

Rosmarinic acid in perilla extract inhibits allergic inflammation induced by mite allergen, in a mouse model

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Summary

Background Perilla and its constituent rosmarinic acid have been suggested to have anti-allergic activity. However, few studies have examined the effects on allergic asthma.

Objective The purpose of this study was to evaluate the effect of oral administration of perilla leaf extract, which contains high amount of rosmarinic acid, on a murine model of allergic asthma induced by house dust mite allergen.

Methods C3H/He mice were sensitized by intratracheal administration of *Dermatophagoides farinae* (Der f). Mice were orally treated with rosmarinic acid in perilla extract (PE) (1.5 mg/mouse/day).

Results Der f challenge of sensitized mice elicited pulmonary eosinophilic inflammation, accompanied by an increase in lung expression of IL-4 and IL-5, and eotaxin. Daily treatment with rosmarinic acid in PE significantly prevented the increases in the numbers of eosinophils in bronchoalveolar lavage fluids and also in those around murine airways. Rosmarinic acid in PE treatment also inhibited the enhanced protein expression of IL-4 and IL-5, and eotaxin in the lungs of sensitized mice. Der f challenge also enhanced allergen-specific IgG1, which were also inhibited by rosmarinic acid in PE.

Conclusion These results suggest that oral administration of perilla-derived rosmarinic acid is an effective intervention for allergic asthma, possibly through the amelioration of increases in cytokines, chemokines, and allergen-specific antibody.

Keywords allergic asthma, eosinophils, mite allergen, perilla, rosmarinic acid

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Introduction

There has been an increasing prevalence of allergic diseases such as asthma, allergic rhinitis, food allergy, and atopic dermatitis over the past two to three decades [1–3]. Currently, more than 130 million people suffer from asthma [4]. Allergens from the house-dust mite (HDM) are a major trigger factor of allergic disorders. Mite allergens have been involved in airway sensitization and allergic diseases. Several studies have suggested that exposure to HDM allergen in infancy increases the risk of developing asthma [5]. *Dermatophagoides farinae* (Der f) is a dust mite allergen which can induce allergic inflammation in mice [6–8]. Increased accumulation of eosinophils in inflamed tissues is a characteristic feature of the major allergic diseases such as asthma [9]. Cytokines and chemokines, including IL-5, are implicated in the pathogenesis of allergic diseases by recruiting and/or activating eosinophils [10, 11].

Despite steady advances in conventional therapies for allergy symptoms, phytochemicals are widely used as alternative medicine without clinical evidence [12]. The leaves of *Perilla frutescens* are one of the most common garnishes in Japan (Fig. 1). Dried red perilla leaves have been used as folk medicine in Japan and China. Perilla leaf extract has been reported to exhibit anti-inflammatory [13, 14], anti-allergic [15, 16], and anti-oxidative activity [17] with therapeutic potential [18]. Rosmarinic acid (Fig. 2), a polyphenolic phytochemical, is one of the most important constituents of perilla leaf extract and inhibits allergic reactions in mice [19]. Anti-oxidant and anti-inflammatory activities of rosmarinic acid have been well recognized [20–22]. A recent study has shown that oral treatment with rosmarinic acid in perilla extract (PE) inhibits hepatic inflammation in mice [23] and also inhibits 12-*O*-tetradecanoylphorbol 13-acetate-induced ear oedema in mice [24]. More recently, we have reported that oral administration of rosmarinic acid inhibits lung injury, which is characterized by neutrophil sequestration and interstitial oedema, induced by diesel exhaust particle (DEP) [25]. We also find that rosmarinic acid inhibits expression of inflammatory cytokines enhanced by DEP.

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Fig. 1. A picture of leaves of *Perilla frutescens*.

However, no study has evaluated the effects of rosmarinic acid in PE on allergic inflammation sensitized by intratracheal allergen. In the present study, we investigated the effect of daily oral supplementation with rosmarinic acid in PE on allergic airway inflammation induced by intratracheal instillation of Der f in mice. Furthermore, we elucidated its effects on the allergen-enhanced lung expression of cytokine and chemokine and on the allergen-specific Ig production.

