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Effect of Polyphenols in Sea Buckthorn Berry on Chemical Mediator Release from Mast Cells

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ABSTRACT: Sea buckthorn (*Hippophae rhamnoides* L.) is a deciduous shrub of the Elaeagnaceae family and is widely distributed in northern Eurasia. Sea buckthorn berry (SBB) has attracted attention for its use in many health foods, although its physiological function remains unknown. In this study, we investigated the inhibitory effect of SBB extract and its fractions on Type-I allergy using mast cell lines. Among these fractions, SBB fraction with the highest amount of antioxidant polyphenols significantly inhibited the release of chemical mediators such as histamine and leukotriene B_4 (LTB_4) from the stimulated mast cells. This fraction also inhibited the influx of calcium ions (Ca^{2+}) and the phosphorylation of tyrosine residues in proteins, including spleen tyrosine kinase, which is associated with signal transduction during the release of chemical mediators. The active SBB fraction contained isorhamnetin as its major flavonol aglycon. Isorhamnetin inhibited histamine and LTB_4 release from the stimulated cells and suppressed intracellular Ca^{2+} influx. These results indicate that isorhamnetin is the primary substance responsible for the antiallergic activity in SBB. In conclusion, SBB may alleviate Type-I allergy by inhibiting the release of chemical mediators from mast cells, and polyphenols may contribute to this effect.

Keywords: allergy, isorhamnetin, mast cells, polyphenols, sea buckthorn

INTRODUCTION

Allergy is a disorder involving an excessive immune response to harmless foreign substances and is commonly classified into four types based on their mechanisms (Warrington et al., 2011; Dispenza, 2019). Type-I allergy is characterized by immediate hypersensitivity to various antigens, such as pollen and food antigens (Duangmee et al., 2022), with mast cells and basophilic leukocytes playing an essential role in the response (Razin et al., 1995; González-de-Olano and Álvarez-Twose, 2018). The antigen binds specifically to immunoglobulin E (IgE), bound to Fcε receptor I (FcεRI) on the cell membrane, and crosslinks IgEs. The crosslinking triggers intracellular signaling, such as the phosphorylation cascade of proteins and the subsequent influx of calcium ions (Ca^{2+}) into the cytoplasm. This stimulation causes degranulation, releasing histamine stored in the granules into the extracellular space (Beaven et al., 1984; Siraganian, 2003; Amin et al., 2005). Additionally, the intracellular signaling activates phospholipase A_2, which releases arachidonic acids from the membrane phospholipids. The arachidonic acids are then converted to leukotrienes (LTs), such as LTB_4, through cascade reactions mediated by 5-lipoxygenase (LOX), and these LTs are secreted into the extracellular space (Lee et al., 1984). Histamine and LTs act as chemical mediators that promote vasodilation, vascular permeability, smooth muscle contraction, and leukocyte chemotaxis, inducing various allergy symptoms such as mucus secretion and sneezing (Amin, 2012). Therefore, compounds that can prevent allergy symptoms are evaluated based on their inhibitory effects on mast cell degranulation and the arachidonate cascade. However, due to their different mechanisms of action, the inhibitory potency of the compounds against these pathways may differ. For example, soy isoflavones and their metabolites have been shown to inhibit LTB_4 production but not histamine release (Takasugi et al., 2014).

In recent years, research on bioactive components, such as flavonoids in herbs and natural plant resources, has attracted considerable attention (Kumar and Pandey, 2013; Castell et al., 2014; Ko et al., 2018; Mashhadi Akbar Boojar, 2020). Functional foods developed through these studies are expected to alleviate allergy symptoms with

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reduced side effects (Kawai et al., 2007). In addition, many natural products have been used in the clinical treatment of chronic diseases, offering potential alternatives to conventional therapies (Chin et al., 2006; Zhang et al., 2013).

Sea buckthorn (*Hippophae rhamnoides* L.) is a deciduous shrub of the Elaeagnaceae family, native to northern Eurasia, and cultivated in Hokkaido, Japan (Sabir et al., 2003; Wang et al., 2022). Sea buckthorn berry (SBB), the yellow-orange fruit of the sea buckthorn, is rich in vitamins, carotenoids, and flavonoids (Ciesarová et al., 2020) and are used in various food processing applications, including brewing (Vilas-Franquesa et al., 2020; Belcar and Gorzelany, 2022). SBB has been shown to exhibit antioxidant, antibacterial, and antiviral effects (Olas et al., 2016; Yue et al., 2017; Kurskaya et al., 2022) and is expected to have a high therapeutic and medicinal potential (Suryakumar and Gupta, 2011). However, the immunomodulatory effects of SBB have been little studied (Geetha et al., 2002; Żuchowski, 2023). In previous studies, many compounds including polyphenols, have been isolated from SBB, with its flavonoid profile primarily comprising isorhamnetin, quercetin, and kaempferol glycosides (Fang et al., 2013; Gu et al., 2022). In this study, we evaluated the inhibitory activities of SBB extract and its fractions on the release of chemical mediators from mast cells using cell lines associated with Type-I allergy. Furthermore, we discussed the mechanism and causative substances of the suppressive effect of SBB on allergic reactions.

MATERIALS AND METHODS

Analysis of nutritional composition

The nutritional composition of SBB was analyzed using various methods based on the Japanese Agricultural Standards. The crude protein and fat in the sample were determined by the Kjeldahl (Sapan et al., 1999) and the Soxhlet (Saini et al., 2021) methods, respectively. The moisture and crude ash contents were measured by the atmospheric heat drying method and the burning method, respectively.

Preparation of extract and fractions

Frozen SBB was purchased from a local agricultural cooperative (Shihoro). SBB (1 kg) was homogenized with 2 L of 70% methanol (MeOH), sonicated for 10 min, and kept at 4°C overnight. The extract was centrifuged at 5,600 *g* for 20 min at 4°C and filtered through filter paper. The residue was re-extracted following the same procedure. The combined extracts were evaporated and subsequently lyophilized. The resulting extract was dissolved in water and subjected to reversed-phase column chromatography using Diaion HP-20 (50×200 mm, 250 μm, Mitsubishi Chemical Corp.). The extract was eluted twice with 200 mL of water and 200 mL of 20～100% aqueous MeOH with a stepwise gradient, producing seven fractions, which were then lyophilized.

