

RESEARCH ARTICLE

# Alleviation of allergic rhinitis symptoms with Pyeongwee-San extract (KMP6)

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## Abstract

**Context:** Allergy is characterized by the overreaction of the immune system. Pyeongwee-San is a traditional Korean medicine which has been used for the treatment of the allergic disorder but the mechanism of action is not clear.

**Objective:** To investigate the effect of Pyeongwee-San extract (KMP6) and its component, hesperidin (HES) in the allergic rhinitis (AR) animal model.

**Method:** We sensitized mice on 1, 5, and 14 days by intraperitoneal injections of 100 µg ovalbumin (OVA) emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received KMP6 and HES before the intranasal OVA challenge for 10 days.

**Results:** The number of nose rubs after the OVA challenge in the OVA-sensitized mice was significantly higher than that in the OVA-unsensitized mice. The increased number of nose rub was inhibited by the oral administration of KMP6 or HES. The increased levels of IgE and histamine level in serum of the OVA-sensitized mice were reduced by KMP6 or HES administration. The level of interferon-γ was enhanced while the level of IL-4 was reduced on the spleen tissue of the KMP6 or HES-administered AR mice. Inflammatory proteins level was reduced by KMP6 or HES administration in the nasal mucosa tissue of the OVA-sensitized mice. In the KMP6 or HES-administered mice, mast cells and eosinophils infiltration increased by OVA-sensitization was decreased.

**Conclusion:** These results indicate that KMP6 and HES ameliorate the allergic inflammatory reactions such as AR.

**Keywords:** KMP6, allergic rhinitis, macrophage-inflammatory protein-2, intercellular adhesion molecule-1, cyclooxygenase-2

## Introduction

Allergic rhinitis (AR) is a chronic disease of the nasal mucosa, affecting approximately 500 million people worldwide.<sup>(1)</sup> Although not life-threatening, AR can deteriorate the quality of life and can be a major risk. Despite its clinical and socioeconomic impact, advances in its treatment still have a long way to go.<sup>(2)</sup> Many of the symptoms of patients with AR, sneezing, itching, and respiratory obstruction cause a lot of pain. However, the symptoms of AR do not end here. If prolonged, AR can cause problems in the nasal voice box and can cause very severe eye and ear symptoms.<sup>(3)</sup> These symptoms are due

to the release of histamine and other active substances by mast cells, which stimulate the dilation of blood vessels, irritate nerve endings and increase the secretion of tears.<sup>(4)</sup>

Since the discovery by Coffman and colleagues of two distinct types of Th in mice, mutual regulation between Th1 cells and Th2 cells has been considered important for homeostatic maintenance of the immune system in the whole body.<sup>(5)</sup> Dysregulated Th1 and Th2 responses lead to excessive Th1 cell or Th2 cell activation, resulting in the development of autoimmune diseases associated with the accumulation of Th1 cells or in an

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(Received 22 April 2011; revised 03 May 2011; accepted 07 May 2011)

induction of allergic diseases due to the accumulation of Th2 cells, respectively.<sup>(6)</sup> In response to exposure to allergens, patients with AR present an inflammatory IgE-mediated response characterized by a Th2 immunologic pattern with mast cells and eosinophils activation and the release of inflammatory mediators, interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$ .<sup>(7,8)</sup> Leukotrienes and prostanoids produced by the 5-lipoxygenase and cyclooxygenase (COX)-2 pathways have potent proinflammatory and vascular actions that implicate them in allergic and inflammatory reactions.<sup>(9)</sup> Eosinophils are innate effector cells that are important in immune responses against helminth parasitic infections and contribute to the pathology associated with allergic inflammatory conditions. Mast cells contribute to the induction and/or maintenance of eosinophilic inflammation by a variety of mechanisms, including IgE-dependent and IgE-independent processes.<sup>(10)</sup> The recruitment of these mast cells to inflammatory sites occurs in response to chemotactic and activation signals.<sup>(11)</sup> Intercellular adhesion molecule (ICAM)-1, a member of the immunoglobulin gene superfamily of adhesion molecules, is expressed on the surface of a variety of cells, including vascular endothelial cells, epithelial cells, alveolar macrophages, and fibroblasts.<sup>(12-14)</sup> Mast cell-derived TNF- $\alpha$  contributes to allergic reactions through production of an ICAM-1.<sup>(15,16)</sup> Macrophage-inflammatory protein-2 (MIP-2) is a potent chemoattractant for immune cells.<sup>(17,18)</sup>

