Original Article

DOI: 10.5582/ddt.2024.01016

Shiikuwasha leaf and peel extracts inhibit allergic reactions by suppressing degranulation in RBL-2H3 rat basophilic leukemia cells and immunoglobulin production in mouse spleen lymphocytes

Takeaki Okamoto[1], Sayaka Yokoyama[2,3], Hinako Ushimaru[3], Mamoru Tanaka[3,4,*]

[1] Faculty of Education, Ehime University, Ehime, Japan;
[2] Department of Food and Nutritional Environment, Kinjo Gakuin University, Aichi, Japan;
[3] Graduate School of Bioscience and Biotechnology, Chubu University, Aichi, Japan;
[4] Department of Food and Nutrition Sciences, College of Bioscience and Biotechnology, Chubu University, Aichi, Japan.

SUMMARY This study aims to investigate the antiallergic effects of Shiikuwasha (*Citrus depressa* Hayata) leaf and peel extracts by examining the regulation of degranulation and inflammatory cytokine production from rat basophilic leukemia (RBL-2H3) cells and antigen-specific antibody production in sensitized mouse spleen lymphocytes. *In vivo* antiallergic activity was evaluated using the passive cutaneous anaphylaxis (PCA) reaction model. Extracts of Shiikuwasha leaves and peel were prepared using 80% methanol and dissolved in dimethylsulfoxide. The dinitrophenyl-human serum albumin-induced β-hexosaminidase levels in immunoglobulin (Ig) E-sensitized RBL-2H3 cells were assessed using enzymatic assays. Cytokine production was measured by enzyme-linked immunosorbent assay. Antibody production capacity was evaluated using lymphocytes isolated from spleens of type I allergy model mice. Lymphocytes were cultured for 72 h with Shiikuwasha extracts, and ovalbumin-specific IgE, IgG1, and IgG2a levels were measured. Shiikuwasha leaf and peel extract significantly reduced β-hexosaminidase release and suppressed interleukin-4 and tumor necrosis factor-α production from RBL-2H3 cells. Ovalbumin-specific IgE and IgG1 production decreased in Shiikuwasha extract-treated lymphocytes. These extracts also significantly suppressed the PCA reaction. Shiikuwasha leaf and peel extract reduce degranulation in RBL-2H3 cells and antibody production in spleen-derived lymphocytes and therefore exhibit antiallergic effects.

Keywords Shiikuwasha, spleen lymphocytes, immunoglobulin, degranulation, antiallergic

1. Introduction

Shiikuwasha (*Citrus depressa* Hayata), a fruit native to the southwest of the Japanese archipelago and Taiwan, has been reported to have anticancer (*1,2*) and procognitive effects (*3*). The main commercial product obtained from Shiikuwasha is fruit juice; however, the extraction efficiency is around 50%, and considerable amounts of by-products such as leaves and peel are discarded. Making productive use of these by-products poses a major challenge (*4*). Shiikuwasha juice is a rich source of bioactive compounds such as ascorbic acid and flavonoids, flavone glycosides, and polymethoxyflavones (PMFs). The squeezed peel and leaf residue also contains PMFs such as nobiletin (NOB), tangeretin (TNG), and sinensetin (SNT), as well as flavonoid glucosides such as hesperidin, which are physiologically active (*5-7*). Recent studies have described the antiallergic mechanism of NOB (*8*). Thus, the Shiikuwasha leaves and peel, which are often unused, might have antiallergic effects.