Determination of total polyphenols

The total polyphenol content in the SBB extract and its fractions was determined by the Folin-Ciocalteu method (Chapin, 1921). The SBB extract and its fractions dissolved in 200 μL of water were mixed with 200 μL of 1 N Folin-Ciocalteu's reagent (Nacalai Tesque Inc.) and incubated for 3 min at room temperature. Subsequently, 200 μL of 10% Na_2CO_3 (w/v) was added, and the mixture was incubated for 30 min at 30°C in the dark. The absorbance of the reaction mixture at 760 nm was measured and calculated using a standard curve of gallic acid obtained in the same experiment. The results were expressed as milligrams of gallic acid equivalent (GAE).

Radical scavenging assay

The radical scavenging abilities of the SBB extract and its fractions were evaluated by the method described previously with 2,2-diphenyl-1-picrylhydrazyl (DPPH, FUJIFILM Wako Pure Chemicals) (Blois, 1958; Zorig et al., 2021). The samples (20 μg/mL) were mixed with 100 μM DPPH in 75% ethanol and incubated for 30 min at 30°C in the dark. After centrifugation at 20,000 *g* for 5 min at 20°C, the absorbance of the reaction mixture was measured at 517 nm.

Analysis of flavonol aglycones

The flavonol aglycones in the fraction were measured by reversed-phase high-performance liquid chromatography (HPLC) based on the method proposed by Olszewska with some modifications (Olszewska, 2008). The sample was dissolved in 70% EtOH and incubated with 650 mM HCl for 60 min at 90°C. The hydrolyzed sample was analyzed on an InertSustain C18 column (4.6×250 mm, 5 μm, GL Sciences) at 40°C. The elution was conducted with a mobile phase comprising 0.5% phosphoric acid (v/v, A) and MeOH (B) at a flow rate of 0.7 mL/min. The gradient was as follows: 0～10 min, 40～60% B; 10～21 min, held at 60% B; 21～23 min, 60～40% B; 23～38 min, held at 40% B. The absorbance of the eluate was monitored at 370 nm. Commercially available kaempferol, quercetin, and isorhamnetin (Extrasynthese) were used as standards.

Cell culture

A rat basophilic leukemia cell line (RBL-2H3) and a mouse mast cell line (PB-3c) were obtained from the JCRB Cell Bank. RBL-2H3 cells were cultured in Eagle's minimal essential medium (MEM, FUJIFILM) containing 10% fetal bovine serum (FBS; HyClone Laboratories),

100 units/mL penicillin, and 100 μg/mL streptomycin. PB-3c cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (FUJIFILM) containing 10% FBS, 2 mM L-glutamine, 25 mM HEPES (FUJIFILM), 1% MEM non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 0.0035 μL/mL 2-mercaptoethanol, 2 ng/mL interleukin-3 (FUJIFILM), 100 units/mL penicillin, and 100 μg/mL streptomycin. The cultures were maintained at 37°C in 5% CO_2. The cytotoxicity of the samples was measured by the trypan blue dye exclusion assay, the most common test for cell viability.

Histamine release assay

The inhibitory activity of the SBB extract and its fractions against histamine release from the cells was evaluated using the method described previously (Matsuo et al., 1997; Li et al., 2005; Zorig et al., 2021). RBL-2H3 cells were incubated with a mouse anti-2,4-dinitrophenyl (DNP) IgE monoclonal antibody (Yamasa) in the MEM at a density of 4×10^5 cells/well for 20 h using 24-well cell culture microplates. The cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated with an appropriate amount of the samples in 450 μL of Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.6 mM glucose; pH 7.2) containing 0.05% bovine serum albumin (BSA, Sigma-Aldrich) for 10 min at 37°C. The cells were stimulated by adding 50 μL of 2 μg/mL DNP-BSA (Millipore) in Tyrode buffer for 20 min at 37°C. To measure the amount of histamine release without stimulation and the total amount of histamine in the cytoplasmic granules, 50 μL of Tyrode buffer and 5% Triton X-100 were added instead of DNP-BSA, respectively. An aliquot (100 μL) of the cell supernatant was mixed with 50 μL of 8 μM 1-methylhistamine as an internal standard and 50 μL of 200 mM *N*-acetyl-L-cysteine. The histamine in the solutions was determined by reversed-phase HPLC using a polymer-based column (Shodex ODP-50-4E, 4.6× 250 mm, 5 μm, Showa Denko) at 50°C with fluorescence detection (excitation, 340 nm; emission, 450 nm) (von Vietinghoff et al., 2006). The sample was isocratically eluted with MeOH/water (35:65, v/v) containing 0.2 mM *o*-phthalaldehyde and 30 mM $Na_2B_4O_7$ at a 0.7 mL/min flow rate.

LTB_4 production assay

The inhibitory activity of the SBB extract and its fractions against LTB_4 production by mast cells was evaluated according to the method described previously (Takasugi et al., 2018). PB-3c cells were cultured for 48 h in the RPMI-1640 medium supplemented with 50 μM of arachidonic acid. After washing twice with PBS, 4×10^6 cells were incubated with an appropriate amount of the samples in 180 μL of Tyrode buffer for 10 min at 37°C. The cells were stimulated by adding 20 μL of 10 μM calcium ionophore A23187 (Sigma-Aldrich) for 20 min at 37°C. Acetonitrile/MeOH (30:25, v/v) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbic acid, and 1 μM prostaglandin B_2 (Cayman Chemical) as an internal standard was added to terminate the reaction. After centrifugation at 20,000 *g* for 15 min at 4°C, LTB_4 in the supernatant was determined through reversed-phase HPLC using an ODS-A column (6.0×150 mm, 5 μm, YMC) at 40°C with a UV detector monitored at 280 nm. The sample was isocratically eluted with 5 mM CH_3COONH_4/acetonitrile/MeOH (30:25:45, v/v/v) at a 1.0 mL/min flow rate.

Analysis of cytoplasmic calcium ions

The change in Ca^{2+} concentration during the stimulation of RBL-2H3 cells was monitored using the Calcium Kit II-Fluo 4 (Dojindo Laboratories Co., Ltd.) as described by Zorig et al. (2021). RBL-2H3 cells were cultured at 3×10^4 cells/100 μL with anti-DNP IgE in 96-well clear-bottomed black microplates for 20 h at 37°C. The cells were incubated with an appropriate amount of the samples in the loading buffer containing 5 μg/mL Fluo 4-AM, a Ca^{2+} fluorescent probe, for 1 h at 37°C. Subsequently, they were stimulated with DNP-BSA (400 ng/mL), and the fluorescence intensity (excitation, 485 nm; emission, 520 nm) was measured continuously using a fluorescence microplate reader (Wallac 1420 ARVO MX/Light, Perkin-Elmer Corp.) at 37°C.

