

***Rosae Multiflorae* Fructus Hot Water Extract Inhibits a Murine Allergic Asthma Via the Suppression of Th2 Cytokine Production and Histamine Release from Mast Cells**

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ABSTRACT Mast cell-mediated anaphylactic reactions are involved in many allergic diseases, including asthma and allergic rhinitis. In Korea, where it has been used as a traditional medicine, *Rosae Multiflorae* fructus (RMF) is known to have potent antioxidative, analgesic, and anti-inflammatory activities and to have no obvious acute toxicity. However, its specific effect on asthma is still unknown. In this study, we evaluated whether or not RMF hot water extracts (RMFW) could inhibit ovalbumin (OVA)-induced allergic asthma and evaluated compound 48/80-induced mast cell activation to elucidate the mechanisms of asthma inhibition by RMFW. Oral administration of RMFW decreased the number of eosinophils and lymphocytes in the lungs of mice challenged by OVA and downregulated histological changes such as eosinophil infiltration, mucus accumulation, goblet cell hyperplasia, and collagen fiber deposits. In addition, RMFW significantly reduced T helper 2 cytokines, TNF- α , IL-4, and IL-6 levels in the BAL fluid of mice challenged by OVA. Moreover, RMFW suppressed compound 48/80-induced rat peritoneal mast cell degranulation and inhibited histamine release from mast cells induced by compound 48/80 in a dose-dependent manner. These results suggest that RMFW may act as an antiallergic agent by inhibiting Th2 cytokine production from Th2 cells and histamine release from mast cells, and could be used as a therapy for patients with Th2-mediated or mast cell-mediated allergic diseases.

KEYWORDS: • Asthma • Cytokines • Histamine • Mast cell • *Rosae Multiflorae* fructus • TNF- α

INTRODUCTION

ALL MAST CELL-MEDIATED inflammatory reactions such as food allergies and asthma are characterized by mast cell accumulation in inflammatory sites.^{1,2} Apart from their function in the release of inflammatory mediators, mast cells also play multiple immunopathological roles in a number of inflammatory disorders by releasing multiple chemokines for recruitment of different leukocyte subtypes. Activated mast cells can produce histamine, along with a wide variety of other inflammatory mediators, such as proteoglycans, proteases, and proinflammatory and chemotactic cytokines.^{3,4} Released histamine and variable inflammatory mediators could contribute to bronchoconstriction, airway hyperresponsiveness, eosinophil infiltration, and mucus hypersecretion.^{5–7} The mechanism by which drugs alleviate allergic conditions has been demonstrated to be either via

their blocking of histamine release or via their preventing degranulation of mast cells as therapeutic targets.⁸

Rosa multiflora Thunberg (RM), commonly known as Multiflora Rose which is native to Korea, Japan, and China, where hips of this plant have been used as a traditional medicine for rheumatism, joint inflammation, edema, and beriberi.^{9,10} In Western countries, the rosehips of some species, especially *Rosa canina*, have been shown to be clinically effective for treating osteoarthritis.^{11,12} Likewise, *Rosae Multiflorae* fructus (RMF) has very low toxicity and has been used to make a variety of food products such as jam and juice.^{13–15} RMF is rich in quercetin glycosides,¹⁶ multiflorins,¹⁷ carotene, vitamin E, essential fatty acids, and minerals.¹⁸ In recent studies, condensed tannins such as procyanidin B-3 and catechin were isolated and identified from the root of RM, which were found to have potent antioxidative and anti-inflammatory activities *in vitro* and *in vivo*.^{19,20} In addition, ethanol extracts and petroleum ether fractions of RMF were shown to possess anti-inflammatory and analgesic effects and to have no obvious acute toxicity.^{21–23} In this study, we provide biological data that support the antiallergic effects of RMF hot water extract (RMFW) in

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an ovalbumin (OVA)-induced murine asthma model to evaluate the therapeutic efficacy of RMFW in allergic asthma via the suppression of Th2 cytokine production from Th2 cells and histamine release from mast cells.