Pyeongwee-San is a traditional Korean medicine which has been used as a basic prescription of allergic disorder. To clarify the efficacy of Pyeongwee-San extract (KMP6) for the treatment of patients with AR, this study investigated the effects of KMP6 and its component, hesperidin (HES), in the ovalbumin (OVA)-induced AR mice model.

## Materials and methods

### Materials

HES, dexamethasone (DEX), terfenadine (TER), OVA, O-phthalaldehyde (OPA), avidin peroxidase, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) tablets substrate (ABTS), and other reagents were purchased from Sigma (St. Louis, MO). Anti-mouse IgE/TNF- $\alpha$ /IL-1 $\beta$ /MIP-2/ICAM-1 antibody (Ab), biotinylated anti-mouse IgE/TNF- $\alpha$ /IL-1 $\beta$ /MIP-2/ICAM-1 Ab, and recombinant mouse (rm) IgE/TNF- $\alpha$ /IL-1 $\beta$ /MIP-2/ICAM-1 were purchased from Pharmingen (Sandiego, CA). Ab for COX-2 and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The caspase-1 assay kit was supplied by R&D Systems Inc. (Minneapolis, MN).

### Preparation of drugs

KMP6 was provided by the Korea Medi Inc. (Seoul, Republic of Korea). To compare the effect of KMP6, we obtained the Han Shin Pyeongwee-San (HS-PS, an over-the-counter drug) from Han Kook Shin Yak

pharmaceutical Co., LTD. (Nonsan, Republic of Korea). The KMP6 was dissolved in distilled water and filtered with 0.22  $\mu$ m syringe filter. HS-PS granules were prepared by dissolving in distilled water and being autoclaved for the sterilization and kept at 4°C. Dilutions were made in saline then filtered through 0.45- $\mu$ m syringe filter. KMP6 is composed of *Atractylodes japonica*, *Machilus thunbergii*, *Citrus Sunki*, *Zizyphus jujube*, *Glycyrrhiza uralensis*, and *Zingiber officinale*. The amounts of KMP6 for adult Korean are about 100–150 g. The yield of powered extraction is commonly about 5% (w/w). Therefore, an adult (average body weight 60 kg) takes about 0.1 g/kg. The dose of KMP6 for an adult person can be 0.1 g/kg. The dose range of 0.01–1 g/kg was chosen to see the dose-dependency. Dose of HS-PS (2 g/kg) was decided twice stronger than KMP6 (1 g/kg) because HS-PS granules (3.5 g) contain some excipients (1.7 g). Major constituents in KMP6 are HES, glycyrrhizin, and liquiritin. Contents of HES and glycyrrhizin in the KMP6 by HPLC are about 5.26 and 2.60 mg/g (data not shown). In this study, HES used as a major compound.

### OVA-induced AR animal model

We maintained 6-week-old female BALB/c (Charles River Technology) mice under pathogen-free conditions. Mouse care and experimental procedures were performed under approval from the Animal Care Committee of Kyung Hee University. We sensitized the mice on 1, 5, and 14 days by intraperitoneal (i.p.) injection of 100  $\mu$ g OVA emulsified in 20 mg aluminum hydroxide (Sigma) and we challenged the mice with 1.5 mg OVA. KMP6, HS-PS, HES, TER, and DEX were administrated orally before the intranasal (i.n.) OVA challenge for 10 days. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in the 10 min after OVA i.n. provocation at 10 days after challenge.

### Histamine assay

The histamine content of serum was measured by an OPA spectrofluorometric procedure. The fluorescent intensity was measured at 460 nm (excitation at 355 nm) using a spectrofluorometer.

