surface of basophils and mast

cells [3]. Next, the antigen invades the body and cross-links with IgE antibodies, which induces degranulation and the release of histamines, cytokines, and proteases from basophils and mast cells [4]. Finally, these chemical mediators and cytokines cause allergic symptoms, including edema, itching, dyspnea, and sometimes anaphylactic shock. Rat basophilic leukemia RBL-2H3 cells, which are established in the peripheral blood with basophilic leukemia, express FcεRI and release IgE-mediated histamines [5] along with β -hexosaminidase (β -Hex) upon mast cell degranulation. Therefore, the β -Hex enzyme is typically used as an *in vitro* marker of mast cell degranulation. RBL-2H3 cells are widely used to develop anti-allergy drugs and the identification of anti-allergic compounds that reduce allergic symptoms [6,7]. *In vivo*, the inhibitory effects of compounds and food extracts are examined by the passive

Abbreviations: BW, body weight; BSHE, black soybean hull extract; Cy, cyanidin; C3G, cyanidin-3-O-glucoside; DMSO, dimethyl sulfoxide; DNP-Alb, albumin-dinitrophenyl; FcεRI, high-affinity IgE receptor; UPLC-DAD–ESI–MS/MS, ultra-performance liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry; HBS, HEPES buffer solution; HEPES, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IC₅₀, half maximal inhibitory concentration, MS, mass spectrometry; PCA, passive cutaneous anaphylaxis; UV-Vis, Ultraviolet–visible.

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cutaneous anaphylaxis (PCA) reaction in an animal model of IgE-mediated allergic responses [8,9].

Studies of bioactive compounds in food have been actively ongoing over the last 20 years. Polyphenols in plant-based foods, such as catechins and flavonoids, are reportedly beneficial for chronic diseases, such as cancers, cardiovascular events, and diabetes [10,11]. Various studies of anti-allergy compounds have focused on the effects of polyphenolic compounds, such as catechins in green tea [12] and rosmarinic acid derived from *Perilla* leaves [7].

Soybeans (*Glycine max*) have high protein and oil contents, and are thus considered a nutritionally rich food [13]. Besides, soybeans contain isoflavones, a type of polyphenol, which are phytochemicals with potential health benefits against heart disease, cancer, and osteoporosis [14]. Therefore, soybeans are widely utilized a source of phytochemicals. The most typical soybean has a yellow seed coat, while black soybeans have a dark black seed coat. The pigment, which is derived from anthocyanins, such as cyanidin-3-glucoside, delphinidin-3-glucoside, and pelargonidin-3-glucoside [15,16], accumulates in the epidermis palisade layer of the seed coat [16]. Furthermore, black soybean hull extract (BSHE) is rich in polyphenols, such as procyanidin and epicatechin [17].

Anthocyanins, which are widely present in the plant kingdom as pigment components in fruits such as berries, flower petals [18], and black rice [19], have anti-oxidant and various other activities that modulate or prevent chronic diseases [20], such as obesity, cancer [19], and cardiovascular disease [21]. For example, anthocyanins and procyanidins in BSHE have been reported to prevent obesity and glucose intolerance [17], and to convey hypoglycemic [22] and hypolipidemic effects in diabetic mice [23]. However, there have been relatively few reports of the suppressive effects of anthocyanins derived from BSHE on type I allergies. In addition, there are many reports of anti-allergic effects of food components *in vitro*, but relatively few *in vivo* [24].

Kuromanin®-10, which is a dried powder of acidic ethanol extract from the seed coat of black soybean produced in China by neutralization, filtration, desalting, and spray-drying, is composed of 10% or more of anthocyanins and more than 75% of proanthocyanidins [25]. Kuromanin®-10 is highly water-soluble with excellent coloration properties and is, thus, used as a colorant and been shown to have antioxidant effects and improve ocular function [25,26].

The aim of the present study was to evaluate the effects of BSHE with the use of Kuromanin®-10 on IgE-mediated type I allergic reactions. The effects were examined using antigen-stimulated RBL-2H3 cells and the PCA reaction in mouse ears. The inhibitory effect of Kuromanin®-10 on the degranulation of RBL-2H3 cells was compared with *Perilla* seed extract [27] and *Rubus suavissimus* (Tencha) leaf extract [28], which are known to have anti-allergic effects. In addition, the active compound in BSHE was isolated based on the inhibitory effect of β -Hex released from RBL-2H3 cells and identified by ultra-performance liquid chromatography coupled to diode array detection and electrospray ionization tandem mass spectrometry (UPLC-DAD-ESI-MS/MS). Furthermore, the effects of the active compound of BSHE were examined both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals, materials, and reagents

The following chemicals, materials, and reagents were used in this study: cyanidin-3-O-glucoside (C3G) chloride, cyaniding (Cy) chloride, luteolin (Extrasynthese, Genay Cedex, France), ketotifen fumarate salt, *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside, *o*-phthalaldehyde (Wako Pure Chemical Industries, Ltd., Aichi, Japan), β -glucosidase derived from almonds, mouse monoclonal anti-dinitrophenyl (DNP) IgE antibody (anti-DNP IgE), DNP-albumin (DNP-Alb), fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), Eagle's minimal essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), glutamine, penicillin,

streptomycin (Invitrogen Corporation, Carlsbad, CA, USA), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) (Gibco, Carlsbad, CA, USA), F-Kit Glucose (Roche Diagnostics GmbH, Mannheim, Germany), and high-performance liquid chromatography (HPLC)-grade acetonitrile (Merck KGaA, Darmstadt, Germany).

2.2. Plant extracts and preparation

Kuromanin®-10 was provided by Sozai Kinou Laboratory Co., Ltd. (Tokyo, Japan). *Perilla* seed extract and Tencha extract were obtained from Oryza Oil & Fat Chemicals Co., Ltd. (Tokyo, Japan) and Maruzen Pharmaceuticals Co., Ltd. (Hiroshima, Japan), respectively. Sample preparation was performed from plant extracts with the use of 30 mg of Kuromanin®-10 dissolved in 1 mL of HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1.0 mM CaCl₂ and 10 mM HEPES, pH 7.4) (HBS) and mixed with a vortex. The insoluble fractions were removed by centrifugation at 15,000 rpm for 5 min at 4 °C. The supernatant was adjusted to 30 mg (Kuromanin®-10 equivalent)/mL with HBS and the extract was defined as BSHE. *Perilla* seed and Tencha extracts were also prepared by the same method as the BSHE for use with the cell degranulation assay.