MATERIALS AND METHODS

Animals

Six-week-old male Balb/c mice for a murine asthma model and Sprague-Dawley rats (aged 8 weeks) for acquisition of rat peritoneal mast cells (RPMCs) were purchased from Damool Science (Daejeon, Korea). These animals were housed in an air-conditioned room ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$), under standard 12 h light/dark cycles and allowed free access to food and tap water. All animal experiments were performed in accordance with the Guidelines for Animal Care and Use and were approved by the Institutional Animal Care and Use Committee of Chonbuk National University Laboratory Animal Center (CBN 2013-0011).

Preparation of RMFW

The RMF used in this study was purchased from Kyeong-Dong Oriental Pharmacy Market (Seoul, Korea). Samples of RMF were extracted with 10× volumes of distilled water under heating condition and then the extract supernatants were collected. This process was repeated again. Next, the collected

supernatants were concentrated and lyophilized. The dried RMFW powders were kept at 4°C until needed and managed with specimen number (KFRI-SL-2157-W) at the Korea Food Research Institute. The RMFW was dissolved and freshly diluted in HEPES Tyrode buffer (136 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 11 mM NaHCO_3 , 0.6 mM NaH_2PO_4 , 2.75 mM MgCl_2 , 5.4 mM HEPES, 1.0 mg/mL BSA, 1.0 mg/mL glucose, 0.1 mg/mL heparin, and pH 7.4) before use.

Induction of the murine asthma model

Mice were divided into four groups according to treatment: (1) Saline as a vehicle control, (2) OVA-induced asthmatic mice, (3) 200 mg/kg/day RMFW in OVA-induced asthmatic mice, and (4) 1.25 mg/kg/day Dexamethasone (Dex) in OVA-induced asthmatic mice as a reference drug for positive control. Mice of groups (2), (3), and (4) were immunized by intraperitoneal injection of 50 μg OVA with 1 mg Inject Alum in a total volume of 200 μL on days 1 and 14. On days 27, 28, and 29 after the beginning of the sensitization period, these mice were challenged for 30 min with an aerosol of 5% (wt/vol) OVA in saline using ultrasonic nebulization (NE-U12; Omron Crop., Tokyo, Japan). On days 15–26, the treatment groups were also orally treated once daily with 200 mg/kg RMFW or 1.25 mg/kg Dex (Fig. 1). Vehicle control group and OVA-induced asthma groups received only saline. Animals were sacrificed 24 h