Analysis of intracellular signaling proteins

The intracellular signaling proteins in RBL-2H3 cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described (Laemmli, 1970; Zorig et al., 2021). RBL-2H3 cells were cultured and stimulated by the antigen-IgE reaction on a 24-well microplate as described above. The cells were washed twice with cold Tris-buffered saline (TBS, pH 7.4) containing 1 mM EDTA. Afterward, 100 μL of RIPA lysis buffer (EzRIPA Lysis Kit, ATTO) containing 1% protease inhibitor and phosphatase inhibitor was added to the cells and incubated for 15 min on ice. The cell lysate was collected with a cell scraper and centrifuged at 20,000 *g* for 20 min at 4°C. The supernatant was mixed with Laemmli sample buffer (Bio-Rad Laboratories) and denatured for 5 min at 95°C. The samples were subjected to SDS-PAGE using a gradient polyacrylamide gel (AnykD TGX gel, Bio-Rad Laboratories). The proteins on the gel were transferred to a polyvinylidene difluoride membrane, which was treated with a blocking buffer (LI-COR) for 1 h. After washing with 0.05% Tween-TBS, the membrane was incubated with antibodies against phospho-tyrosine (p-Tyr, mouse monoclonal: 4G10, Millipore), phospho-spleen tyrosine kinase

(p-Syk, rabbit polyclonal, Abcam), or β-actin (mouse monoclonal: 8H10D10, Cell Signaling Technology) in an immunoreaction enhancer solution (TOYOBO) for 1～2 h. After washing with 0.05% Tween-TBS, the membrane was incubated with polyclonal antibodies against mouse or rabbit IgG conjugated with IRDye (800CW or 680RD, LI-COR). The immunoreactivity was measured by near-infrared fluorescence at 800 nm and 680 nm using the Odyssey CLx (LI-COR).

Statistical analysis

Data (n=3) were presented as mean±standard deviation. The statistical significance of differences was analyzed by the Tukey-Kramer multiple comparison test using Mac statistical analysis ver. 3.0 (Esumi Co., Ltd.). The differences with *P*-values less than 0.05 were considered statistically significant. The experiments were repeated several times to confirm the reproducibility of the results.

RESULTS

Nutritional composition of SBB

The crude protein, crude fat, carbohydrate, moisture, and crude ash contents in 100 g of frozen SBB were 1.6, 3.7, 12.4, 81.9, and 0.4 g, respectively.

Fractionation of SBB extract

The extraction yield of SBB prepared with 70% MeOH was 5.65%. Seven fractions were isolated from the SBB extract using chromatography, and their yields are shown in Table 1. Fractions (Fr.) 1～3, containing hydrophilic substances such as sugars, exhibited high yields, while Fr. 4～7, containing hydrophobic substances, exhibited relatively low yields.

Table 1. The yield of sea buckthorn berry fractions

Fractions	Solvent ratio (methanol:water, v/v)	Yield (%)
1	0:100	27.40
2	0:100	38.98
3	20:80	9.90
4	40:60	1.20
5	60:40	1.90
6	80:20	2.43
7	100:0	0.40

Polyphenol contents and radical scavenging abilities of SBB extract and its fractions

The total polyphenol content of the SBB extract and Fr. 1～3 was 25～50 mg GAE/g of dry weight (Fig. 1A). On the other hand, Fr. 5～7 showed significantly higher polyphenol contents than those of the extract and Fr. 1～3, with values of 500～600 mg GAE/g. The DPPH radical scavenging abilities of Fr. 5 and 6 were approximately 90%, significantly higher than those of the extract and other fractions (Fig. 1B).

Effects of SBB extract and its fractions on chemical mediator release from mast cells

The inhibitory activities of the SBB extract and its fractions on histamine release from RBL-2H3 cells by antigen-IgE stimulation were examined at 200 μg/mL to evaluate their antiallergic activity. Fr. 5 and 6 significantly suppressed histamine release from the stimulated cells, representing approximately 39% and 23% of the control, respectively (Fig. 2A). However, the extract and the other fractions showed no inhibitory effect at 200 μg/mL. The inhibitory activity of Fr. 6 tended to be stronger than that of Fr. 5. The SBB extract and its fractions were also evaluated for their inhibitory effect on LTB_4 production by PB-3c cells. Fr. 5 and 6 significantly suppressed LTB_4 production by the stimulated cells, approximately 18% of the control at 1 mg/mL (Fig. 2B). Fr. 7 also appeared to suppress LTB_4 production. In contrast, the SBB extract and

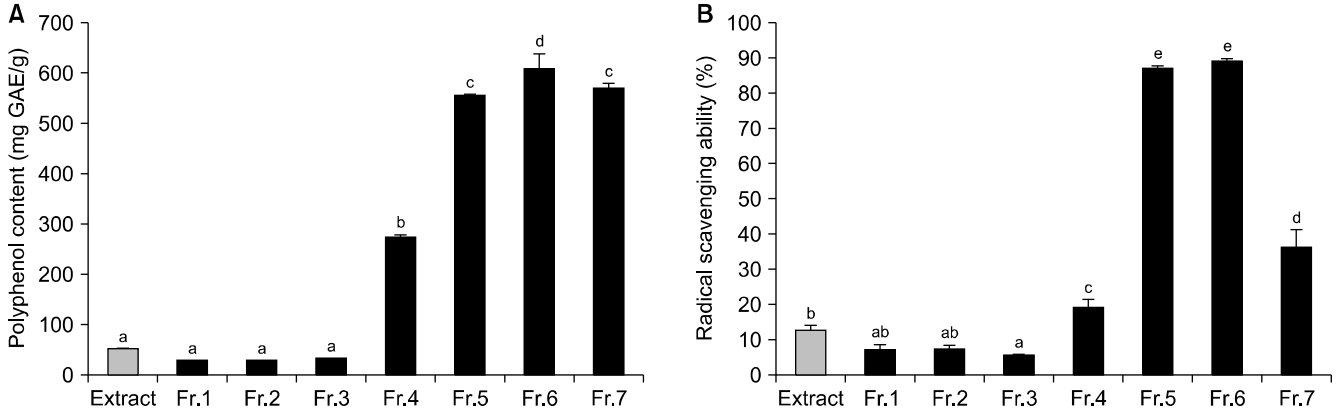

Fig. 1. Polyphenol contents and radical scavenging abilities of sea buckthorn berry extract and its fractions (Fr.). (A) Polyphenol contents. The results were expressed as mg gallic acid equivalent (GAE). (B) Radical scavenging ability. Data are presented as mean±SD (n=3). Different letters (a-e) indicate significant differences (P<0.05) between groups.