The active compounds of BSHE were identified and quantified. BSHE samples for the PCA reaction were prepared using 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (phosphate-buffered saline, PBS) instead of HBS at 30 min prior to administration to the mice. Briefly, 600 mg of Kuromanin®-10 was dissolved in 10 mL of PBS and mixed for 5 min. After centrifugation, the supernatant was adjusted to 60 mg/mL with PBS.

2.3. Culturing of rat basophils

RBL-2H3 mast cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and cultured in Eagle's minimal essential medium supplemented with 4 mM glutamine, 10% fetal bovine serum, 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 10% sodium bicarbonate under an atmosphere of 5% CO₂/95% air at 37 °C. The culture medium was exchanged at 3 and 5 days after passage and the cells were seeded into the wells of 96-well microplates on day 6. The cells were used for the experiments at 2 days after seeding.

2.4. β -Hex and histamine release by RBL-2H3 cells

An antigen-stimulated β -Hex release assay was performed with RBL-2H3 cells in accordance with the method described by Matsuda *et al.* [6] RBL-2H3 cells were seeded in the wells of 96-well plates (1×10^5 cells/well), cultured for 48 h, and then sensitized with anti-DNP IgE (0.25 μ g/mL) for 1 h at 37 °C. After removing the medium and washing, the cells were pretreated with the vehicle (0.1% dimethyl sulfoxide, DMSO) in HBS for 10 min and then treated with the sample solution or vehicle for 10 min at 37 °C. Afterward, the cells were stimulated by the addition of DNP-Alb (0.25 μ g/mL) for 30 min at 37 °C. The reaction was stopped by the addition of ice-cold DMSO for 10 min. To determine the total accumulated intracellular β -Hex activity, 0.5% Nonidet P-40 was added to the wells, which were untreated with the sample and HBS in place of the antigen. Then, the cells were lysed and the lysate was used to prepare a calibration curve for the measurement of enzymatic activity. To assess histamine release, RBL-2H3 cells were seeded in the wells of a 24-well plate (5×10^5 cells/well) and reacted with or without the sample solution in the same manner as β -Hex assay. Furthermore, the effects of plant extracts on cell viability at 125, 250, and 500 μ g/mL or the vehicle (0.1% DMSO) without sensitization were assessed with the trypan blue dye exclusion method [29]. Cell viability is expressed as a percentage relative to the vehicle.

2.5. Measurement of β -Hex released from RBL-2H3 cells

Released β -Hex activity was measured according to the method described by Matsuda *et al.* [6]. The supernatants of the antigen-stimulated cell cultures were transferred to new plates. Then, 1.5 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside in 100 mM citrate buffer (pH 4.5) was added to each well and the plates were incubated at 37 °C for 60 min. The reaction was stopped by the addition of 0.5 M carbonate buffer (pH 10.0). The supernatant of antigen-unstimulated wells with the sample solution was used as a blank. Absorbance was measured at 405 nm (reference wavelength, 595 nm) using a Powerscan® HT multi-detection microplate reader (Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan). The rate of β -Hex release in the sample was determined from a calibration curve with a 5%–50% solution of the cell lysate recovered with Nonidet P-40. The β -Hex release inhibition rate was calculated as follows:

Inhibition (%) = $[1 - (T - B) / (C - N)] \times 100$ for the following four conditions:

Test sample (T): DNP-Alb (+), test sample (+)

Blank (B): DNP-Alb (–), test sample (+)

Control (C): DNP-Alb (+), test sample (–)

Normal (N): DNP-Alb (–), test sample (–).

2.6. Inhibition of β -Hex activity by extracts

Inhibition of the enzymatic activity of the extracts was examined using cell lysate of total accumulated intracellular β -Hex. For measurement of β -Hex activity, HBS with or without the sample was added to the cell lysate to prepare a 15% solution. The inhibition rate of β -Hex activity by the sample was calculated with the following formula that in the absence of inhibitors as 100%.

Inhibition (%) = $[1 - (\beta\text{-Hex activity with the sample} / \beta\text{-Hex activity without sample})] \times 100$

2.7. Measurement of histamine release by RBL-2H3 cells

The released histamine was extracted as described below. The antigen-stimulated cell medium was centrifuged at 3,000 rpm and 4 °C for 1 min. Then, the supernatant was collected and mixed with NaCl and 1 N NaOH. Following the addition of *n*-butanol and chloroform (3:2, v/v), the mixture was vigorously mixed for 5 min. Next, the organic layer obtained by centrifugation at 1,400 rpm for 5 min at 4 °C was mixed with 0.1 N HCl and *n*-heptane and shaken. Afterward, the aqueous layer was assayed using the *o*-phthalaldehyde spectrofluorometric method [30]. The fluorescence intensity was measured at excitation wavelength of 360 nm and emission wavelength of 450 nm using an F-2000 spectrofluorophotometer (Hitachi, Ltd., Tokyo, Japan). The histamine content was calculated from a calibration curve of a histamine standard solution. The inhibition rate of histamine release was calculated in the same manner as for β -Hex release.

2.8. Isolation and quantification of active compounds by HPLC

BSHE diluted with the mobile phase was filtered through a 0.45- μ m Millex-LG filter (EMD Millipore Corporation, Billerica, MA, USA) and subjected to HPLC analysis using an Atlantis™ dC₁₈ column (5 μ m, i.d. 3.9 $\times$ 150 mm; Waters Corporation, Milford, MA, USA) and an LC-10 AT pump (Shimadzu Corporation, Kyoto, Japan) under the following conditions: injection volume, 20 μ L; flow rate, 0.5 mL/min; and column detection wavelength, 280 nm. Compounds were eluted using a mobile phase (A) consisting of 0.05% trifluoroacetic acid and (B) 80% acetonitrile/0.05% trifluoroacetic acid. The gradient was programmed as follows: initial 0% B; 0%–8% B for 0–10 min; 8%–18% B for 10–140 min; and 80% B isocratic for 10 min. Fractions collected every 10 min were lyophilized and dissolved in HBS for use in the β -Hex release assay to identify the active compounds of BSHE.