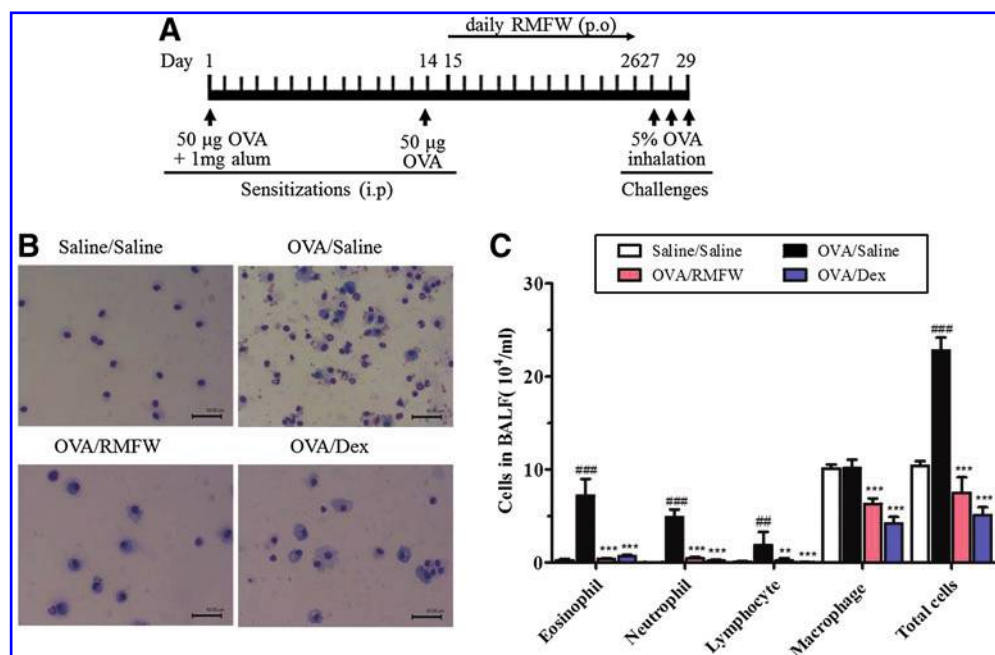


FIG. 1. Effect of RMFW on cellular changes in BALF. (**A**) Schematic diagram of the asthma induction protocol. Mice were sensitized on days 1 and 14, and challenged on days 27, 28, and 29 by OVA. Mice in control (Saline/Saline) group were sensitized and challenged by saline respectively. Mice in the RMFW treatment group were also administered orally once a day at 200 mg/kg BW for 12 days. Infiltration of inflammatory cells (**B**), and differential cellular components and total cells (**C**) in BALF. Values are presented as the mean $\pm$ SD ($n = 10$ per group). Data were analyzed using ANOVA followed by Student's t test. ## $P < .01$, ### $P < .001$, significantly different from the value of control group. ** $P < .01$, *** $P < .001$, significantly different from the value of OVA/Saline group. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; RMFW, *Rosae Multiflorae* fructus hot water extract. Color images available online at www.liebertpub.com/jmf

after the last challenge on day 30 to investigate the inhibitory effect of RMFW.

Collection and analysis of bronchoalveolar lavage fluid

Twenty-four hours after the final OVA challenge, bronchoalveolar lavage fluid (BALF) was collected by cannulating the upper part of the trachea and lavaging, as described previously.⁷ The total number of viable cells in BAL fluid was determined by trypan blue exclusion using a hemocytometer. Differential cell counts were determined using cytopsin (Centrifuge 5403; Eppendorf, Hamburg, Germany) preparations, followed by Diff Quik staining (Sysmex Co., Kobe, Japan).

Histopathologic analysis

Histopathologic analysis of lung was performed as previously described.⁷ Animals were sacrificed with an overdose of ether 24 h after the last OVA challenge and histologic specimens were collected. Using routine histologic procedures for light microscopic evaluation, tissue specimens were taken from the mid zone of the lung of the mice, and fixed in 10% formalin and embedded in paraffin. Serial 5 μ m thick sections were then cut, and stained with hematoxylin and eosin (H&E) for inflammatory cells, congo red for eosinophils, periodic acid-Schiff for goblet cells, and mucus and Masson trichrome for collagen fiber deposits.

Measurement of Th2-related cytokine levels

Th2-related cytokines such as TNF- α , IL-4, and IL-6 levels in the BALF from each mouse were measured using enzyme-linked immunosorbent assay (ELISA) as described previously.²⁴ Briefly, recovered supernatants and standard solution were transferred to 96-well plates precoated with the appropriate monoclonal antibodies raised against each of the target cytokines, and then incubated at room temperature for 2 h. After thorough washing with the washing buffer included in the kit, horseradish peroxidase-conjugated secondary antibody was added to each well, and incubation was continued at room temperature for 2 h. After removal of the secondary antibody, the substrate solution for the enzymatic reaction was added, and samples were then incubated for another 30 min in the dark. The reaction was terminated by adding stop solution, followed by measurement of absorbance at 450 nm in a microplate reader (Model-550; Bio-Rad, Tokyo, Japan).