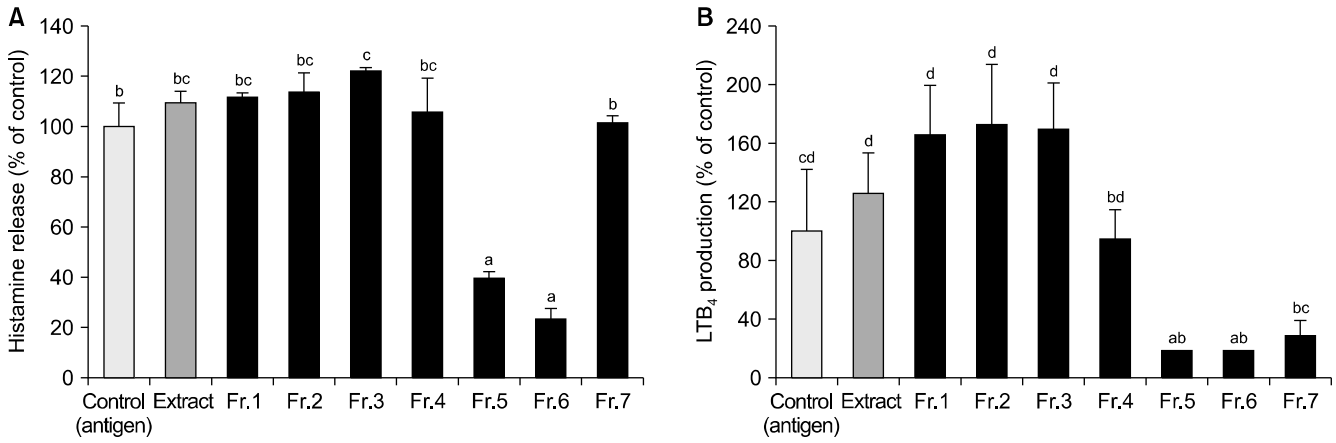

Fig. 2. Effect of sea buckthorn berry extract and its fractions (Fr.) on chemical mediator release from mast cells. (A) Histamine release from RBL-2H3 cells. (B) Leukotriene B_4 (LTB_4) production by PB-3c cells. Data are presented as mean±SD (n=3). Different letters (a-d) indicate significant differences ($P<0.05$) between groups.

the other fractions tended to increase LTB_4 production, although the effects were not statistically significant. The trypan blue assay confirmed that the SBB extract and its fractions were not cytotoxic against RBL-2H3 and PB-3c cells at 400 μg/mL and 2.0 mg/mL, respectively (data not shown).

Effects of Fr. 6 on allergic reactions in mast cells

Fig. 3 shows the effects of Fr. 6 on chemical mediator release. Fr. 6 dose-dependently inhibited histamine release from the stimulated RBL-2H3 cells at 50, 100, and 200 μg/mL (Fig. 3A). Similarly, Fr. 6 dose-dependently inhibited LTB_4 production by the stimulated PB-3c cells at 0.5, 1.0, and 2.0 mg/mL (Fig. 3B). Fr. 6 was not cytotoxic to RBL-2H3 and PB-3c cells at 1.0 mg/mL and 2.0 mg/mL, respectively (data not shown).

Fig. 4A shows the effect of Fr. 6 on changes in cytoplasmic Ca^{2+} concentration in RBL-2H3 cells stimulated by the antigen-IgE reaction. The relative fluorescence intensity of the control increased gradually after the stimulation (arrowhead), whereas no change was observed without the stimulation. The relative fluorescence intensity after the stimulation was dose-dependently suppressed by Fr. 6 at 0.5 and 1.0 mg/mL and by more than 50% of the control at 1.0 mg/mL. Fig. 4B shows a representative result of Western blot analysis of p-Tyr (upper panel), p-Syk (middle panel), and β-actin (lower panel) in stimulated RBL-2H3 cell lysates. Compared to the unstimulated cells (Lane 1), cell stimulation increased tyrosine-phosphorylated proteins from 38 to 160 kDa and phosphorylated Syk (control, Lane 2). Using anti-p-Syk antibody, an overlay detection against the membrane identified the control band of p-Tyr with a molecular weight of approximately 70 kDa (upper panel, arrowhead) as p-Syk at 72 kDa (middle panel). Fr. 6 (400 μg/mL) inhibited the phosphorylation of tyrosine and Syk in the stimulated cells (Lane 3). No differences were observed in the band intensities of β-actin, a housekeeping protein, detected at 42 kDa among unstimulated, control (stimulated), and Fr. 6 lanes. Fig. 4C and 4D show the relative band intensities of p-Tyr and p-Syk normalized to that of β-actin, respectively, with the experiment performed in triplicate. Fr. 6 significantly suppressed the phosphorylation of tyrosine and Syk compared to the control.

Effects of major flavonol aglycones in Fr. 6 on chemical mediator release from mast cells

The major flavonol aglycones (Fig. 5A) in Fr. 6 were ana-

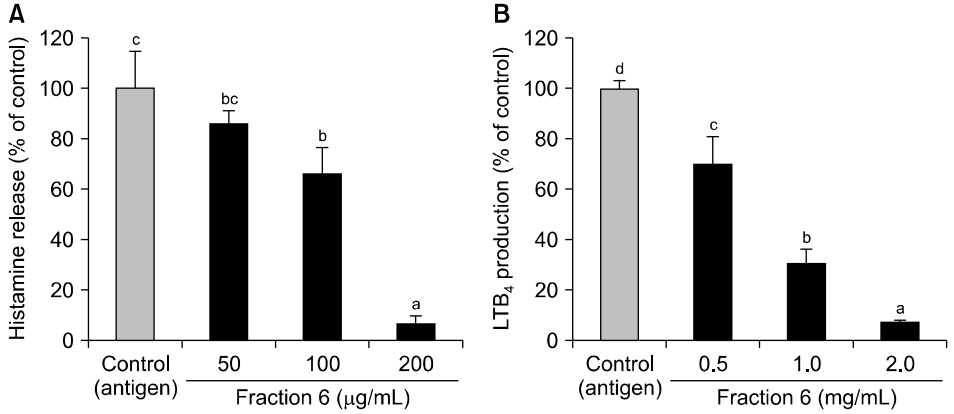

Fig. 3. Dose-dependent inhibitory effect of fraction 6 on chemical mediator release. (A) Histamine release from RBL-2H3 cells. (B) Leukotriene B_4 (LTB_4) production by PB-3c cells. Data are presented as mean±SD (n=3). Different letters (a-d) indicate significant differences ($P<0.05$) between groups.