Materials and methods

Animals

Male C3H/He mice (7 weeks old) were obtained from Japan Clea Co. (Tokyo, Japan). The studies have been carried out in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health. All animal studies were approved by the Institutional Review Board.

Preparation of rosmarinic acid in PE

Rosmarinic acid in PE was prepared as previously described [23]. Rosmarinic acid concentration was determined as 68% w/w by high-performance liquid chromatography (Fig. 2).

Study protocol

Crude extract of Der f was purchased from LSL Co., Ltd (Tokyo, Japan) and 1 µg of Der f was dissolved in 100 µL of phosphate-buffered saline (PBS) solution (Nissui Pharmaceu-

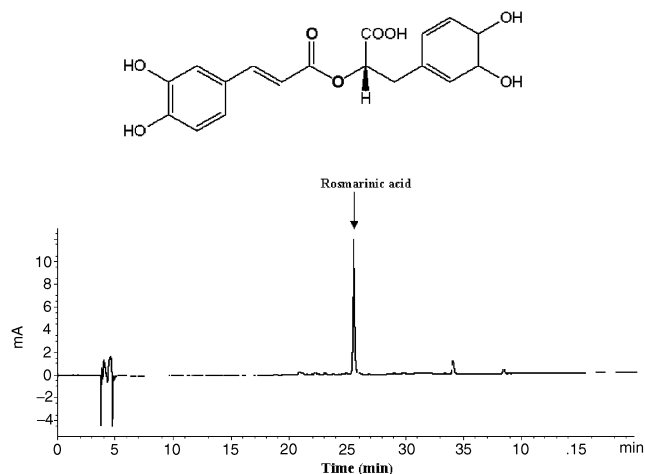


Fig. 2. Chemical structure of rosmarinic acid and chromatogram of rosmarinic acid in perilla extract monitored by ultraviolet detector.

tical, Tokyo, Japan) at pH 7.4 containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan). Each mouse was anaesthetized with 4% halothane, and the solution was instilled via an intratracheal cannula [26]. The animals were randomized into three experimental groups. The vehicle group intratracheally received 100 µL PBS containing 0.05% Tween 80 four times at 2-week intervals. The Der f group and the PE+Der f group intratracheally received 1 µg of Der f in the same vehicle. Der f extract was injected intratracheally four times at 2-week intervals. The PE+Der f group was treated orally with rosmarinic acid at a dose of 1.5 mg/day in 100 µL of 5% ethanol. The vehicle and Der f groups were given equal volumes of 5% ethanol. Daily oral administration was continued from 3 days prior to first Der f treatment throughout the experiment. All mice were killed by deep anaesthesia using diethylether 24 h after the final Der f instillation.

Histological evaluation, bronchoalveolar lavage (BAL), and measurement of cytokines and chemokines

The lungs were fixed and stained with haematoxylin and eosin as previously described [27]. All bronchi of the whole sections of randomly selected lung were examined, and the intensity of inflammation was graded in blinded fashion as previously reported [28]. The sections were also stained with periodic acid-Schiff (PAS) to evaluate the degree of proliferation of goblet cells. BAL and cell counts were conducted as previously described [26]. The lungs were homogenized and centrifuged as previously described [26]. ELISAs for IL-5, eotaxin, and keratinocyte chemoattractant (KC) were conducted according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The ELISA for IL-4 was also conducted according to the manufacturer's instructions (Amersham, Buckinghamshire, UK).

Der f-specific Ig determination

Der f-specific IgG1 antibodies in plasma were measured by ELISA with solid-phase antigen as previously reported [6].

Statistical analysis

Data are reported as mean with standard errors. Differences were considered significant at values of $P < 0.05$. Differences in the score of eosinophilic inflammation were determined with non-parametric analysis. All statistical calculations were performed with the Winstat 3.0 EXCEL plug-in software from R. Fitch (<http://www.winstat.com/>, Kalmia, Cambridge, MA, USA).