Type 1 allergic responses are evoked by the antigen-induced activation of the high-affinity immunoglobulin E (IgE) receptor FcεRI expressed on mast cells and basophils (*9,10*). Antigens presented by antigen-presenting cells are recognized by T cells, leading to cellular activation and cytokine production. B cells are also activated by antigens to produce various antibodies. IgE produced from B cells binds to FcεRI on the surface of mast cells and basophils in the skin, gut, and respiratory and cardiovascular systems, priming them for reactivity upon re-exposure to the allergen. The elicitation of classic allergic symptoms occurs minutes after allergen exposure when the IgE-bound mast cells and basophils recognize the allergen and are activated (*11*). Antigen binding to IgE is

essential and is the first step in triggering the signaling cascades that lead to degranulation. There are several phases in the pathogenesis of type 1 allergic responses, and allergy symptoms may be arrested by blocking the response at any point. In this study, we investigate the antiallergic action of Shiikuwasha leaf and peel extract by testing its effects on IgE/mast cell-dependent degranulation and inflammatory cytokine production in a mast cell line and antibody production by sensitized mouse splenic lymphocytes. In addition, we test the antiallergic activity of Shiikuwasha leaf and peel extracts *in vivo*.

2. Materials and Methods

2.1. Plant extract preparation

The Shiikuwasha leaves and fruit used in this study were collected in Kochi Prefecture, Japan. Leaves and fruit peel were air dried at 50°C for 6-12 h and then ground coarsely. Samples of the dried Shiikuwasha leaves and peel (100 g) were extracted with 1 L of 80% methanol (Nacalai Tesque, Tokyo, Japan) for 72 h. To obtain a solid extract of Shiikuwasha leaves and peel, the methanol solution was filtered using filter paper into different conical flasks, dried over Na_2SO_4 (Nacalai Tesque), and evaporated until dry.

2.2. Analysis of polymethoxyflavones in Shiikuwasha leaf and peel extracts

NOB, TNG, and SNT were analyzed using high-performance liquid chromatography (HPLC) with a C18 reverse-phase column.

2.3. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were obtained from the Health Science Resources Bank (Tokyo, Japan) and were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) with 10% (*v/v*) fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin (Nacalai Tesque), and 100 µg/mL streptomycin (Nacalai Tesque) at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. β-hexosaminidase release activity assay

To evaluate IgE-mediated degranulation, a β-hexosaminidase release assay was used as described previously (*12*). RBL-2H3 cells were seeded in a 24-well plate (2.5×10^5 cells/well) in DMEM with 10% FCS and cultured overnight at 37°C. Cells were then washed twice with phosphate-buffered saline (PBS) (Nacalai Tesque) and sensitized by treatment with 500 µL of 50 ng/mL dinitrophenyl (DNP)-specific IgE (Sigma-Aldrich) for 2 h. The cells were then washed with modified Tyrode's (MT) buffer. Shiikuwasha samples were diluted in MT buffer at 250 µg/mL, and 490 µL of each sample solution or MT buffer with 0.5% dimethylsulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan) as a control was added to the culture for 10 min. Cells were then challenged by adding DNP-human serum albumin (HSA) (Sigma-Aldrich; final concentration 50 ng/mL) to the culture for 30 min. The supernatant was collected, and the cells were lysed with MT buffer containing 0.1% Triton X-100 (Wako Pure Chemicals). Aliquots of each supernatant and cell lysate were incubated with 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide (Wako Pure Chemicals) solubilized in 0.1 M citrate buffer (pH 4.5) for 30 min at 37°C. The enzyme reaction was terminated by adding 2 M glycine buffer (pH 10.4), and the absorbance was measured at 405 nm using a microplate reader (xMark™ Microplate Absorbance Spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA). The percentage of β-hexosaminidase activity released from RBL-2H3 cells was calculated using the following equation: Enzyme release activity (%) = absorption of cell supernatant/(absorption of cell supernatant + absorption of cell lysate) × 100.

2.5. Cytokine production assay

RBL-2H3 cells were seeded in a 12-well plate (1.0×10^6 cells/well) in DMEM with 10% FCS and cultured overnight at 37°C. The cells were washed twice with PBS and sensitized by treatment with 1 mL (50 ng/mL) DNP-specific IgE for 2 h. Cells were then washed with MT buffer. Shiikuwasha extract was diluted in MT buffer at 250 µg/mL, and 490 µL of each solution or MT buffer with 0.5% DMSO as a control was added to the culture for 10 min. After this incubation, 10 µL of DNP-HSA (final concentration 50 ng/mL) was added, and the culture was incubated for 4 h. The levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-4 in the supernatant were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The percentage of TNF-α and IL-4 production from RBL-2H3 cells was calculated using the following equation: Cytokine production (%) = level with Shiikuwasha sample/level with control sample × 100.