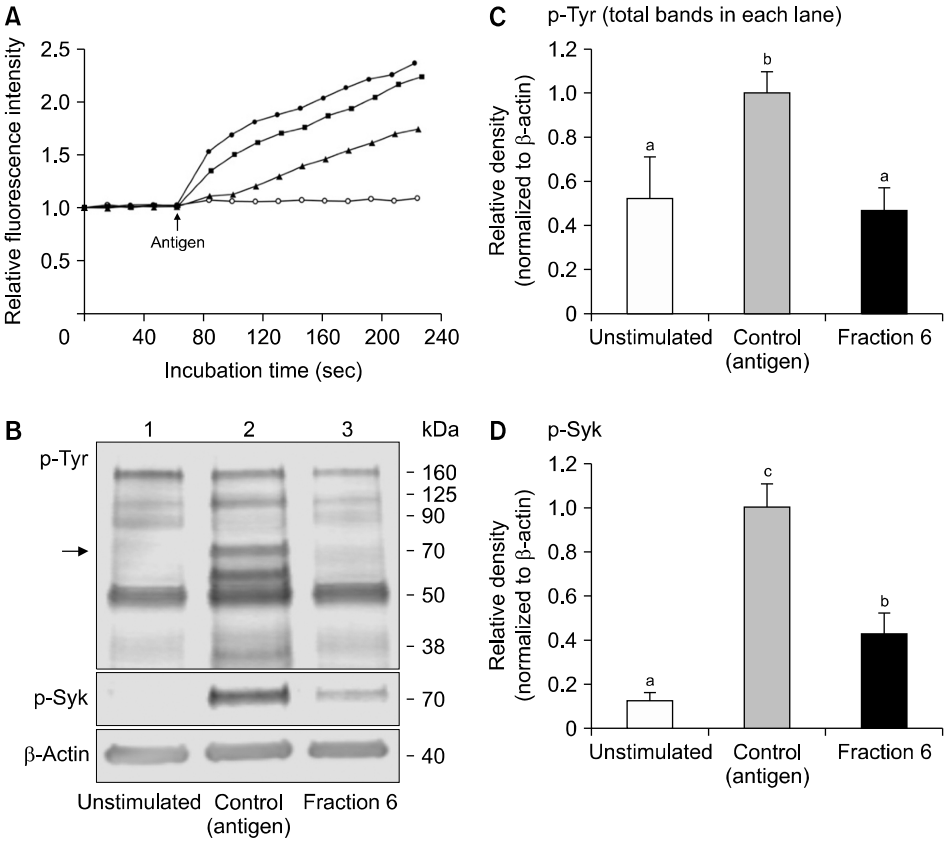

Fig. 4. Effect of fraction (Fr.) 6 on mast cell signaling. (A) Time-course analysis of cytoplasmic Ca^{2+} concentration. Unstimulated (○), control (●), 0.5 mg/mL Fr. 6 (■), and 1.0 mg/mL Fr. 6 (▲). (B) Phosphorylation of tyrosine (Tyr) and spleen tyrosine kinase (Syk). Lane 1, unstimulated; Lane 2, control; and Lane 3, Fr. 6 (400 μg/mL). The experiments were performed in triplicate, and a representative result was presented. (C and D) The relative densities of p-Tyr (total bands in each lane) and p-Syk (72 kDa) normalized to β-actin, respectively. Data are presented as mean±SD (n=3). Different letters (a-c) indicate significant differences (P<0.05) between groups.

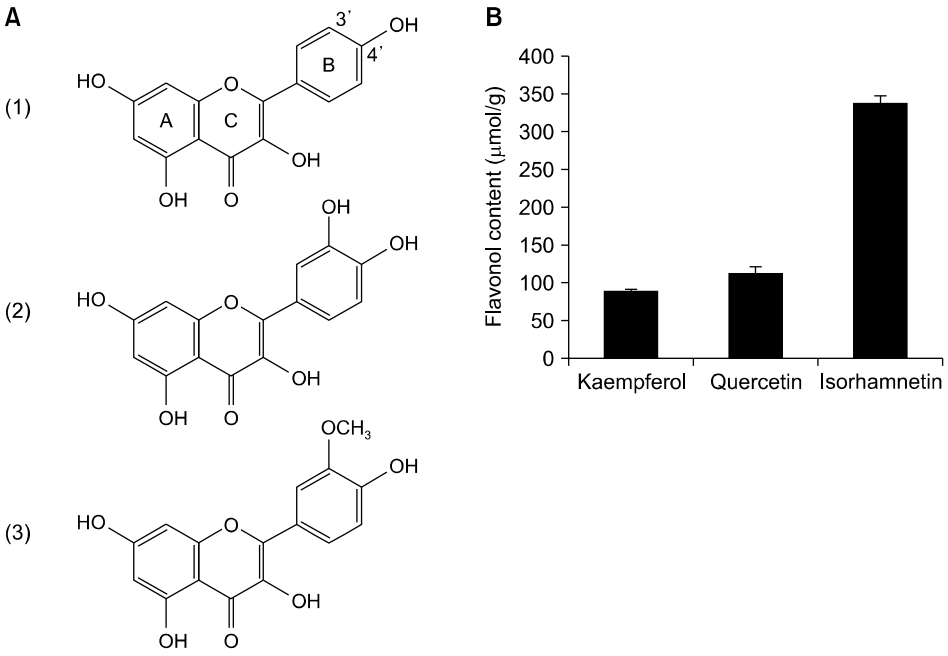

Fig. 5. Major flavonols in fraction (Fr.) 6. (A) The structures of kaempferol (1), quercetin (2), and isorhamnetin (3). (B) The contents of major flavonols. The results were reported as μmol per gram of Fr. 6. Data are presented as mean±SD (n=4).

lyzed by HPLC, with isorhamnetin being the most abundant (337.9 μmol/g dry weight of Fr. 6) as shown in Fig. 5B. The amounts of kaempferol (89.1 μmol/g) and quercetin (111.7 μmol/g) were lower than that of isorhamnetin.