To determine the contents of the active compounds in BSHE, a calibration curve was plotted using the peak area at 520 nm from 0.3125 to 20 μ M of authentic C3G solution. The standard solutions exhibited linear relationships between the peak area and the amounts injected ($R^2 = 0.9994$).

2.9. Acid and β -glucosidase hydrolyses

The isolated compounds were subjected to hydrolysis with acid and β -glucosidase. For acid hydrolysis, the isolated compounds were dissolved in 0.6 N HCl containing 2% 2-mercaptoethanol and then hydrolyzed at 90 °C for 60 min. For β -glucosidase hydrolysis, 2 U/ μ L of β -glucosidase was added to an equal volume of 1.0 mM isolated compound dissolved in 50 mM citrate buffer (pH 3.0) and the solution was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 0.2 N HCl. The reactants were diluted with solvent prior to UPLC–DAD–ESI–MS/MS analysis. In addition, the hydrolysate with β -glucosidase was used for quantification of released D-glucose with an F-Kit Glucose in accordance with the manufacturer's instructions. A glucose standard curve was used as a reference.

2.10. UPLC–DAD–ESI–MS/MS analysis

The isolated compound and hydrolysates were diluted with mobile phase A at a ratio of 1:10 and filtered through a 0.2- μ m Millex-LG filter prior to UPLC–MS/MS analysis with the use of an Acquity UPLC system coupled to a DAD and Xevo tandem quadrupole mass detector (Waters Corporation) equipped with a Z-spray ESI source, operating in positive and negative modes for direct injection analyses. The analyses were performed on an Acquity UPLC HSS T3 column (1.8 μ m, i.d. 2.1 $\times$ 100 mm; Waters Corporation) using a gradient mobile phase comprising (A) 0.1% formic acid and (B) 80% acetonitrile/0.1% formic acid. The gradient was programmed as follows: initial 0% B; 0%–30% B for 0–10 min; 30%–95% B for 10–12 min; and 95% B isocratic for 3 min. The UPLC conditions were set as follows: injection volume, 5 μ L; sample storage, 4 °C; flow rate, 0.3 mL/min; column temperature, 40 °C; and detection wavelength, 200–600 nm. The ionization working conditions for the Xevo tandem quadrupole mass detector were set as follows: capillary voltage, 1 kV; collision energy, 30 V; cone gas flow rate, 0.5 mL/min; desolvation temperature, 400 °C; and desolvation gas flow rate, 650 L/h. Data acquisition was performed using MassLynx™ v 4.1 software (Waters Corporation). The peaks of the compound and its products were identified on the basis of the retention times (Rt), ultra-violet–visible (UV–Vis) spectra, and MS and MS/MS spectral data as compared with those of pure authentic standards. C3G and Cy were used as the active compound and its hydrolysate.

2.11. Animals

Male Institute of Cancer Research mice (5-week-old) were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and acclimated for one week under a 12-h light/12-h dark cycle at 25 °C $\pm$ 2 °C and relative humidity of 45%–55% in an animal facility with access to commercial CE-2 diet (CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee and conducted in accordance with the Animal Experiment Guidelines of Okayama Prefectural University (permission nos. 19–1 and 23–8).

2.12. PCA reactions

The PCA reaction was performed as described by Inagaki *et al.* [8], but with slight modifications. The mice ($n = 4$ or 6/group) were sensitized to anti-DNP IgE (100 ng/ear) in PBS by intradermal injection in the ears under pentobarbital anesthesia. The left ear was sensitized with anti-DNP IgE and the right ear was injected with PBS as blank non-

sensitized control. After 24 h, PCA reactions were induced by intravenous injection of DNP-BSA in PBS (140 µg/mouse) containing 1% Evans blue azo dye. Samples or PBS, as a control, were orally administered by a feeding needle at 1 h before antigen challenge. The test samples used were BSHE [500 and 1,000 mg/kg body weight (BW)], C3G (100 and 200 µmol/kg BW), and ketotifen (0.5 µmol/kg BW) for oral administration, and BSHE (100 mg/kg BW) and C3G (40 mg/kg BW) for intravenous administration. At 30 min after the antigen challenge, the mice were euthanized and their ears were removed with a pair of scissors.

For measurement of Evans blue extravasation, extraction of the dye was performed according to the method described by Kawagoe et al. [9]. The mouse ears were dissolved in 1 N KOH at 37 °C for 24 h, were mixed with 0.6 N phosphoric acid/acetone (5:13, v/v), and the extracts were filtered through a 0.45-µm filter. The absorbance of the extract was measured at 620 nm. The dye leakage rate was calculated based on the relative value of the dye exudation of the test group to the amount of dye in the control group according to the following equation.

Extravasation of the dye (%) = $(T - B) / (C - N) \times 100$

Test sample (T): anti-DNP Alb IgE (+), test sample (+)

Blank (B): anti-DNP Alb IgE (-), test sample (+)

Control (C): anti-DNP Alb IgE (+), test sample (-)

Normal (N): anti-DNP Alb IgE (-), test sample (-).

2.13. Statistical analysis

Data are presented as the mean ± standard deviation (SD) of three independent experiments for the *in vitro* assays and as the mean ± SD of four or five mice for the *in vivo* assays, respectively. Statistical analyses were performed using one-way analysis of variance followed by the Tukey–Kramer multiple comparison post-hoc test for the *in vitro* assays.