2.6. Animals

BALB/cCrSlc mice (5-week-old females weighing 15-20 g) were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan) and used as a murine type 1 allergy model. Mice were housed in a room with a 12 h light/dark cycle maintained at 24°C ± 3°C with 55% ± 10% humidity and *ad libitum* access to standard laboratory rodent feed (Oriental Yeast, Tokyo, Japan) and water. The experimental design

followed the guidelines for animal experimentation and was approved by the University of Kochi Animal Experimental Committee (authorization number 2016-003).

2.7. Analyses of splenic lymphocytes

The sensitized mice were anesthetized with isoflurane and injected intraperitoneally with 50 µg/mL ovalbumin (OVA) (Sigma-Aldrich) and 4 mg aluminum hydroxide (Imject Alum; Pierce, Rockford, IL) in 0.2 mL of PBS at pH 7.0 on days 1, 8, and 15. Mice were euthanized under anesthesia, and spleens were collected on day 22. The isolation and culture of spleen lymphocytes were performed as previously reported (*13*). Spleen lymphocytes obtained from mice on day 22 were collected and washed with RPMI-1640 medium (Nacalai Tesque) containing 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% FCS. Cells were cultured in 24-well culture plates (2×10^6 cells/well) for 72 h at 37°C (5% CO_2), and supernatants were collected. The levels of OVA-specific IgE, IgG1, and IgG2a spleen lymphocyte supernatants were analyzed by enzyme-linked immunosorbent assay, as described previously (*14*). Ninety-six-well microtiter plates were precoated with 100 µL of OVA (100 µg/mL) in a carbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were then washed with PBS containing 0.05% Tween 20 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) (PBS-T), and 1% bovine serum albumin (Wako Pure Chemicals) in PBS-T was added to each well. The plate was incubated for 1 h at 37°C, the spleen lymphocyte culture supernatant was diluted threefold with 1% bovine serum albumin/PBS-T, and 100 µL aliquots were added to each well. The plate was then incubated for 1 h at 37°C. The secondary antibodies used were mouse anti-IgE-horseradish peroxidase (HRP) (Southern Biotech, Birmingham, AL, USA) diluted 1000-fold, mouse anti-IgG1-HRP (Southern Biotech) and mouse anti-IgG2a-HRP (Southern Biotech) diluted 5000-fold, and 100 µL was added to each well and incubated for 1 h at 37°C. Then, 100 µL of *o*-phenylenediamine (0.4 mg/mL) in citrate-phosphate buffer (pH 5.0) containing 0.006% H_2O_2 (aq.) (Nacalai Tesque) was added to each well. The reaction was quenched with 2.5 M H_2SO_4 (Nacalai Tesque) after 3-5 min at room temperature, and color development was measured by colorimetric photometry at 490 nm.

2.8. Passive cutaneous anaphylaxis reaction

IgE-mediated passive cutaneous anaphylaxis (PCA) reaction was evaluated as described previously (*12*). BALB/c mice were lightly anesthetized, the right ears were injected intradermally with 1 µg anti-DNP IgE in 20 µL PBS, and the left ears were injected with 20 µL PBS as control. After 23 h, 100 µL of 50 mg/mL Shiikuwasha extract samples diluted with PBS to a DMSO concentration of 10% were orally administered. The untreated control group and the DNP-HSA antigen-treated IgE group were orally administered 200 µL of PBS as a control. After another hour, mice were injected intravenously with 200 µL of 1% Evan's blue dye (Wako Pure Chemicals) containing 100 µg DNP-HSA. Ear swelling was observed for 30 min after the DNP-HSA challenge. Subsequently, the mice were anesthetized and euthanized, and their ears were removed and incubated in 1 mL formamide (Nacalai Tesque) at 63°C for 48 h. The intensity of the absorbance was measured at 610 nm.