Fig. 6 shows the effects of the major flavonol aglycones found in Fr. 6 on histamine release from the stimulated RBL-2H3 cells (Fig. 6A) and LTB_4 production by the stimulated PB-3c cells (Fig. 6B) using authentic standards of kaempferol, quercetin, and isorhamnetin at 20 μM. The three flavonols significantly suppressed the histamine release, with isorhamnetin exhibiting stronger inhibitory activity than kaempferol and quercetin. The three flavonols also inhibited LTB_4 production, with isorhamnetin and quercetin exhibiting stronger inhibitory activities than kaempferol. The three flavonols were not cytotoxic

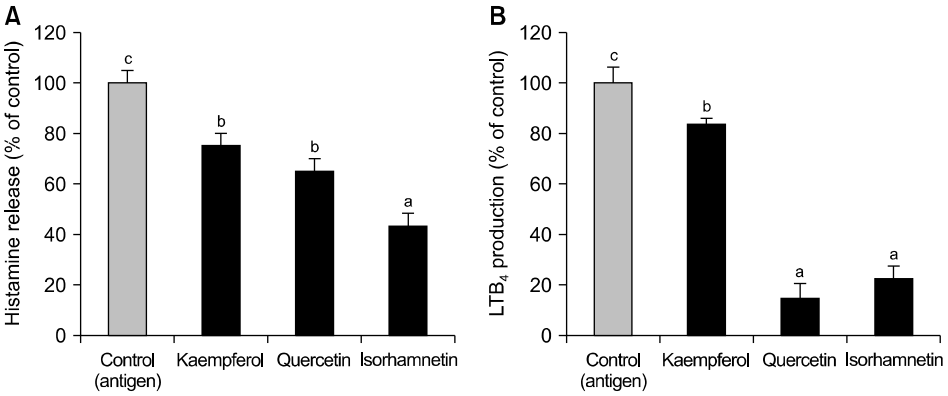

Fig. 6. Effects of major flavonols in fraction 6 on chemical mediator release from mast cells. (A) Histamine release from RBL-2H3 cells. (B) Leukotriene B_4 (LTB_4) production by PB-3c cells. Data are presented as mean±SD (n=3). Different letters (a-c) indicate significant differences (P<0.05) between groups.

to RBL-2H3 and PB-3c cells at 20 μM (data not shown).

Effects of isorhamnetin on allergic reactions in mast cells

Fig. 7 shows the effects of isorhamnetin on the chemical mediators. Isorhamnetin significantly suppressed histamine release from the stimulated RBL-2H3 cells in a dose-dependent manner at 5, 10, and 20 μM (Fig. 7A), with approximately 40% of the control at 20 μM. Isorhamnetin also significantly inhibited LTB_4 production by the stimulated PB-3c cells in a dose-dependent manner at 2, 5, and 10 μM (Fig. 7B), with approximately 5% of the control at 10 μM.

Fig. 8A illustrates the effect of isorhamnetin on changes in cytoplasmic Ca^{2+} concentration in the stimulated RBL-2H3 cells. The relative fluorescence intensity of the control increased poststimulation (arrowhead). Isorhamnetin dose-dependently inhibited the increase in fluorescence intensity at 5, 10, and 20 μM. Fig. 8B shows the effect of isorhamnetin on the phosphorylation of Tyr and Syk in the stimulated RBL-2H3 cells. A representative result of Western blot analysis indicates that tyrosine-phosphorylated proteins (upper panel) and phosphorylated Syk (middle panel) were increased in the stimulated cells (control, Lane 2) compared to the unstimulated cells (Lane 1). The band intensities of β-actin in the cell lysates ensure equal protein loading in the unstimulated, control (stimulated), and isorhamnetin (20 μM) lanes. The suppression of Tyr and Syk phosphorylation by 20 μM isorhamnetin was much less effective than that by 400 μg/mL Fr. 6. Fig. 8C and 8D show the relative band intensities of p-Tyr and p-Syk normalized to that of β-actin, respectively. There was a trend toward suppression of Tyr and Syk phosphorylation following treatment with 20 μM isorhamnetin; however, the difference was not statistically significant.

DISCUSSION

Common allergies, such as hay fever and food allergies, are immediate-type hypersensitivity reactions, classified as Type-I. In immediate-type allergies, mast cells and basophilic leukocytes play an important role in releasing chemical mediators that cause symptoms. Therefore, inhibiting these cellular responses is key to alleviating allergic reactions (Duangmee et al., 2022). Recently, natural plant compounds, such as polyphenols found in edible and medicinal plants, have been expected to safely alleviate allergy symptoms compared to medicines with potential side effects such as headaches and drowsiness (Bellik et al., 2012). SBB is rich in bioactive compounds and has been traditionally used in China and Mongolia to treat asthma and skin conditions (Singh et al., 2011). Flavonols such as kaempferol, quercetin, and isorhamnetin in SBB are associated with its physiological functions (Żuchowski, 2023). We aimed to clarify the antiallergic effects of SBB using cell lines and identify the active com-

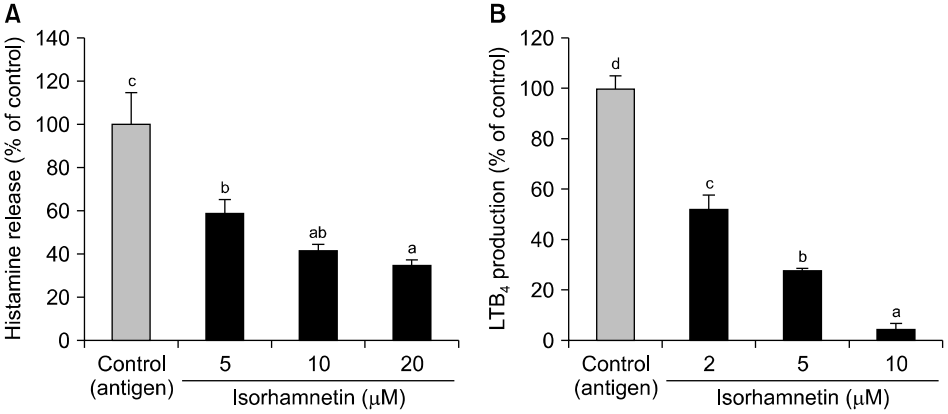

Fig. 7. Dose-dependent inhibitory effect of isorhamnetin on chemical mediator release. (A) Histamine release from RBL-2H3 cells. (B) Leukotriene B_4 (LTB_4) production by PB-3c cells. Data are presented as mean±SD (n=3). Different letters (a-d) indicate significant differences (P<0.05) between groups.