### Histological examination

Tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. Sections of the nasal mucosa samples were 4  $\mu$ m thick. Each section was stained with hematoxylin and eosin (H&E, for eosinophils), alcian blue and safranin O (A&S, for mast cells) before dewaxing and dehydration. The numbers of eosinophils and mast cells on both sides of the septal mucosa were counted. Sections were coded and randomly analyzed by two blinded observers.

### Enzyme-linked immunosorbent assay

A modified enzyme-linked immunosorbent assay (ELISA) was used to measure the cytokine on tissue protein. The

ELISA was performed by coating 96-well plates with 6.25 ng/well of capture Ab. Before the subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of cytokine, as provided by the manufacturer. After exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies, and avidin peroxidase, and ABTS substrate solution containing 30%  $\text{H}_2\text{O}_2$ . The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Production IgE in serum, cytokine levels in the spleen and nasal mucosa were divided according to the total protein. Protein levels were determined using a bicinchoninic acid.

### Western blot analysis

Nasal mucosa tissue samples were heated at 95°C for 5 min and briefly cooled on ice. Following the centrifugation at 15,000×g for 5 min, 50 µg aliquots were resolved by 10% SDS-PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerol, 20% methanol at 25 V. Blots were blocked for at least 2 h with 1 × PBS containing 0.05% Tween-20 containing 5% nonfat dry milk and then incubated with primary antibodies (COX-2, 1:500 dilution) for 1 h at room temperature. Blots were developed by peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

### Statistical analysis

The results shown are a summary of the data from at least three experiments and are presented as the mean ± standard error of the mean (SEM). Statistical evaluation of the results was performed by an independent *t*-test and ANOVA with Tukey *post hoc* test. The results were considered significant at a value of  $P < 0.05$ .

## Results

### Effect of KMP6 and HES on clinical symptoms, IgE production, histamine release, and IFN-γ and IL-4 production in the AR animal model

To investigate the inhibitory effect of KMP6 in the AR model, we sensitized mice on days 1, 5, and 14 by i.p. injections of 100 µg OVA emulsified in 20 mg aluminum hydroxide and challenged mice with 1.5 mg OVA. KMP6, HS-PS, HES (a main component of KMP6), DEX, or TER were administered orally before i.n. OVA challenge for 10 days. We also tested the effect of KMP6 compared with DEX or TER used as a positive control in this study. The number of nasal rubs after the OVA challenge in the OVA-sensitized mice was significantly higher than