Preparation of RPMCs

RPMCs were isolated as previously described.²⁴ In brief, rats were anesthetized with ether by administering a peritoneal injection of 10 mL of Ca²⁺-free HEPES-Tyrode buffer, following which the abdomen was gently massaged for approximately 90 s. The peritoneal cavity was opened, and fluid was aspirated using a Pasteur pipette. RPMCs were purified using a Percoll density gradient, as described in detail elsewhere.²⁵ RPMC preparation was approximately 95% pure as assessed by toluidine blue staining and at least 98% of these cells were viable as assessed by trypan blue exclusion.

Observation of mast cell degranulation

Purified RPMCs (1×10^6 cells/mL) were resuspended in HEPES-Tyrode buffer. The RPMCs were pretreated with RMFW (0–1 mg/mL) for 10 min at 37°C and observed within 10 min of addition of compound 48/80 (5 μ g/mL) under phase contrast microscopy and photographed as described by Cochrane *et al.*²⁶ The mast cells were classified ($\times 1000$) as follows: (1) extensively degranulated (>50% of the cytoplasmic granules exhibiting fusion, staining alterations, and extrusion from the cell), (2) slight to moderately degranulated (10–50% of the granules exhibiting fusion or discharge), or (3) normal.

Histamine assay

RPMCs (2×10^5 cells/well) were preincubated with RMFW (0–1 mg/mL) at 37°C for 10 min and then incubated with compound 48/80 (5 μ g/mL) for 30 min. The cells were separated from the released histamine by centrifugation at 150 g for 10 min at 4°C. Residual histamine in the cells was released by boiling cells. After centrifugation, histamine content was measured by using ELISA. The percent inhibition of histamine release was calculated using the following formula: % inhibition = [(histamine release without RMFW - histamine release with RMFW) / histamine release without RMFW] $\times 100$.

Statistical analysis

Results were expressed as mean $\pm$ SD for the number of experiments. Student's *t*-test and ANOVA with Dunnett's test were used for statistical comparison among the groups. Results with *P* < .05 were considered statistically significant.

RESULTS

RMFW decreased the number of eosinophils and other inflammatory cells in BALF

As shown in Figure 1, the number of inflammatory cells in BALF of mice treated with OVA was significantly increased compared with that of mice treated with saline alone. In addition, 200 mg/kg/day RMFW treatment significantly inhibited the increase in eosinophils and lymphocytes and had inflammatory cells counts similar to those of the saline-treated mice (Fig. 1B, C).

RMFW inhibited OVA-induced histopathological changes in lung tissue

Histopathological analyses revealed typical pathological features of asthma in OVA-sensitized and OVA-challenged mice. In the lung tissues of mice treated with OVA, there was increased infiltration of numerous inflammatory cells including eosinophils around bronchioles and blood vessels, accumulation of mucus in the lumen of bronchioles, and hyperplasia of goblet cells at the epithelium of bronchioles compared to saline-treated mice. In addition, deposits of collagen fibers among inflammatory cells and between