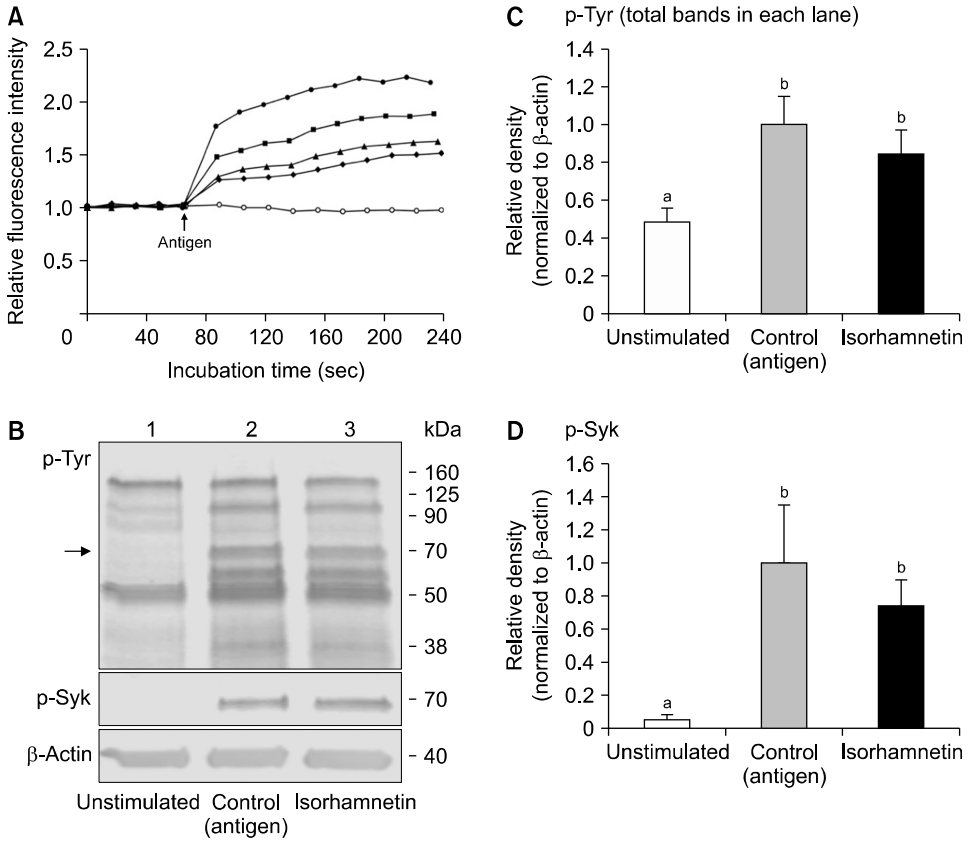

Fig. 8. Effect of isorhamnetin on mast cell signaling. (A) Time-course analysis of cytoplasmic Ca^{2+} concentration. Unstimulated (○), control (●), 5 μM isorhamnetin (■), 10 μM isorhamnetin (▲), and 20 μM isorhamnetin (◆). (B) Phosphorylation of tyrosine (Tyr) and spleen tyrosine kinase (Syk). Lane 1, unstimulated; Lane 2, control; Lane 3, isorhamnetin (20 μM). The experiments were performed in triplicate, and a representative result was presented. (C and D) The relative band intensities of p-Tyr and p-Syk normalized to β-actin, respectively. Data are presented as mean±SD (n=3). Different letters (a,b) indicate significant differences (P<0.05) between groups.

pounds that contribute to the inhibitory activity.

SBB was extracted with 70% MeOH and fractionated by reversed-phase column chromatography, resulting in seven fractions. The polyphenols in SBB have been shown to be involved in its antioxidant activity (Chen et al., 2007, 2013). For example, epigallocatechin gallate, a polyphenol abundant in green tea, has shown antioxidant activity and strong antiallergic effects (Yamada and Tachibana, 2000). Yamada et al. (1999) reported that the antioxidant activity of phenolic compounds is involved in the inhibitory activity of LTB_4 production by mast cells. Therefore, we determined the polyphenol content and antioxidant activity of the SBB extract and its fractions. Among the fractions, Fr. 1 and 2 eluted with water accounted for significant weight fractions (Table 1), while the fractions exhibited lower polyphenol content and antioxidant activity. In contrast, the polyphenol content and antioxidant activity increased in Fr. 4 eluted with 40% MeOH, and Fr. 5 eluted with 60% MeOH and Fr. 6 eluted with 80% MeOH contained very high amounts of polyphenols (55~60%, w/w) and showed high antioxidant activity (Fig. 1).

In many studies, the antiallergic activity of natural ingredients is commonly evaluated by measuring β-hexosaminidase activity in the culture medium as an index of basophilic leukocyte degranulation following stimulation with antigens. In this study, we evaluated the antiallergic activity of the SBB extract and its fractions by directly measuring the release of histamine, one of the chemical mediators that cause allergy symptoms (Matsuo et al., 1997; von Vietinghoff et al., 2006; Zorig et al., 2021), from RBL-2H3 cells stimulated by the antigen-IgE reaction. Fr. 5 and 6 strongly inhibited histamine release from RBL-2H3 cells, while the SBB extract and other fractions showed no inhibitory effect (Fig. 2A). This result suggests that the inhibitory effect of Fr. 5 and 6 on histamine release is due to the polyphenols, which exhibit antioxidant properties (Lin et al., 2014).

LTB_4 is another chemical mediator produced by the antigen-stimulated mast cells through the reaction of 5-LOX with arachidonic acids. This reaction, which is a form of lipid peroxidation, has been suggested to be inhibited by antioxidants (Santangelo et al., 2007; Schneider et al., 2007; Yahfoufi et al., 2018). In a previous study, we developed a method to evaluate the inhibitory effect of food ingredients on LTB_4 production using the mast cell line PB-3c (Takasugi et al., 2018). Applying this method, we evaluated the antiallergic activity of the SBB extract and its fractions and found that Fr. 5, 6, and 7 showed sig-

nificant inhibition of LTB_4 production (Fig. 2B). This result suggests that polyphenols with antioxidant activity may contribute to the inhibitory activity of Fr. 5 and 6 on LTB_4 production as well as histamine release. On the other hand, the mechanism of LTB_4 suppression and its active compounds of Fr. 7 may differ from those of Fr. 5 and 6, as Fr. 7 exhibited relatively low antioxidant activity.