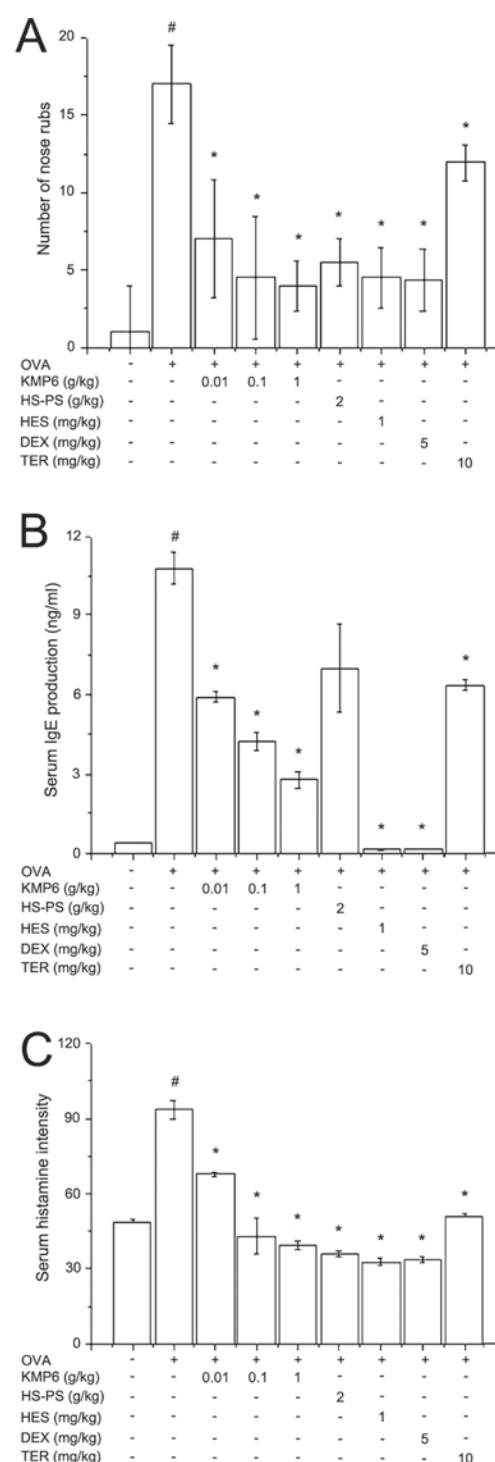


Figure 1. Effect of KMP6 and HES on clinical symptoms, IgE level, and histamine release in the AR model. We sensitized mice on 1, 5, and 14 days by i.p. injections of 100 µg OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received KMP6, HS-PS, HES, DEX, and TER before the intranasal OVA challenge for 10 days. (A) The number of the nasal rubs that occurred in the 10 min after the OVA intranasal provocation. (B) IgE was measured by ELISA. (C) Serum was isolated from blood and then assayed about histamine.  $P < 0.05$ : significantly different from the OVA-unsensitized mice.  $*P < 0.05$ : significantly different from the OVA-sensitized mice. KMP6, Pyeongwee-San; HS-PS, Pyeongwee-San from Han Kook Shin Yak pharmaceutical Co.; HES, hesperidin; DEX, dexamethasone; TER, terfenadine.  $N = 5$ .

those in the OVA-unsensitized mice. Increased rub score was inhibited by the treatment of KMP6, HS-PS, HES, DEX, or TER (Figure 1A). Levels of IgE in the OVA-sensitized mice were significantly higher than those in the serum of the OVA-unsensitized mice (Figure 1B). Increased IgE levels in serum were significantly reduced by KMP6, HES, DEX, or TER but not HS-PS. Histamine levels in the serum were also reduced by KMP6, HS-PS, HES, DEX, or TER (Figure 1C). To identify the Th1/Th2 immune reaction in KMP6, HS-PS, HES, DEX, or TER-administered mice, we measured IFN- $\gamma$  and IL-4 production. IFN- $\gamma$  levels were significantly reduced in the OVA-sensitized mice. IFN- $\gamma$  level was significantly increased in the KMP6, HES, DEX, or TER-administered AR mice (Figure 2). IL-4 level in the spleen of AR mice was significantly increased compared with that in OVA-unsensitized mice (Figure 2). IL-4 level was significantly decreased in the KMP6, HS-PS, HES, DEX, or TER-administered AR mice. The inhibitory effect of KMP6 (1 mg/mL) is similar to the effect of DEX and TER in the AR models.

### Effect of KMP6 and HES on inflammatory protein levels in the AR animal model

To evaluate the regulatory effect of KMP6, HS-PS, HES, or DEX on inflammatory cytokine production, we measured the protein levels of TNF- $\alpha$ , IL-1 $\beta$ , MIP-2, and ICAM-1 in the nasal mucosa tissue were increased in the OVA-sensitized mice compared to OVA-unsensitized mice (Figure 3A–D). However, protein levels of TNF- $\alpha$ , IL-1 $\beta$ , MIP-2, and ICAM-1 were significantly inhibited in the KMP6, HS-PS, HES, or DEX-administered mice compared to the OVA-sensitized mice ( $P < 0.05$ ). In addition, expression of COX-2 was inhibited by administration of

KMP6, HS-PS, HES, or DEX in the nasal mucosa tissue (Figure 3E and 3F).

### Effect of KMP6 and HES on eosinophils and mast cells infiltration in the nasal mucosa tissues

The respective numbers of inflammatory cells (eosinophils and mast cells) in the nasal mucosa in the AR mice were significantly higher than those in the OVA-sensitized mice. In the KMP6, HS-PS, HES, or DEX-administered mice, eosinophil and mast cell infiltration increased by OVA-sensitization was decreased (Figure 4A and 4B).

### Discussion

In this study, KMP6 and HES, major component of KMP6 reduced the allergic and inflammatory reaction in the AR animal model.