The Student's *t*-test was performed to compare two groups for the cell viability and *in vivo* assays. A probability (*p*) value of < 0.05 was considered statistically significant. The results were calculated using IBM SPSS Statistics v.24 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Inhibition of β-Hex and histamine release by BSHE

The effect of BSHE extract on the degranulation of RBL-2H3 cells was evaluated (Fig. 1). BSHE inhibited the release of β-Hex from RBL-2H3 cells in a dose-dependent manner in the range of 1 to 1,000 µg/mL (Fig. 1A). The half maximal inhibitory concentration (IC₅₀) of the BSHE was 130 µg/mL, and those of the *Perilla* seed and *Tencha* extract were 290 and 35 µg/mL, respectively. The *Perilla* seed extract had no inhibitory effect on β-Hex activity. BSHE and *Tencha* extract were found to significantly inhibit β-Hex activity at concentrations of greater than 500 and 125 µg/mL, respectively (*p* < 0.05), but there was no inhibition at the IC₅₀ value, suggesting that the inhibition of β-Hex release by the extracts was specific, absent inhibition of enzymatic activity. These results revealed that the inhibition of β-Hex release by BSHE extract was stronger than that of *Perilla* seed extract, but weaker than that of *Tencha* extract. Furthermore, the effects the extracts on histamine release were examined (Fig. 1B). Histamine release was inhibited by the extracts in a concentration-dependent manner. The IC₅₀ values of BSHE, *Perilla* seed, and *Tencha* extracts were 130, 180, and 24 µg/mL, respectively. The inhibitory order was consistent with that of β-Hex. Together, these results demonstrate that BSHE inhibited antigen-stimulated degranulation of RBL-2H3 cells. The cell viabilities in response to treatment with *Perilla* seed were 100.7% or 98.7% and *Tencha* extracts were 99.9% or 99.2% at

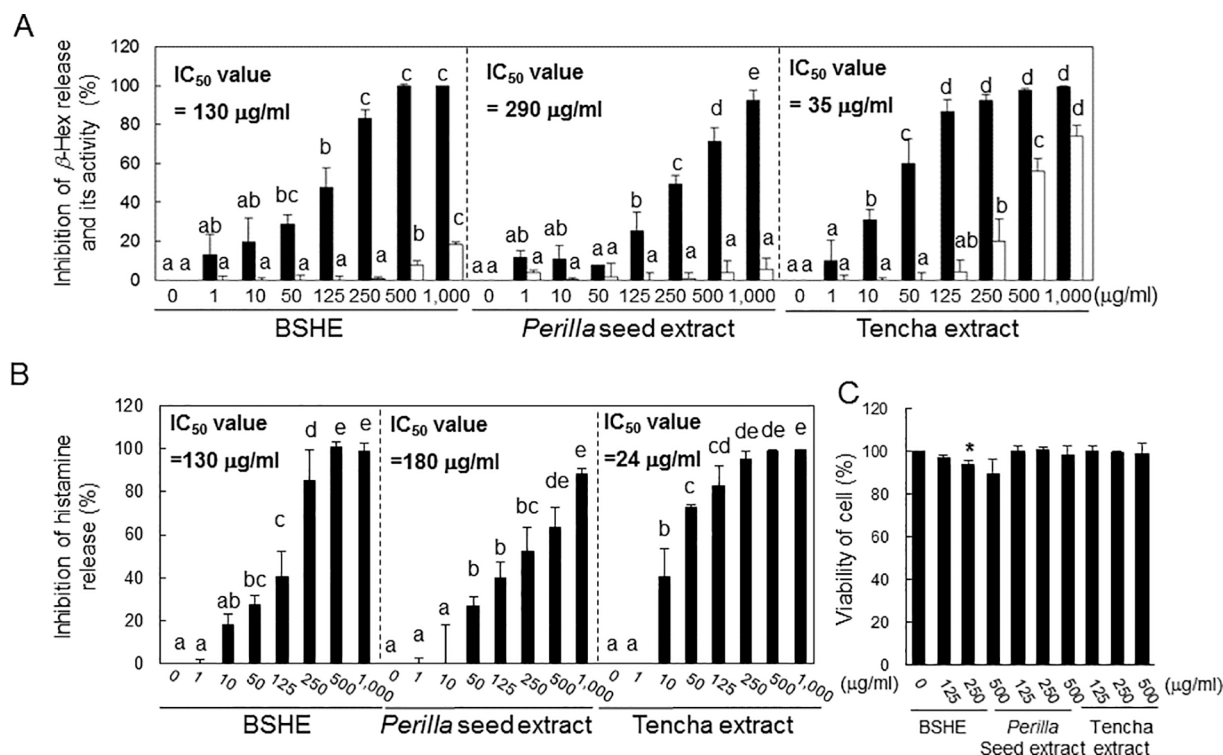


Fig. 1. Effects of plant extracts on β-Hex release (A), histamine release (B), and cell viability (C) by RBL-2H3 cells. RBL-2H3 cells were sensitized with anti-DNP IgE for 12 h and pre-treated with the sample (BSHE, *Perilla* seed extract, or *Tencha* extract) for 10 min. An allergic reaction was induced by the addition of DNP-Alb. β-Hex and histamine that were released into the supernatant were assayed. The results are expressed as the inhibitory percentages of β-Hex release (black bar) and β-Hex activity (white bar) in the presence of the plant extract to the amount of β-Hex released in the absence of the plant extract. The results of the histamine assay are expressed as the percentage of histamine release in the presence of the plant extract to the amount of histamine released in the absence of the plant extract. Values are expressed as the mean ± SD of three determinations, and different letters indicate significant differences among extracts, *p* < 0.05 (Tukey–Kramer multiple-range test). IC₅₀ of plant extracts are shown in each inside panel. Cell viability is expressed as a percentage relative to vehicle. Data are presented as the mean ± SD of triplicate wells and significant differences were detected using Student's *t*-test at **p* < 0.05 to vehicle.

250 µg/mL or 500 µg/mL, respectively, indicating no cytotoxic effect at the IC₅₀ values (Fig. 1C). Conversely, BSHE cell viability showed no cytotoxicity at 125 mg/mL (97.4%), but there was a significant difference between the viability of BSHE at 250 µg/mL (94.2%) and that of the vehicle.