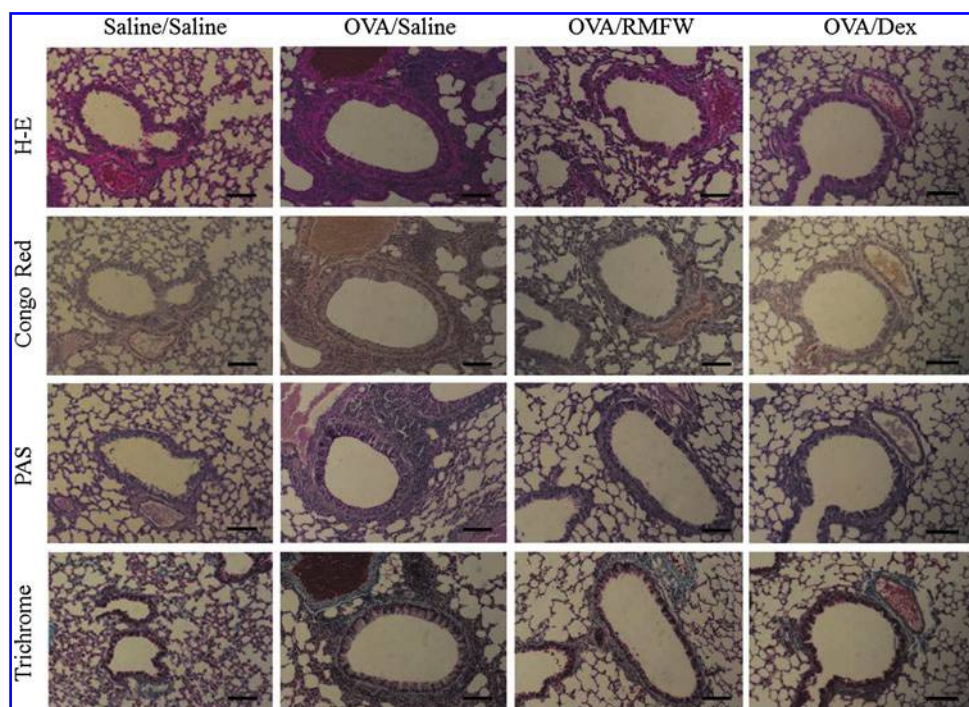


FIG. 2. Effect of RMFW on histopathologic changes in lung tissues. Mice were sensitized on days 1 and 14, and challenged on days 27, 28, and 29 by OVA. Mice in the RMFW treatment group were also administered orally once a day at 200 mg/kg BW for 12 days. Lung tissues from each group were stained with H&E for inflammatory cells, congo red for eosinophils, PAS for goblet cells and mucus, and Masson trichrome for collagen fiber deposits. H&E, hematoxylin and eosin; PAS, periodic acid-Schiff.

submucosal and muscular layers were observed in lung tissues of OVA-sensitized and OVA-challenged mice (Fig. 2). However, RMFW treatment significantly decreased the infiltration of inflammatory cells, the accumulation of mucus in the lumen of bronchioles, and hyperplasia of goblet cells in the epithelium of bronchioles of lung induced by OVA. Also RMFW treatment significantly attenuated the deposits of collagen fibers among inflammatory cells and between submucosal and muscular layers of lungs induced by OVA (Fig. 2). These results suggest that RMFW may inhibit allergic asthma.

RMFW inhibited the secretion of Th2-related cytokines in BALF and lung tissue induced by OVA

Next, Th2-related cytokines including TNF- α , IL-4, IL-5, and IL-6 have been known to regulate IgE-mediated allergy and asthma²⁷ were also investigated in the BALF of OVA-sensitized and OVA-challenged mice using ELISA. As shown in Figure 3, the level of TNF- α , IL-4, and IL-6 in BALF of OVA-sensitized and OVA-challenged mice were significantly increased compared with that of mice treated with saline alone. However, RMFW treatment significantly attenuated the increases in TNF- α , IL-4, and IL-6 in BALF of mice induced by OVA (Fig. 3). These data indicated that RMFW might effectively regulate Th2-immune response on the OVA-induced murine asthma model.

RMFW suppressed the mast cell degranulation and histamine release from RPMCs induced by compound 48/80

Mast cells in asthma have been known to exert their pathophysiological role through histamine release induced

by degranulation.²⁸ To investigate whether RMFW directly suppressed histamine release from RPMCs, we used compound 48/80 that has been known as a stimulator of the degranulation of RPMCs through perturbation of the membrane.²⁹ Compound 48/80-treated RPMCs had cytoplasmic