2.9. Statistical analysis

Data are expressed as the mean ± standard error of measurement. All statistical analyses were performed using IBM SPSS Statistics software version 21.0 (IBM Japan, Tokyo, Japan). Analysis of variance was used to determine differences between the control and treated mice. Tukey tests (β-hexosaminidase release activity, TNF-α, and IL-4) and Dunnett's test (OVA-specific IgE, IgG1, and IgG2a) were also performed *post hoc*. Data were considered statistically significantly different when *P*-values were < 0.05.

3. Results

3.1. Determination of three PMFs in Shiikuwasha leaf and peel extracts

HPLC analysis showed that the NOB content in the methanolic extract of Shiikuwasha leaf and peel was 1.8×10^3 mg/100 g and 9.1×10^2 mg /100 g, respectively. The TNG content was 1.5×10^3 mg/100 g and 4.6×10^2 mg/100 g, respectively, and the SNT content was 2.6×10^2 mg/100 g and 1.2×10^2 mg/100 g, respectively (Table 1). The NOB and SNT contents were twice as high in the leaf extract than in the peel extract, and the TNG content was three times higher in the leaf extract than in the peel extract.

3.2. Inhibition of degranulation and cytokine production by Shiikuwasha leaf and peel extracts

RBL-2H3 cells are commonly employed as model mast cells, and β-hexosaminidase is frequently used

Table 1. Quantification of nobiletin, tangeretin, and sinensetin in 100 g of Shiikuwasha leaf and peel extracts

	Leaf (mg/ 100g)	Peel (mg/ 100g)
Nobiletin	1.8×10^3	9.1×10^2
Tangeretin	1.5×10^3	4.6×10^2
Sinensetin	2.6×10^2	1.2×10^2

as an indicator of mast cell degranulation, given its abundance in mast cell granules and its activation and release in response to chemical mediators such as histamine (*8,12,15*). To investigate the effect of Shiikuwasha leaf and peel extract on IgE/mast cell-dependent degranulation and inflammatory cytokine production, we used the mast cell line RBL-2H3. Compared with the control, Shiikuwasha leaf and peel extracts significantly inhibited the release of β-hexosaminidase from RBL-2H3 cells (Figure 1A).

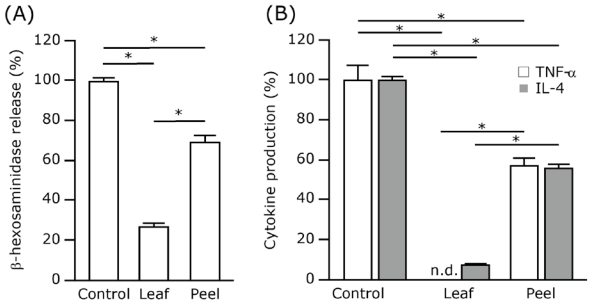

Figure 1. Shiikuwasha leaf and peel extracts reduce immunoglobulin E (IgE)-induced allergic responses and inflammatory cytokine production in a rat basophilic leukemia mast cell line (RBL-2H3). (**A**) The β-hexosaminidase levels released from dinitrophenyl (DNP)-specific IgE-sensitized RBL-2H3 cells pre-treated with control or Shiikuwasha extract (250 mg/mL) and challenged with DNP-human serum albumin (HSA) are shown. The percentage of β-hexosaminidase activity released from RBL-2H3 cells is shown. (**B**) TNF-α and IL-4 levels released by DNP-specific IgE-sensitized RBL-2H3 cells pretreated with control or Shiikuwasha extract (250 mg/mL) and challenged with DNP-HSA are shown. The percentage of TNF-α and IL-4 produced by RBL-2H3 cells is shown. Data are presented as the mean ± standard deviation (n = 3). Analysis of variance was used to determine differences between the control and other groups. Tukey's test was performed post hoc. The asterisk (*) indicates $P < 0.05$; n.d., not detected.