Results

Rosmarinic acid in PE inhibits allergic inflammation with mucus cells induced by Der f

We first examined whether rosmarinic acid in PE could inhibit lung injury induced by intratracheal administration of Der f (Fig. 3). In histological study, intratracheal instillation with Der f led to the recruitment of eosinophils and neutrophils around the airways (Figs 3b and e), whereas that with vehicle did not (Figs 3a and d). Oral treatment with rosmarinic acid in PE strongly inhibited the allergic inflammation induced by Der f as indicated by a lack of recruitment of inflammatory cells (Figs 3c and f). PAS-positive mucus cells were not present in the bronchial epithelium of lungs from the vehicle group (Figs 4a and d). In contrast, numerous PAS-positive mucus cells were present in the bronchial epithelium of lungs from the Der f group (Figs 4b and e). Rosmarinic acid in PE treatment decreased the mucus-containing airway epithelial cells induced by Der f challenge (Figs 4c and f). Furthermore, we evaluated the magnitude of eosinophil recruitment around the airways in the lung specimens. Quantitative analysis showed that the number of eosinophils was increased after Der f challenge (Fig. 5, $P < 0.01$ vs. vehicle). The intensity of the infiltration was significantly prevented by PE treatment ($P < 0.05$ vs. Der f).

Effects of rosmarinic acid in PE on cell count in BAL fluid

We also investigated the cellular profiles of BAL fluid. The numbers of eosinophils and neutrophils in BAL fluid were

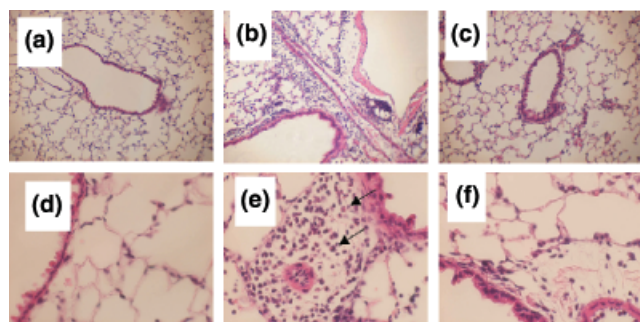


Fig. 3. Rosmarinic acid in perilla extract (PE) inhibits allergic inflammation in the lung induced by *Dermatophagoides farinae* (Der f). C3H/He mice were randomized into three experimental groups; one that received intratracheal inoculation of vehicle and two that received Der f inoculation, with and without oral pretreatment with rosmarinic acid in PE throughout the experiment at a dose of 1 mg/day. Lung specimens were stained with haematoxylin and eosin 24 h after the final intratracheal instillation of Der f. Lung sections were obtained from mice instilled with vehicle alone (a, d), Der f (b, e), or Der f pretreated with rosmarinic acid in PE (c, f). Eosinophils are indicated by arrows. Original magnification, $\times 80$ (a–c), $\times 400$ (d–f).

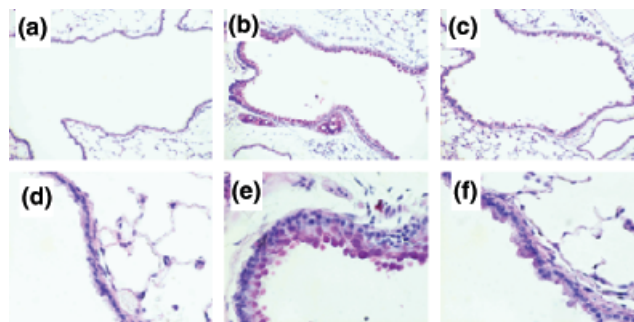


Fig. 4. Rosmarinic acid in perilla extract (PE) inhibits mucus cell accumulation. We performed periodic acid-Schiff staining of lung sections 24 h after the final intratracheal instillation of *Dermatophagoides farinae* (Der f). Lung sections were obtained from mice instilled with vehicle alone (a, d), Der f (b, e), or Der f pretreated with rosmarinic acid in PE (c, f). Goblet cell hyperplasia and mucus hypersecretion are observed in the airway epithelium. Original magnification, $\times 80$ (a–c), $\times 400$ (d–f).