Of the constituents of *Perilla* seed extracts, the flavonoid apigenin is reported to inhibit histamine release from mast cells, while luteolin and quercetin inhibit lipoxygenase activity [27]. On the other hand, galloyl-oxygen-diphenyl-type ellagitannin, a plant-specific polyphenol [28], is an anti-allergic compound of Tencha extract.

3.2. Identification of the active components of BSHE on cell degranulation

In a preliminary experiment, BSHE was added to a polyvinyl-pyrrolidone column, which adsorbs polyphenols well. The active compounds were undetectable in any fraction eluted with 10% to 100% ethanol, suggesting that the active compound was a polyphenol adsorbed on the column. BSHE was fractionated by HPLC and the active compounds were investigated based on the inhibition of β -Hex release (Fig. 2A). Notably, fractions 8 and 15 strongly inhibited the release of β -Hex (Fig. 2B). Multiple peaks were observed for fraction 15, whereas fraction 8 had a single peak. Therefore, we focused on the active compound indicated by an arrow in fraction 8, which showed high inhibition at a single peak.

3.3. Identification of an active component in BSHE

UPLC profiles and UV-Vis spectra of an isolated compound (A), authentic C3G (B), acidic or β -glucosidase hydrates of the isolated compound (C and D), and authentic Cy (E) are shown Fig. 3. The compound was predicted from aglycones produced by hydrolysis of glycosides. The UPLC-MS and UPLC-MS/MS data are shown in Figures S1 and S2, respectively. The isolated compound was identified using Rt, UV-Vis spectra, and UPLC-MS and UPLC-MS/MS data of hydrolysates and authentic compounds (Table 1) as follows:

The isolated compound: Rt (min) 6.57. UV-Vis: λ_{max} (nm) 281,

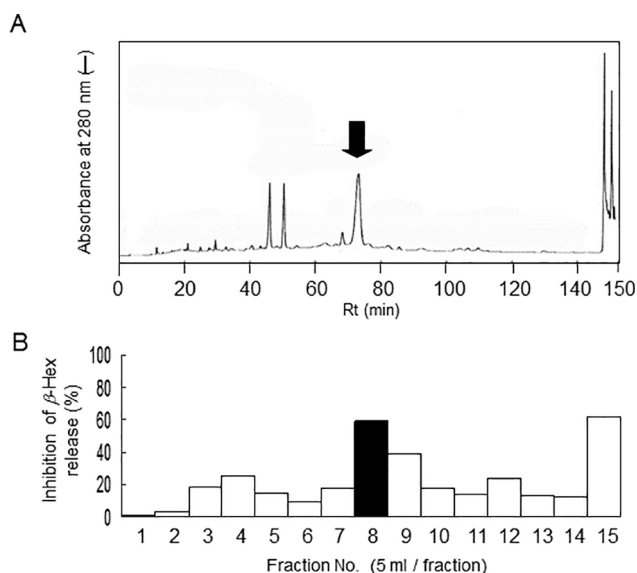


Fig. 2. Screening of active components in BSHE that inhibit the degranulation of RBL-2H3 cells based on the β -Hex assay. The active components in BSHE were separated by HPLC on an AtlantisTM dC₁₈ column with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid and monitored at 280 nm (A). The eluents were collected every 10 min and the fractions concentrated by freeze-drying were subjected to the β -Hex release assay in the same manner as described in the text (B). The arrow shows the peak corresponding to the active component.

515.7. MS: m/z 449.4 [M]⁺ (molecular ion peak, Cy + 1 mol glucose) and m/z 447.3 [M - 2H]⁻ (molecular ion peak, Cy + 1 mol glucose), which yielded MS/MS product ion m/z 287.2 [M]⁺ (Cy, [M - 162]⁺, loss of hexosyl unit) and m/z 285 [M - 2H]⁻ (Cy, [M - 162]⁻), respectively. The C3G standard corresponded with this peak; therefore, this compound was assumed to be C3G [19,31–33]. The acidic hydrolysed product: Rt (min) 8.81. UV-vis: λ_{max} (nm) 281, 523.7. MS: m/z 287.2 [M]⁺ (molecular ion peak, Cy) and m/z 285.2 [M - 2H]⁻ (molecular ion peak, Cy). The Cy standard corresponded with these hydrolysed peaks; therefore, these products were assumed to be Cy [31,32].

Furthermore, β -glucosidase treatment yielded the aglycon Cy from the glycoside C3G (Fig. 3D). In addition, hydrolysis of 20 nmol of the active compound yielded 18.4 nmol of D-glucose, suggesting that the active compound has 1 M of D-glucose bound to β -glucoside. These results demonstrate that the active compound of BSHE is C3G (chemical structure shown in Fig. 4). The contents of BSHE dissolved in HBS or PBS by HPLC analysis using authentic C3G were 4.9 and 5.4 g per 100 g of Kuromanin®-10, respectively.