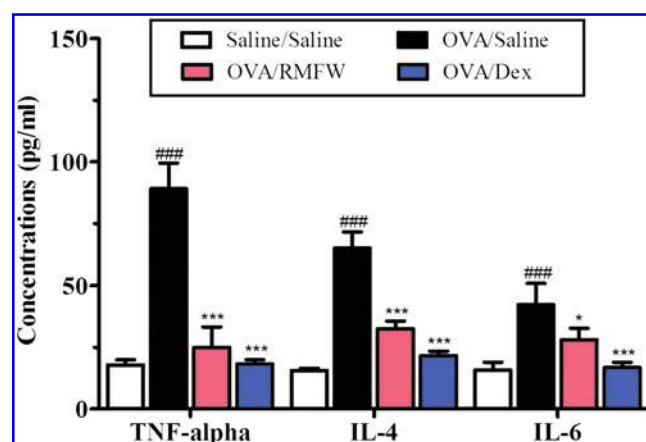


FIG. 3. Effect of RMFW on Th2 cytokines in BALF. Mice were sensitized on days 1 and 14, and challenged on days 27, 28, and 29 by OVA. Mice in the RMFW treatment group were also administered orally once a day at 200 mg/kg BW for 12 days. Levels of TNF- α , IL-4, and IL-6 in BALF of OVA-sensitized and OVA-challenged mice were significantly increased compared with that of mice treated with saline alone. However, RMFW treatment significantly attenuated the increases of TNF- α , IL-4, and IL-6 in BALF of mice induced by OVA. Values are presented as the mean $\pm$ SD ($n = 10$ per group). Data were analyzed using ANOVA followed by Student's t test. ### $P < .001$, significantly different from the value of control (Saline/Saline) group. * $P < .05$, *** $P < .001$, significantly different from the value of OVA/Saline group.

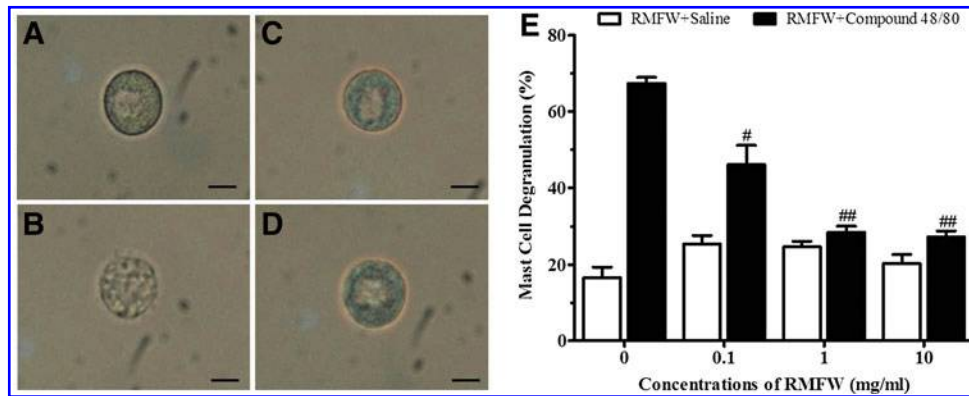


FIG. 4. Suppressive effect of RMFW on compound 48/80-induced degranulation of RPMCs. RPMCs were preincubated with RMFW E (0.1–10 mg/mL) at 37°C for 10 min before incubation with compound 48/80. The RPMCs were micrographed within 10 min after the addition of saline or compound 48/80 by using inverted phase contrast microscopy. (A) Controls: Normal RPMCs in HEPES–Tyrode buffer. (B) Degranulated RPMCs after stimulation with compound 48/80 (10 μ g/mL). (C) RPMCs after addition of 10 mg/mL RMFW. (D) RPMCs pretreated with 10 mg/mL RMFW and subsequently stimulated with compound 48/80 (10 μ g/mL). (E) The percentage of mast cell degranulation is shown in graph. Values are presented as the mean $\pm$ SD ($n=5$ per group). Data were analyzed using ANOVA followed by Student's t test. [#] $P<.01$, ^{##} $P<.05$, ^{###} $P<.001$ significantly different from the value of compound 48/80-treated group. RPMC, rat peritoneal mast cell. Color images available online at www.liebertpub.com/jmf