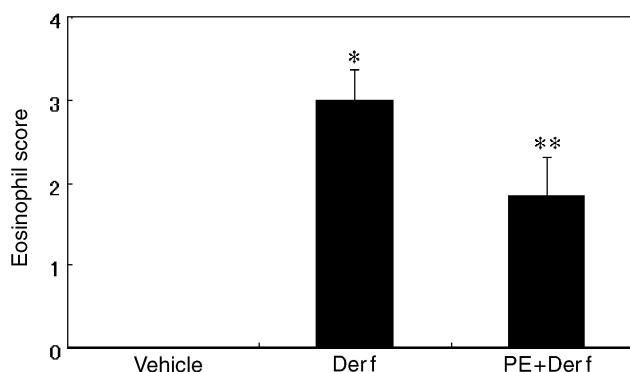


Fig. 5. Rosmarinic acid in perilla extract (PE) inhibits eosinophilic inflammation in the lung. Degree of eosinophilic infiltration into submucosal layer was estimated as follows: not present, 0; very slight, 1; slight, 2; moderate, 3; moderate to marked, 4; or marked, 5. Very slight was defined as $< 10\%$ of the airway associated with eosinophilic reaction, slight as 20–30% of the airway, moderate as 30–50%, moderate to marked as 50–70%, and marked as $> 70\%$ or more of the airway ($n = 6$ for each group). Values are the mean $\pm$ SE. * $P < 0.01$ vs. vehicle, ** $P < 0.01$ vs. *Dermatophagoides farinae* (Der f).

significantly greater in the Der f group than in the vehicle group (Fig. 6, $P < 0.01$). Rosmarinic acid in PE inhibited the influx of both eosinophils and neutrophils in BAL fluid (Figs 6a and b, $P < 0.01$ vs. Der f).

Effect of PE on lung expression of cytokine proteins

To examine the effects of rosmarinic acid in PE on the local expression of cytokines, we measured protein levels of IL-5 and IL-4 in lung tissue supernatants and in the BAL supernatants (Table 1). Instillation of Der f resulted in significant elevations of IL-5 concentrations in both lung tissue supernatants and in BAL supernatants compared with vehicle instillation ($P < 0.01$). Pretreatment with rosmarinic acid in PE inhibited the increased expression of IL-5 in lung tissue supernatants ($P < 0.05$ vs. Der f) and in BAL supernatants ($P < 0.01$ vs. Der f). Similarly, IL-4 was elevated in the lung tissue supernatants and BAL supernatants after Der f challenge ($P < 0.05$ vs. vehicle). Treatment with rosmarinic acid in PE inhibited the elevation of IL-4 in the BAL supernatants ($P < 0.05$ vs. Der f).