Fr. 6, which had the strongest inhibitory effect on chemical mediators and the highest yield, was further investigated for its antiallergic activity. It showed a dose-dependent inhibitory effect on histamine release from the stimulated RBL-2H3 cells and LTB_4 production by the stimulated PB-3c cells (Fig. 3). In mast cells, an increase in intracellular Ca^{2+} concentration due to influx from the endoplasmic reticulum is the final step in signal transduction and is essential for degranulation and induction of the arachidonic acid cascade. Therefore, inhibiting this increase is important in the antiallergic mechanism of food components (Zhang et al., 2023). We examined the inhibitory effect of Fr. 6 on this increase during stimulation and found that Fr. 6 inhibited Ca^{2+} influx in a concentration-dependent manner (Fig. 4A). In the mast cell signaling, a phosphorylation cascade reaction of protein tyrosine residues occurs upstream of the Ca^{2+} influx (Gilfillan and Rivera, 2009). Antigen-IgE crosslinking activates Lyn, which binds to the β-chain of FcεRI and phosphorylates ITAM on the β- and γ-chains of the FcεRI complex. Syk is phosphorylated and activated by binding to the chains, which in turn phosphorylates the downstream LAT upon activation. This sequential phosphorylation ultimately induces degranulation and the arachidonic acid cascade through an increase in intracellular Ca^{2+} concentration (Siraganian, 2003). To further elucidate the mechanism of chemical mediator inhibition by Fr. 6, we solubilized the stimulated RBL-2H3 cells and analyzed protein phosphorylation using Western blotting. The fluorescence intensity of the total bands throughout each lane with molecular weights ranging from 38 to 160 kDa was normalized to the fluorescence intensity of the loading control β-actin. Fr. 6 was found to significantly inhibit the phosphorylation of tyrosine residues of the proteins (Fig. 4B, upper panel and Fig. 4C). In particular, Fr. 6 strongly inhibited the phosphorylation of proteins around 60 and 70 kDa, presumed to be Lyn (56 kDa) and Syk (72 kDa), respectively (Siraganian, 2003). Subsequently, we double-stained the band with an anti-p-Syk antibody after detecting it with an anti-p-Tyr antibody. The band around 70 kDa was identified as p-Syk (Fig. 4B, middle panel), indicating that the tyrosine residue of Syk is phosphorylated. Normalizing the fluorescence intensity of the p-Syk band to β-actin, we observed that Fr. 6 significantly inhibited the phosphorylation of Syk (Fig. 4D). These results indicate that the suppression of chemical mediator release from RBL-2H3 cells by Fr. 6 is due to the inhibition of Syk phosphorylation and Ca^{2+} influx in cell signaling. However, further investigation is needed to clarify the behavior of intermediate signaling molecules.

SBB reportedly contains three major flavonoid glycosides: kaempferol, quercetin, and isorhamnetin (Fig. 5A) (Arimboor et al., 2008; Ma et al., 2016). As mentioned above, Fr. 6 contains high levels of polyphenols with antioxidant activity, presumed to be the active components inhibiting the allergic reaction. HPLC analysis of the flavonoid aglycon content of Fr. 6 showed that it contained a much higher amount of isorhamnetin compared to quercetin and kaempferol (Fig. 5B). Hence, we evaluated the inhibitory activity of these three flavonols on chemical mediator release. The order of inhibitory activity on histamine release from the stimulated RBL-2H3 cells was isorhamnetin>quercetin=kaempferol (Fig. 6A). Similarly, the order of inhibitory activity on LTB_4 production by the stimulated PB-3c cells was isorhamnetin=quercetin >kaempferol (Fig. 6B). Comparing the antichemical mediator activities of kaempferol and quercetin showed that the activity may increase with the number of phenolic hydroxyl groups in the B ring. Previous studies have reported that the methylation of epigallocatechin gallate (a polyphenol abundant in Benifuuki green tea), in which a hydroxyl group at the C-3" or C-4" position is replaced by a methoxy group, enhances its antiallergic activity (Maeda-Yamamoto et al., 2004, 2012). Isorhamnetin, a methylated form of quercetin with a hydroxyl group replaced by a methoxy group at the C-3' position, is assumed to be responsible for its high antihistaminic activity. On the other hand, no effect of methoxylation was observed on anti-LTB_4 activity. Quercetin has been reported to strongly inhibit LT production (Kimata et al., 2000). In our study, isorhamnetin showed activity comparable to that of quercetin.

Finally, isorhamnetin, a major polyphenol with the highest chemical mediator inhibitory activity among the three flavonols in Fr. 6, was examined in detail as a candidate responsible for the observed antiallergic effects. Isorhamnetin showed a dose-dependent inhibitory effect on histamine release from the stimulated RBL-2H3 cells and LTB_4 production by the stimulated PB-3c cells (Fig. 7). These results indicate that the inhibitory activity of Fr. 6 on chemical mediator release is attributable to isorhamnetin. Previous studies have reported the inhibitory effects of isorhamnetin on degranulation and quercetin on LT production (Kwon et al., 2011; Wu et al., 2022), and we have previously reported the inhibitory effects of quercetin and kaempferol on LTB_4 production (Takasugi et al., 2018). This study is the first to demonstrate the inhibitory effect of isorhamnetin on LTB_4 production. To further clarify the mechanism of chemical mediator inhibition by isorhamnetin, we examined its effect on the increase in intracellular Ca^{2+} concentration in RBL-2H3

cells stimulated by the antigen-IgE reaction and found a concentration-dependent inhibitory effect (Fig. 8A). The phosphorylation of the intracellular proteins tyrosine and Syk showed an inhibitory trend, although the difference was not significant (Fig. 8B～8D). This may be because 20 μM isorhamnetin used in this experiment was lower than the isorhamnetin concentration in 400 μg/mL Fr. 6, which is equivalent to 135 μM isorhamnetin. Other polyphenols, such as kaempferol and quercetin, may also contribute to the inhibitory effect of Fr. 6 on the phosphorylation of the intracellular proteins tyrosine and Syk. In addition, the concentration of isorhamnetin in the SBB extract is 8.2 nmol/mg, which is much lower than the concentration required for effective suppression. This may why the SBB extract did not show inhibitory activity against chemical mediators (Fig. 2). Accordingly, an optimal method for extracting isorhamnetin should be developed to utilize SBB as a functional food material.

In conclusion, the polyphenol-rich fraction of SBB and its major flavonol, isorhamnetin, showed inhibitory effects on the release of chemical mediators from mast cells, and the inhibition of intracellular signal transduction was postulated as the mechanism of action. These results suggest that SBB may serve as a functional food that can alleviate the symptoms of Type-I allergy. Future studies should elucidate more detailed mechanisms, including the effects on signaling molecules other than Syk, using cell lines. Furthermore, *in vivo* feeding studies using animal models of experimental allergy should also be performed to clarify the influence of SBB metabolism on its antiallergic effects.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: HA. Analysis and interpretation: HA, SQ, AZ, MT. Data collection: SQ, AZ, NS, AY, TK. Writing the article: SQ, HA, MT. Critical revision of the article: HA, MT. Final approval of the article: all authors. Statistical analysis: SQ, MT. Overall responsibility: HA.

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