vacuoles, irregular membranes, and extruded granules near the cell surface (Fig. 4). RMFW-treated RPMCs were similar to those seen in saline-treated RPMCs, regardless of the dose of the RMFW. However, treatment with RMFW (0.1–10 mg/mL) inhibited the degranulation of RPMCs stimulated with compound 48/80 (Fig. 4). RMFW might protect the lipid membrane, affecting inhibition of the perturbation induced by compound 48/80. Moreover, RMFW diminished compound 48/80-induced histamine release at concentrations between 0 and 10 mg/mL from RPMCs (Fig. 5). These results suggested that RMFW may alleviate compound 48/80-induced histamine release from mast cells by the suppression of mast cell degranulation.

DISCUSSION

Medicinal herbs have been used as components of traditional medicines in Asian countries such as Korea and China for long times.³⁰ Recently, there is growing interest in the use of herbs and their bioactive compounds because most herbs are relatively cheap and easily available and have no toxicity. As part of our continuing search for biologically active antiallergic agents from medicinal sources, we analyzed the antiallergic effect of RMFW in experimental animal asthma models and evaluated the therapeutic efficacy of RMFW in allergic asthma via suppression of histamine release from mast cells. First of all, the effects of RMFW (10, 20 and 40 mg/mL) on cell viability were assessed by an MTT assay and there was no significant change in cell viability observed in the response to these concentrations (data not shown). On the basis of this result and Zhang et al.'s report,²¹ we used the 200 mg/kg of RMFW in all of our studies. In this study, we demonstrated the inhibitory effect of RMFW on allergic asthma model in Balb/c mice aggravated by histamine release from mast cells. RMFW treatment inhibited eosinophil infiltration into the lungs and

suppressed histopathological changes including infiltration of inflammatory cells, hyperplasia of goblet cells, and deposit of collagen fiber in the lungs of mice with OVA-induced asthma.

Inflammation plays an important role in various diseases with high prevalence within population such as asthma and allergic rhinitis.³¹ Allergic asthma is primarily mediated by Th2 cells, which secrete the characteristic cytokines IL-4, IL-5, and IL-6. During an inflammatory response, TNF- α ,

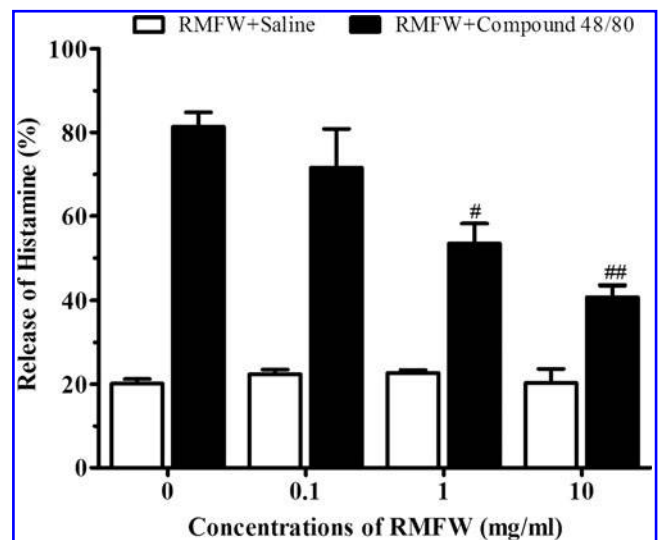


FIG. 5. Inhibitory effect of RMFW on compound 48/80-mediated histamine release of RPMCs. RPMCs were preincubated with RMFW (0.1–10 mg/mL) at 37°C for 10 min before incubation with compound 48/80. RMFW inhibited compound 48/80-induced histamine release in a dose-dependent manner. Values are presented as the mean $\pm$ SD ($n=5$ per group). Data were analyzed using ANOVA followed by Student's t test. [#] $P<.01$, ^{##} $P<.05$, significantly different from the value of compound 48/80 without RMFW.