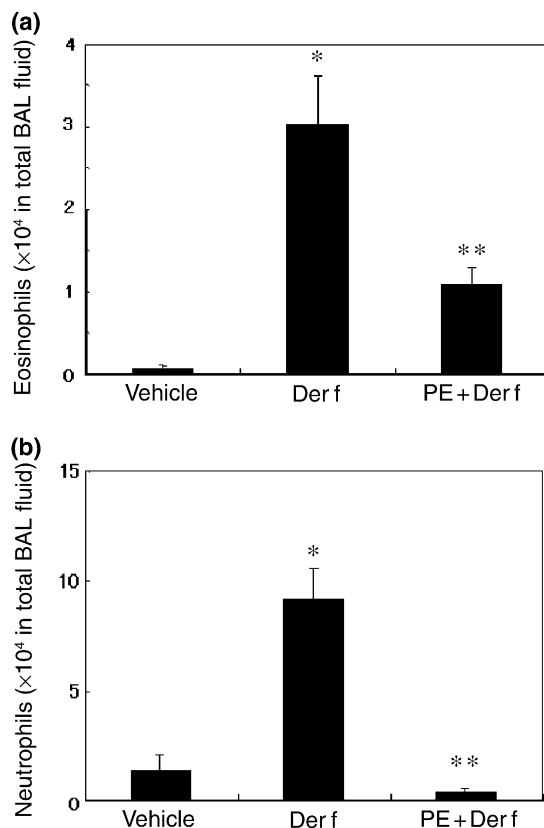


Fig. 6. Rosmarinic acid in perilla extract (PE) inhibits eosinophilic infiltration to bronchoalveolar (BAL) fluid. BAL was performed 24 h after the final intratracheal instillation of *Dermatophagoides farinae* (Der f). Eosinophil (a) and neutrophil (b) counts were assessed on cytological preparations stained with Diff-Quick (a, b) ($n = 8$ for the vehicle group, $n = 12$ for the Der f group, $n = 12$ for the PE+Der f group). Values are the mean $\pm$ standard error. * $P < 0.01$ vs. vehicle, ** $P < 0.01$ vs. Der f.

Table 1. Protein levels of Th2 cytokines in lung tissue and BAL supernatants*

Group	n	Lung tissue supernatants		BAL supernatants	
		IL-5 (pg/total lung tissue supernatants)	IL-4 (pg/total lung tissue supernatants)	IL-5 (pg/total BAL supernatants)	IL-4 (pg/total BAL supernatants)
Vehicle	8	10.8 $\pm$ 2.4	74.9 $\pm$ 4.9	0	97.2 $\pm$ 12.8
Der f	12	96.5 $\pm$ 6.6†	109.0 $\pm$ 5.5†	209.4 $\pm$ 20.5†	126.6 $\pm$ 11.0†
Der f+PE	12	73.5 $\pm$ 7.1‡	101.0 $\pm$ 2.8	152.9 $\pm$ 21.1§	91.4 $\pm$ 12.7‡

BAL, bronchoalveolar lavage; PE, perilla extract; Der f, *Dermatophagoides farinae*.

*Three groups of mice were intratracheally inoculated with vehicle or Der f. BAL was conducted 24 h after the last intratracheal instillation. Results are shown as mean $\pm$ standard error. n = number of animals.

† $P < 0.01$ vs. vehicle. ‡ $P < 0.05$ vs. Der f. § $P < 0.01$ vs. Der f.

Effect of rosmarinic acid in PE on local expression of chemokine proteins

To examine the local expression of chemokines, we also measured protein levels of eotaxin and KC in the lung tissue supernatants and BAL supernatant (Table 2). Instillation of Der f resulted in a significant elevation of eotaxin concentration in the lung tissue supernatants and BAL supernatants

compared with vehicle instillation ($P < 0.01$). Pretreatment with rosmarinic acid in PE inhibited the enhanced expression of eotaxin in the BAL supernatants ($P < 0.05$ vs. Der f). In the BAL supernatants, expression of KC was significantly greater in the Der f group than in the vehicle group ($P < 0.05$). Treatment with rosmarinic acid in PE significantly inhibited the increased expression of KC induced by Der f ($P < 0.05$ vs. Der f).

Effects of rosmarinic acid in PE on allergen-specific Ig production

To examine whether rosmarinic acid in PE treatment inhibits allergen-specific Ig production, we measured Der f-specific IgG1. The levels of Der f-specific IgG1 were significantly greater in the Der f group than in the vehicle group ($P < 0.01$, Fig. 7). Treatment with rosmarinic acid in PE significantly inhibited the elevated levels of Der f-specific IgG1 ($P < 0.05$ vs. Der f).

Discussion

In the present study, mite allergen challenge has caused allergic airway inflammation in mice. Der f-induced allergic airway inflammation has been inhibited by daily oral supplementation with rosmarinic acid in PE. The beneficial effects of rosmarinic acid in PE are associated with a decrease in the enhanced local protein expression of IL-4, IL-5, eotaxin, and KC. Furthermore, allergen-specific production of Ig is decreased by the treatment with rosmarinic acid in PE.