Furthermore, Shiikuwasha leaf extract inhibited the release of β-hexosaminidase more than Shiikuwasha peel extract.

The inflammatory response of mast cells is regulated by cytokines such as IL-4, IL-5, IL-6, and IL-13, and TNF-α is the major cytokine of mast cells (*16*). To test whether Shiikuwasha leaf and peel extract affect the inflammatory response, we measured IL-4 and TNF-α production by RBL-2H3 cells. This showed that IL-4 and TNF-α production was significantly reduced by Shiikuwasha leaf and peel extract compared with the control. (Figure 1B). Furthermore, leaf extract was more inhibitory than peel extract. These results suggest that the production of inflammatory cytokines associated with degranulation and degranulation are inhibited by compounds derived from Shiikuwasha leaves and peel and that the extent of inhibition depends on the part of the Shiikuwasha plant used.

3.3. Inhibition of OVA-specific antibody production by Shiikuwasha leaf and peel extracts

To evaluate the effects of Shiikuwasha leaf and peel extracts on the production of antibodies, we used spleen lymphocytes derived from a type 1 allergy mouse model. Overall, Shiikuwasha leaf and peel extracts decreased the OVA-specific IgE, IgG1, and IgG2a production by spleen lymphocytes (Figure 2). A statistically significant decrease in OVA-specific IgE was observed across the Shiikuwasha leaf extract concentration range of 0.1-500 μg/mL and 5-500 μg/mL by Shiikuwasha peel extract (Figure 2A). A statistically significant decrease in OVA-specific IgG1 was induced by Shiikuwasha leaf extract across the range of 1-500 μg/mL and across the range of 10-500 μg/mL by Shiikuwasha peel extract (Figure

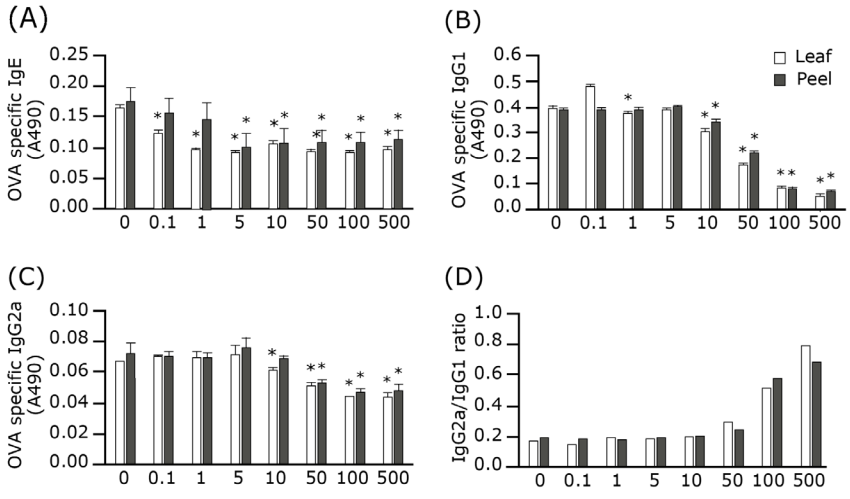

Figure 2. Shiikuwasha leaves and peel extracts reduce the production of OVA-specific antibodies in spleen lymphocytes from OVA-sensitized mice. Immunoglobulin levels secreted by splenic lymphocytes derived from type 1 allergy mice following treatment with either Shiikuwasha leaf or peel extracts are indicated. (**A**) OVA-specific IgE, (**B**) OVA-specific IgG1, (**C**) OVA-specific IgG2a, and (**D**) IgG2a/IgG1 ratio. Data are presented as the mean ± standard deviation (n = 3). Analysis of variance was used to determine differences between 0 μg/mL (control) and the other concentrations. Dunnett's test was performed post hoc. Statistical significance is indicated at the following levels: (*) $P < 0.05$, relative to the control value. IgE, immunoglobulin E; IgG1, immunoglobulin G1; IgG2a, immunoglobulin G2a.