3.4. Effect of C3G on degranulation of RBL-2H3 cells

The effects of commercial C3G, as compared with the anti-allergic compound luteolin, on the release of β -Hex and histamine from RBL-2H3 cells were investigated. As shown in Fig. 5, C3G significantly suppressed the release of β -Hex and histamine ($p < 0.01$), indicating that C3G in BSHE acts as an inhibitor of cellular degranulation. However, the IC₅₀ values of C3G and luteolin on β -Hex release were 110 and 0.8 µM and those on histamine release were 120 and 1.0 µM, respectively. Reportedly, luteolin is the strongest inhibitor of cellular degranulation among flavonoids [24], whereas C3G had no effect [32] on RBL-2H3 cells. Anthocyanins are stable under acidic conditions and form red flavylum cations, but are unstable under neutral and alkaline conditions. Moreover, anthocyanins exist as anionic quinoidal bases at neutral pH [18,34]. Therefore, C3G may undergo structural change or degradation when subjected to cell-based assay. Kay *et al.* [35] reported that incubation of C3G in Dulbecco's modified Eagle's medium for 4 h resulted in a 56% decrease in the inhibitory rate of C3G, as compared to 15% in phosphate buffer at pH 7.4. Meanwhile, there was no change in the inhibitory rate of C3G incubated in HBS for 1 h for the cell assay, suggesting that there would be no loss in this assay. On the other hand, the concentration of C3G in 250 µg/mL of BSHE was 25 µM, as determined by HPLC analysis, demonstrating that the inhibition of β -Hex and histamine release at this concentration reflected only about 15% and 30% of that with 250 µg/mL of BSHE, respectively (Fig. 5AB). These results are suggesting the presence of inhibitors, other than C3G. Peaks detected in fraction 15 in Fig. 2 also exhibited inhibition. In addition, Kuromanin®-10 contains proanthocyanidins [25]. Apple-derived polyphenols containing more than 90% procyanidins inhibited the release of histamine both *in vitro* and *in vivo* through IgE antibody binding to the FcεRI receptor, similar to the IC₅₀ value of ketotifen [36,37]. These findings, together with those from previous reports, suggest that procyanidin is a potential degranulation inhibitor in BSHE. However, the composition of procyanidins in Kuromanin®-10 is unknown, although dimers or trimers occur in soybean seed coat extracts [17].

3.5. Effect on PCA reactions of BSHE and C3G in mouse ears

Dye leakage was decreased by oral administered BSHE (1,000 mg/kg BW) containing 5.4% C3G prior to antigen stimulation, as compared with the control (70.2%, $p = 0.059$, Fig. 6A). In addition, oral administration of 200 µmol/kg of C3G had significantly suppressed the PCA reaction (48.8%, $p < 0.01$) to the same degree as treatment with 1.2 µmol/kg of ketotifen (37.8%, $p < 0.01$), while 100 µmol/kg of C3G had no effect. Because C3G in 1,000 mg/kg of BSHE is equivalent to about 120 µmol (98 mg)/kg, no significant suppression was expected, which supported the experimental findings of C3G administration. These

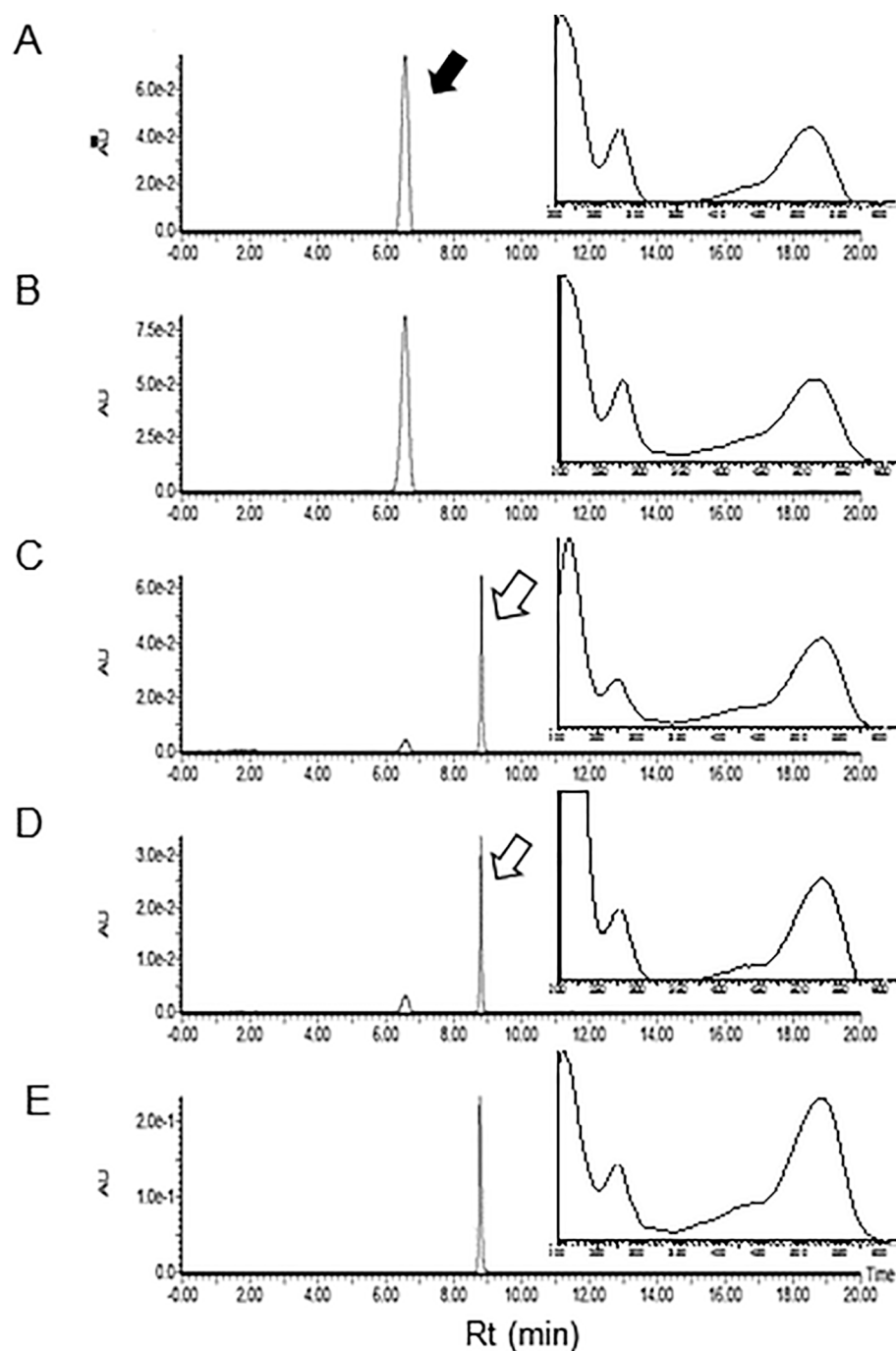


Fig. 3. UPLC profiles at 520 nm and Uv-vis spectra of the active component isolated from BSHE and its hydrolysates using HCl and β -glucosidase. The isolated component and its hydrolysates were separated by UPLC. The UV-Vis spectrum profiles for each peak are shown on the right side of each panel. (A) The isolated active component, (B) authentic C3G, (C) acidic hydrolytic products of the isolated component, (D) β -glucosidase-hydrolysate of the isolated component, and (E) authentic Cy. The closed and open arrows show the peak corresponding to the active compound and its products.