AR is characterized by a two-phase allergic reaction. In the early-phase inflammatory response, the allergen-IgE dependent activation of mast cells and basophils results in the production of pharmacologically active mediators such as histamine, prostaglandins, leukotrienes, and cytokines which produce sneezing, rhinorrhea, and itching.<sup>(19)</sup> The late-phase of AR shows accumulations of mast cells, eosinophils, and basophils in the epithelium and an accumulation of eosinophils in the deeper lamina propria.<sup>(20)</sup> Recruitment of inflammatory cells including eosinophils, mast cells, basophils, and T cells results in further release of histamine and leukotrienes, as well as in the release of other compounds including proinflammatory cytokines, COX-2, and chemokines, sustaining the allergic response and promoting the late-phase response.<sup>(20,21)</sup> In previous studies, polyphenolic

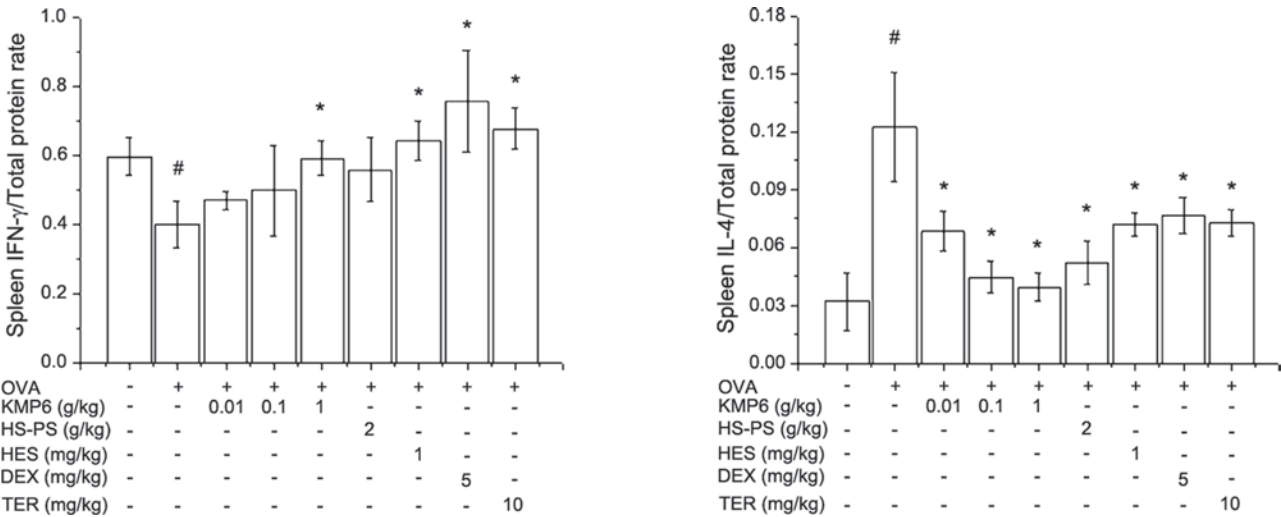


Figure 2. Effect of KMP6 and HES on Th1/Th2 cytokine levels in the AR model. We sensitized mice on 1, 5, and 14 days by i.p. injections of 100  $\mu$ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received KMP6, HS-PS, HES, DEX, and TER before the intranasal OVA challenge for 10 days. IFN- $\gamma$  and IL-4 were measured in spleen tissues by ELISA. All parameters measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. # $P < 0.05$ : significantly different from the OVA-unsensitized mice. \* $P < 0.05$ : significantly different from the OVA-sensitized mice. KMP6, Pyeongwee-San; HS-PS, Pyeongwee-San from Han Kook Shin Yak pharmaceutical Co.; HES, hesperidin; DEX, dexamethasone; TER, terfenadine.  $N = 5$ .