2B). Although a significant decrease in OVA-specific IgG2a was observed at higher extract concentrations, no significant decrease was observed at lower concentrations (Figure 2C).

To evaluate the balance of helper T (Th)1/Th2 cells, we used the IgG2a/IgG1 ratio to indicate antiallergic effects. Th1 cells produce interferon-γ and TNF-α, which induce T-cell-mediated immunity and IgG2a production and downregulate Th2 cells. In contrast, IgE production in mice is induced by IL-4, and IL-5 is secreted by Th2 cells. IgE responses are accompanied by IgG1 production, also induced by IL-4, resulting in allergic diseases (*17*). Inhibiting an allergic response is important for enhancing Th1 immune responses and suppressing Th2 immune responses. The IgG2a/ IgG1 ratio increased with the addition of Shiikuwasha leaf and peel extracts in a concentration-dependent manner (Figure 2D).

A comparison of the effects of Shiikuwasha leaf and peel extracts on antibody production showed significantly lower IgE and IgG1 antibody production following exposure to leaf extract compared with peel extract, even at lower leaf extract concentrations (Figures 2A and B). The effects of Shiikuwasha leaf and peel extract on antibody production differed in that IgE was particularly affected at low concentrations of leaf extract compared with peel extract (Figure 2A).

3.4. Extracts of Shiikuwasha leaf and peel inhibit IgE-mediated PCA

Mice sensitized with DNP-specific IgE and intravenously challenged with the antigen DNP-HSA develop strong PCA, concomitant with rapid capillary dilatation and increased vascular permeability of the ears, as demonstrated by leakage of intravenously injected Evans blue dye into the reaction site. When Shiikuwasha leaf and peel extracts were orally administrated, the vascular permeability of the ears was attenuated (relative to controls), as evaluated by quantification of the amount of Evans blue dye in the ears (Figure 3).

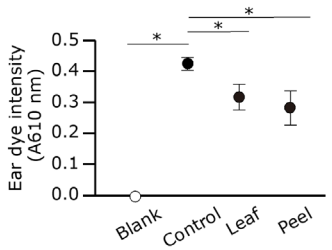

Figure 3. Shiikuwasha leaf and peel extracts reduce passive cutaneous anaphylaxis in mice. BALB/c mice were sensitized with anti-DNP IgE and DNP-HSA to induce cutaneous anaphylaxis. Ears were excised to quantify the extravasated dye, and the absorbance intensity of the Evans blue dye extracted was measured. Quantification of Evans blue dye leakage is presented as mean ± standard error of the mean (n = 8-11).

4. Discussion

Plants contain many bioactive compounds that often go unused, being discarded as food waste. Shiikuwasha is a small green citrus fruit that is popular in Okinawa, Japan, and Taiwan. It is known for its intensely sour taste and strong, distinctive aroma. Despite the presence of potentially valuable bioactive compounds such as PMFs, including NOB and TNG, and flavonoid glucosides such as hesperidin in the peel and leaves (*5,6*), peel and leaves are often discarded (*4*). In this study, Shiikuwasha leaf and peel extracts modulated RBL-2H3 cell degranulation and antibody production in spleen lymphocytes. In addition, Shiikuwasha leaf and peel extracts also suppressed the allergic response in a murine model.

Type 1 allergy is caused by the excessive activation of mast cells and basophils by IgE, resulting in inflammatory responses. Antigens induce the production of antigen-specific IgE that binds to FcεRI with high affinity on the surfaces of mast cells or basophils. FcεRI stimulation of mast cells and basophils results in degranulation and the release of mediators, including histamine, β-hexosaminidase, and other proinflammatory cytokines such as IL-4 and TNF-α (*9,10*). In this study, the release of β-hexosaminidase, IL-4, and TNF-α RBL-2H3 cells was significantly reduced by treatment with Shiikuwasha leaf and peel extracts.