Table 1

UPLC-ESI-MS/MS and UV-Vis spectrum data for an isolated inhibitory compound in BSHE and its hydrolyzed product.

Peak ^a	Compound/Product	Rt (min)	UV λ Max (nm)	MS [M] ⁺	MS/MS [M] ⁺	MS [M - 2H] ⁻	MS/MS [M - 2H] ⁻	Tentative name
A	Isolated active compound	6.57	281, 515.7	449.4	287.2	447.3	285.2	Cyanidin-3-O-glucoside
B	Authentic C3G	6.56	281, 515.7	449.4	287.2	447.3	285.2	—
C	Acidic hydrolysate	8.81	281, 523.7	287.2	— ^b	285.2	— ^b	Cyanidin
D	β -Glycosidase-hydrolysate	8.77	281, 523.7	— ^c	— ^b	— ^c	— ^b	Cyanidin
E	Authentic Cy	8.80	281, 523.7	287.2	— ^b	285.2	— ^b	—

^a Corresponds to the peaks of the chromatogram marked with the letter in Fig. 3.

^b Not analyzed.

^c Not detected.

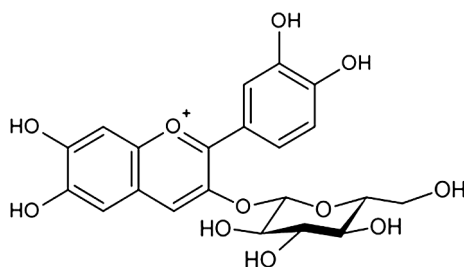


Fig. 4. The structure of C3G.

results indicate that orally administered C3G in BSHE acts as an inhibitor of allergic reactions, although it is not as potent as ketotifen, which is an antihistamine medication. Although 200 $\mu\text{mol/kg}$ of C3G is equivalent to approximately 1,700 mg/kg of BSHE, this dose is lower than 5074.1 mg/kg/day for males and 7617.9 mg/kg/day for females of BSHE, which had no observable adverse effect [38]. Therefore, this dose would provide no clinical effect. On the other hand, ovalbumin-induced allergic inflammation of the airway was suppressed by reducing eosinophil infiltration and Th2 cytokine production in mice fed a diet containing 0.4% of C3G for 2 weeks (i.e., C3G of 1.2 mg per mouse per day) [39]. Administration of C3G during the anti-sensitization period or continuous intake may cause an anti-allergic effect even at low doses.

In contrast, intravenous administration of BSHE at 100 mg/kg significantly suppressed the PCA reaction (27.4%, $p < 0.01$) (Fig. 6B), indicating that the intravenous administration of BSHE more strongly inhibited the PCA reaction at doses at 10% of that orally. In addition, intravenous C3G significantly suppressed PCA reaction at 40 mg/kg (29.4%, $p < 0.01$), indicating that the intravenous administration of C3G was more effective in suppressing the PCA reaction than oral administration, which is similar to the result with BSHE. Dose differences have been observed in experiments on the scratching behaviour of mice following oral and intraperitoneal administration of C3G prior to histamine administration [32]. The major difference between the oral and intravenous effects may be due to poor intestinal absorption of the active

ingredient in BSHE. It is generally accepted that C3G is slightly absorbed in the intestinal tract [40], although Czank *et al.* reported that the relative bioavailability of C3G was $12.38 \pm 1.38\%$ [41]. Moreover, C3G is metabolized to methylation [42] and glucuronic acid/sulfate conjugates [43] or metabolized into catabolites, such as protocatechuic acid, by the microbiota in the large intestine [34,42–44]. In addition, the absorption of procyanidin, presumed to be another active component of BSHE, is thought to be lower, as some of the B-type procyanidin oligomers and (–)-epicatechin are absorbed in the small intestine and exist mainly as conjugates in plasma. Notably, the monomeric unit (–)-epicatechin had the highest bioavailability, followed by procyanidin B2 and procyanidin C1 [45]. A similar phenomenon was reported for commercially available catechins and cocoa polyphenols. Monomers of catechins and epicatechins are well absorbed [46], while dimers are only slightly absorbed [47]. In Fig. 6A, the inhibitory effect of BSHE could be explained by the effect of only C3G contained in BSHE, indicating that the inhibitory effects attributed to substances different from C3G observed *in vitro* were not observed *in vivo* via oral administration. Conversely, in Fig. 6B, the inhibitory effect of C3G required several times the amount that was contained in BSHE. This means that intravenous administration may have been affected by other compounds that are not absorbed by oral administration as seen *in vitro*. Therefore, procyanidins are other potential inhibitor candidates in BSHE, although the absorption of these polymers may be limited. To support the claims about the anti-allergic effects of black bean hulls, the anti-allergic effects and its absorbency of active compounds, other than C3G, must be investigated in future studies.

On the other hand, the soybean is considered an allergy-inducing food [48,49] and soybean allergens have cross-reactivity with fruit allergens and pollen with similar structures, which can lead to pollen food allergy syndrome [49,50]. Therefore, the use of BSHE for patients with soybean allergies or cross-reactive allergy with soybean should be further investigated.