IL-4, and IL-6 are released from the various cells. TNF- α promotes inflammation, leukocyte infiltration, and tissue fibrosis.³² IL-4 plays a key role in the differentiation of Th2 cells from Th0 cells and may be important in initial sensitization to allergens and isotype switching of B cells to producers of IgE.³³ Furthermore, IL-6 induces recruitment and activation of leukocytes into the inflammatory site.³⁴ Also, inducible enzymes such as cyclooxygenase-2 and nitric oxide synthase stimulate the production of high levels of proinflammatory mediators.^{35,36} Various medicinal herbs have been shown to downregulate the secretion of proinflammatory cytokines IL-1 β , IL-6, and TNF- α .³¹ Indeed, treatment with RMFW decreased the expressions of TNF- α , IL-4, and IL-6 in BALF of OVA-treated mice. Decreased expressions of IL-4, IL-6, and TNF- α by RMFW is a key indicator of attenuated allergic asthma symptoms. These results indicated that RMFW may have antiallergic effect by blocking production of proinflammatory cytokines from Th2 cells. These results are similar to those found in previous studies. Park *et al.*¹⁹ reported that two condensed tannins from the RM Thunberg, procyanidin B-3 and *ent*-guibourtinidol-(4 β →6)-catechin significantly reduced the serum levels of eosinophils, IgE, and certain Th2 cytokine. Also, Cheng *et al.*²³ revealed that a herbal formula consisting of RMF and *Lonicerae japonicae* Flos inhibited the secretion of nitric oxide, IL-6, and TNF- α , which were associated with inflammatory diseases. Guo *et al.*²² and Park *et al.*³⁷ investigated extracts of RM root or the petroleum ether fraction of RMF has anti-inflammatory and antioxidant effects via downregulating cyclooxygenase-2 expression and reducing nitric oxide production by inhibiting nitric oxide synthase activity. Based on these results, we used the whole hot water extract of RMF, which is likely to contain the above-mentioned bioactive components. Also, other multiple compounds from RMFW may have anti-inflammatory and antiallergic activities. In the future, we are trying to purify other compounds from RMF to find additional biologically active compounds.

Allergic asthma is the most common mast cell-mediated disease, and the main symptoms are bronchoconstriction, airway hyperresponsiveness, eosinophil infiltration, and mucus hypersecretion in the airways.^{5–7} The pathogenesis of allergic asthma initially involves the interaction of allergens with a specific IgE bound to the surface of mast cells on the bronchial epithelium. As a result, many symptoms associated with asthma are caused by the release of mediators such as histamine, nitric oxide, leukotriene, and cytokines from mast cells and basophils induced by degranulation.^{38,39} Recently, antihistamines have been used as a therapy for asthma to evoke acute bronchodilation.⁴⁰ However, these antihistamines tend to cause side effects and reduce receptor binding affinity and T-cell responses.⁴¹ Due to these reasons, plant-based medicines are increasingly being used as therapies for allergic disorders. We used compound 48/80 that has been known as a stimulator and induces histamine release from RPMCs and investigated whether RMFW has a direct antihistamine effect to evaluate the therapeutic efficacy of RMFW in OVA-induced allergic asthma via sup-

pression of histamine release from mast cells. RMFW significantly inhibited compound 48/80-induced mast cell degranulation and histamine release from RPMC in a dose-dependent manner.

In summary, RMFW effectively decreased production of Th2 cytokines TNF- α , IL-4, and IL-6, and suppressed the infiltration of inflammatory cells, the hyperplasia of goblet cells and the deposit of collagen fiber in the lungs of mice with OVA-induced asthma. Moreover, RMFW significantly inhibited both compound 48/80-induced mast cell degranulation and the histamine release from RPMCs. Our data suggest that RMFW may be used as a therapeutic agent for patients with Th2-mediated or mast cell-mediated allergic diseases.

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AUTHOR DISCLOSURE STATEMENT

These authors declare no conflicts of interest exist.

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