Furthermore, the results from experiments using splenic lymphocytes indicate that titers of antigen-specific IgE and IgG1, which have a role in allergies, were reduced, suggesting that Shiikuwasha leaf and peel extracts may suppress type 1 allergies through a mechanism other than degranulation. Suppressing IgE production by Shiikuwasha leaf and peel extracts would reduce the rate at which IgE binds to FcεRI receptors on mast cells, raising the possibility of suppressing degranulation associated with the onset of type 1 allergic responses. Additionally, our findings indicate that culturing cells in the presence of Shiikuwasha leaf and peel extracts restored the balance of Th1/ Th2 from the Th2 cell dominant state induced by OVA sensitization to a more normal state.

Interestingly, the effects of Shiikuwasha leaf and peel extract differed in that degranulation, IL-4, TNF-α, and IgE levels were reduced at lower leaf than peel extract concentrations. Quantifying PMFs (*5,6*) in the leaves and peel of Shiikuwasha showed that the leaves contained more PMFs than the peels, including NOB, TNG, and SNT. Inhibition of degranulation and cytokine production by NOB and TNG has been reported (*8,18*). We also demonstrated the inhibitory effects of NOB and TNG on the degranulation of RBL-2H3 cells (*19*). The differing potency suggests that Shiikuwasha leaf and peel extracts contain different bioactive components, such as PMFs. Shiikuwasha

leaf extract exhibited more potent antiallergic effects *in vitro*, whereas *in vivo*, both Shiikuwasha leaf and peel extracts demonstrated comparable effects. One possible explanation for these findings is the different rates of absorption and metabolism of compounds *in vivo*. Flavonoids, such as NOB, are commonly found in plants, with the majority being glycosides. However, flavonoids are often inactive in their glycoside form and require hydrolysis into aglycones to become active. Additionally, it has been reported that glycosides are poorly absorbed in the intestinal tract (*20*). Therefore, the observed antiallergic effects of the Shiikuwasha leaf and peel extracts used in this experiment may not be attributed to a single component but to multiple components, including NOB and TNG.

During type 1 allergic reactions, antibodies are produced from B cells after antigen presentation. Sensitization is established by the binding of IgE antibody to mast cells or basophils. Further antigen presentation and binding to IgE activates mast cells and basophils and induces degranulation, leading to allergy symptoms. This study shows that Shiikuwasha leaf and peel extract suppress IgE/mast cell-dependent degranulation, and inhibit inflammatory cytokine production, and antibody production in spleen lymphocytes. Moreover, in an IgE/mast cell-dependent mouse model, Shiikuwasha leaf and peel extracts suppressed allergic reactions. Therefore, Shiikuwasha leaves and peel appear to have antiallergic effects. These effects were stronger at a higher PMF content. While PMFs may have an impact, the specific compound or combination of compounds that suppress antibody production is currently unknown. Although challenges remain, such as ensuring safety and conducting in-patient studies, we propose that further studies of Shiikuwasha leaves and peel may lead to the discovery of a valuable allergy medication. Better use of food waste can also contribute to environmental conservation.

In summary, we have identified antiallergic effects of Shiikuwasha leaf and peel extracts *in vitro* and *in vivo*. Further studies are necessary to determine the active ingredients that mediate the inhibition of degranulation and antibody production and the inhibitory mechanism.

Acknowledgements

We thank Hirotaka Kakita for his assistance with the HPLC analyses of PMFs. We also thank Paul Danielian, PhD, from Edanz (*https://jp.edanz.com/ac*) for editing a draft of this manuscript.

Funding: This study was supported by JSPS KAKENHI Grant Numbers 16K21299 and 19K05880.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received March 14,2024; Revised May 18, 2024; Accepted June 1, 2024.

Address correspondence to:
Mamoru Tanaka, Department of Food and Nutrition Sciences, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan.
E-mail: m-tanaka@isc.chubu.ac.jp

Released online in J-STAGE as advance publication June 6, 2024.