Previous *in vitro* and *in vivo* studies have shown that PE inhibits histamine release, antigen-specific IgE production, and passive cutaneous anaphylaxis (PCA) reaction [15, 16]. Furthermore, Makino et al. have reported that PE suppresses the PCA reaction, which is brought about by rosmarinic acid [19, 29]. Rosmarinic acid is a polyphenolic phytochemical frequently found in herbal plants and is mostly responsible for anti-inflammatory and anti-oxidative activity [21, 22]. There has been evidence that eating more apples and hard fruit, which contain phytochemicals such as flavonoid and non-flavonoid phenolic compounds, can protect against impaired lung function through anti-oxidant, anti-allergic, and anti-inflammatory activities [30, 31]. Recently Lee et al., have reported that Butterbur, a herbal remedy, exhibited protection against adenosine monophosphate-induced nasal responsiveness during the grass pollen season in sensitized patients [32]. These studies have proposed protective role for phytochemicals in respiratory diseases. Here, we show that rosmarinic acid in PE prevent allergic airway inflammation. We believe that this is the first demonstration that PE inhibit eosinophilic inflammation elicited by allergen *in vivo*.

In the present study, we used rosmarinic acid in PE at 1.5 mg/day, because we have already confirmed rosmarinic acid in PE inhibit hepatic inflammation at 1.25–5 mg/day [23]. Furthermore, we have confirmed that rosmarinic acid, the major component of PE used in this study, inhibits DEP-induced lung injury with neutrophilic inflammation and interstitial oedema [25]. In that study, we demonstrated that these effects were associated with changes in pro-inflammatory cytokine and chemokine such as IL-1 β , KC, and

Table 2. Protein levels of chemokine in lung tissue and BAL supernatants*

Group	n	Lung tissue supernatants		BAL supernatants	
		Eotaxin (pg/total lung tissue supernatants)	MCP-1	Eotaxin (pg/total BAL supernatants)	KC
Vehicle	8	89.3 ± 11.4	17.47 ± 4.63	13.85 ± 2.48	87.94 ± 38.44
Der f	12	358.7 ± 24.2†	45.86 ± 3.90†	94.03 ± 20.5†	226.92 ± 33.72†
Der f+PE	12	343.6 ± 25.6	27.70 ± 4.61‡	52.44 ± 7.92§	95.54 ± 23.84‡

BAL, bronchoalveolar lavage; MCP, monocyte chemoattractant protein; PE, perilla extract; Der f, *Dermatophagoides farinae*; KC, keratinocyte chemoattractant. *Three groups of mice were intratracheally inoculated with vehicle or Der f. BAL was conducted 24 h after the last intratracheal instillation. Results are shown as mean ± standard error. n = number of animals.

†P < 0.05 vs. vehicle. ‡P < 0.01 vs. Der f. §P < 0.05 vs. Der f.

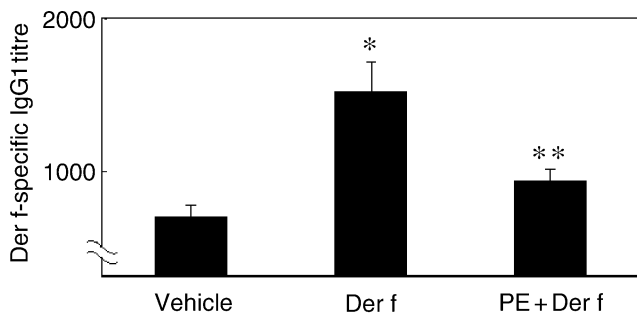


Fig. 7. Rosmarinic acid in perilla extract (PE) inhibits *Dermatophagoides farinae* (Der f)-specific IgG1 antibody levels in the plasma (n = 16 for the vehicle group, n = 24 for the Der f group, n = 22 for the PE+Der f group). Values are given as the mean ± standard error. *P < 0.01 vs. vehicle, **P < 0.05 vs. Der f.

monocyte chemoattractant protein-1 expression that plays a crucial role in the initiation and progression of the inflammatory response. Thus, it is likely that the inhibition of inflammatory cells such as neutrophils and eosinophils together with related inflammatory cytokines and/or chemokines may contribute to the prevention of allergic inflammatory responses.