In conclusion, the results of the present study demonstrated that BSHE has anti-allergic effects both *in vitro* and *in vivo* and one of the active components is C3G. Intravenous administration of BSHE and C3G

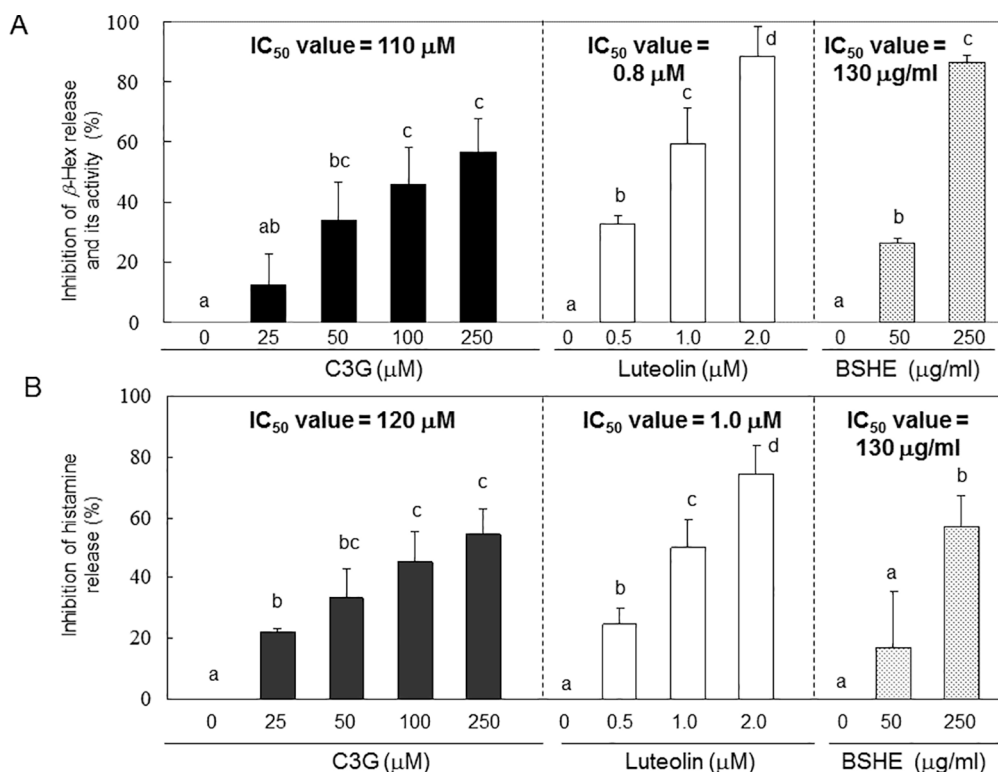


Fig. 5. Inhibitory effect of C3G on β -Hex release (A) and histamine release (B) by RBL-2H3 cells. RBL-2H3 cells sensitized by anti-DNP IgE were incubated with DNP-Alb and the β -Hex and histamine released into the medium were assayed. The results are expressed as the percentages of the amount of β -Hex and histamine released in the presence of C3G and luteolin (positive control) to those in the absence of each. Values are presented as the mean $\pm$ SD of three determinations. IC_{50} of compounds and BSHE are shown in each inside panel.

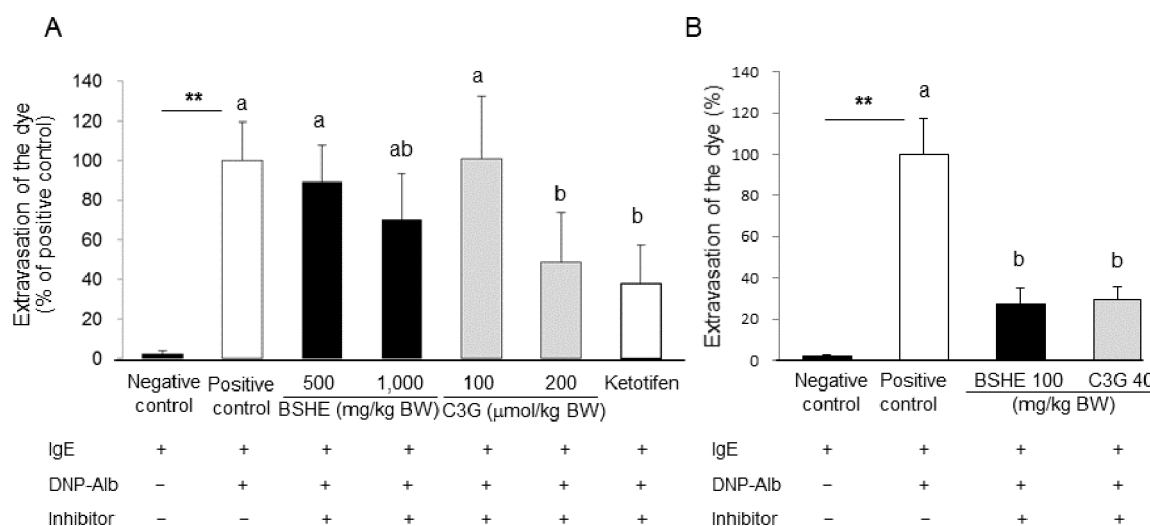


Fig. 6. Effects of BSHE and C3G orally administered (A) and intravenously administered (B) on the PCA reaction. The PCA reaction was conducted as described in the text. Anti-DNP IgE was intravenously injected and sensitized for 24 h. Mice were orally administered BSHE at 500 and 1,000 mg/kg BW, C3G at 100 and 200 µmol/kg BW, ketotifen at 1.2 µmol/kg BW, and PBS (positive control) or intravenous administered BSHE at 100 mg/kg BW, C3G at 40 mg/kg BW, and PBS (positive control). After 60 min, DNP-Alb was intravenously injected and the PCA reaction was induced. The mice were sacrificed at 30 min after ingestion of the antigen and the permeated dye was extracted from the ears. The amount of extravasated dye was determined by measuring the absorbance at 620 nm. Data are presented as means $\pm$ SD for oral and intravenous administrations in six and four mice, respectively. Different letters indicate significant differences among groups, $p < 0.05$ (Tukey–Kramer multiple-range test). Significant differences between the negative and positive controls were detected using the Student's t -test at $**p < 0.01$.

more strongly inhibited PCA responses at lower doses than oral administration, suggesting that the difference would be due to the absorption of BSHE and C3G. This information can prove useful for the design of appropriate formulations of anthocyanin-based products to suppress allergic reactions. This study found that C3G, a pigment of the black soybean hull, has anti-allergic effects for the potential treatment of type I allergies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2021.107394>.

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