Eosinophil accumulation is a distinctive feature of allergic inflammation. Eosinophils have the potential to activate Th2 cells to release disease-modulating cytokines and to assist in priming the immune system for allergic responses. The eosinophil has migrated into an inflamed tissue, it becomes activated and releases toxic mediators such as granule proteins, lipid mediators, oxygen metabolites, proteases, and cytokines [33]. Given the perceived importance of eosinophils in allergic inflammation, they have been logical therapeutic targets [34]. Eosinophil activating chemotactic cytokines and chemokines such as IL-5, eotaxin, and IL-4 are implicated in the pathogenesis of allergic diseases by recruiting and/or activating eosinophils [11, 35]. Many studies have suggested that these eosinophil-activating chemotactic cytokines are associated with the pathogenesis of allergic inflammation caused by mite allergens [6, 36]. IL-5, a Th2-type cytokine, promotes recruitment and activation of eosinophils and stimulates the release of chemical mediators from eosinophils [37]. Many studies have focused on IL-5 as a viable target for the treatment of asthma and allergic diseases [10, 38, 39]. IL-4 is also pivotal in the pathogenesis of allergic disorders through its wide range of effects including the formation of allergen-specific Ig [40]. Inhibitor of IL-4 signalling pathway

has been suggested as a therapeutic target [41, 42]. In the present study, IL-5 and IL-4 levels were significantly increased by Der f, which were inhibited by the treatment with rosmarinic acid in PE. Thus, the inhibition of IL-5 and IL-4 may be related to the suppressed eosinophilic airway inflammation by rosmarinic acid in PE.

Chemokines are effective leucocyte chemoattractants and may play an important role in mediating eosinophil recruitment in various allergic conditions. Eotaxin is an eosinophil-specific chemokine associated with the recruitment of eosinophils to sites of allergic inflammation [11, 35]. In the present study, expression of eotaxin was induced by Der f, which was significantly inhibited by treatment with rosmarinic acid in PE. KC, the murine homologue of human IL-8, has been suggested to induce lung inflammation and mucus accumulation in the airways [43, 44]. It has been suggested that expression of KC is induced in ovalbumin-challenged lungs of mice together with PAS-positive mucus accumulation, indicating that KC is another important molecule for allergic inflammation [45]. Treatment with rosmarinic acid in PE also inhibited KC expression enhanced by Der f. We observed that mRNA expression of IL-5 and KC is inhibited by the treatment with rosmarinic acid in PE as well as protein expression (data not shown), suggesting that inhibitory action of rosmarinic acid in PE on Der f-induced allergic inflammation depends at least in part on its inhibitory effect on mRNA expression.

It has been suggested that *Petasites hybridus*, a well-studied herbal remedy, exhibit *in vitro* inhibition of cysteinyl leukotriene biosynthesis and also exhibit effectiveness in treating with seasonal allergic rhinitis in human [32, 46–48]. In our investigation, rosmarinic acid in PE exhibit inhibition of pulmonary eosinophilic inflammation and of the enhanced expression of IL-4 and IL-5, and eotaxin. Since allergic inflammation is characterized by eosinophils, the beneficial effects of rosmarinic acid in PE on allergic inflammation induced by mite allergen may depend, at least in part, on the inhibition of eosinophil infiltration, accompanied by an increase in lung expression of IL-4 and IL-5, and eotaxin. Recently, we have confirmed that PE (rosmarinic acid 50 mg/day) is effective for seasonal allergic rhinoconjunctivitis in humans [49].

It has been suggested that allergen-specific IgG1 plays an important role in the degranulation of eosinophils and manifestation of allergic disease [50, 51]. In the present study, Der f-specific IgG1 antibody was inhibited by treatment with rosmarinic acid in PE, suggesting that amelioration of allergic

inflammation in this model is at least in part due to the inhibition of allergen-specific antibody. Similarly, allergen-specific IgE was elevated by Der f and inhibited by the treatment with rosmarinic acid in PE (data not shown).

In conclusion, daily oral supplementation with rosmarinic acid in PE prevented eosinophilic airway inflammation induced by HDM allergen in mice. These effects were associated with inhibition of the enhanced local expression of Th2 cytokines and chemokines. Rosmarinic acid in PE also inhibited allergen-specific Ig production enhanced by HDM. Considering the evidence and our results, phytochemicals such as rosmarinic acid may plausibly reduce allergic inflammation.

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