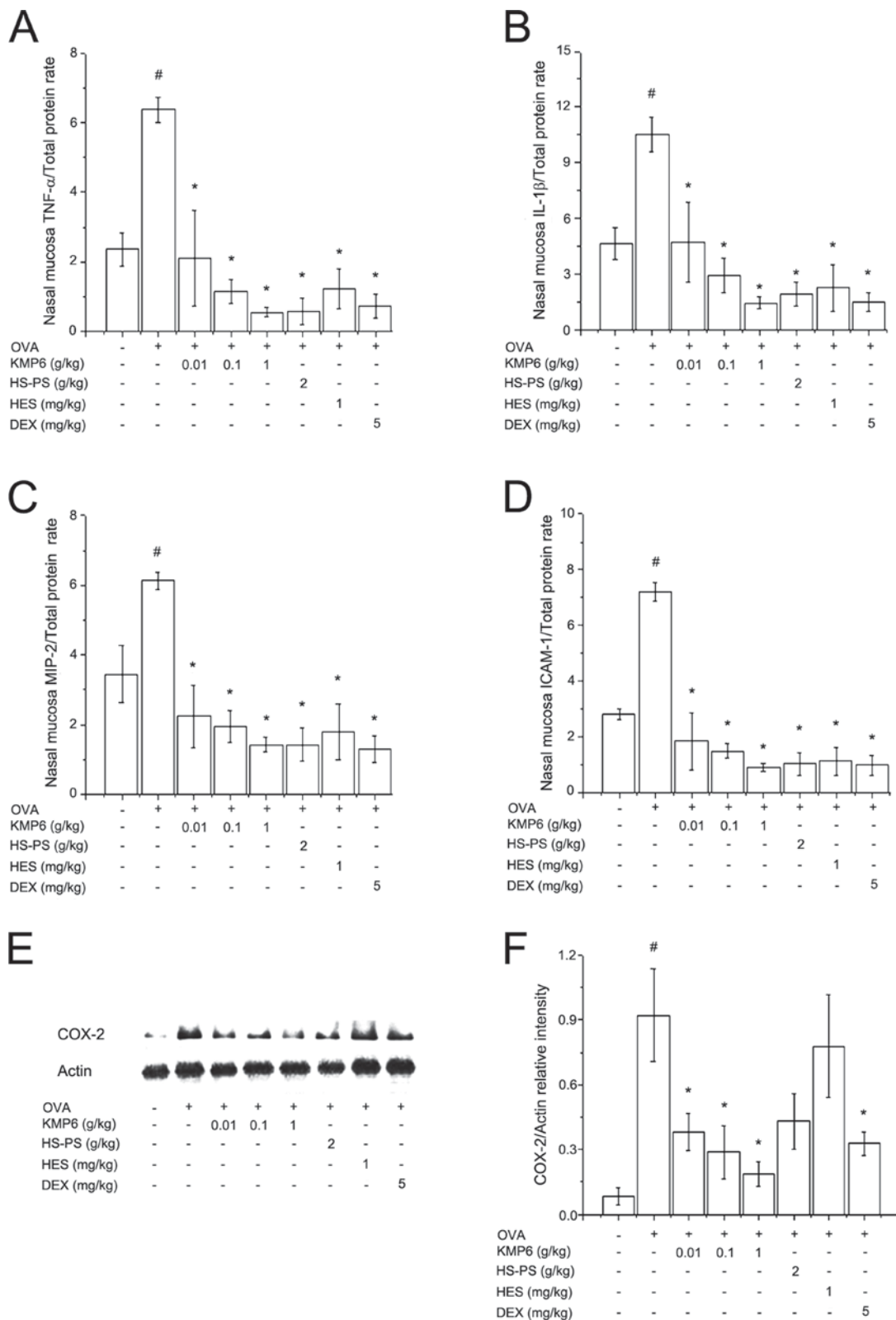


Figure 3. Effect of KMP6 and HES on inflammatory protein levels in the AR model. We sensitized mice on 1, 5, and 14 days by i.p. injections of 100  $\mu$ g OVA emulsified in 20 mg of aluminum hydroxide and we challenged mice with 1.5 mg OVA. Mice received KMP6, HS-PS, HES, and DEX before the intranasal OVA challenge for 10 days. (A–D) TNF- $\alpha$ , IL-1 $\beta$ , MIP-2, and ICAM-1 were measured by ELISA method in nasal mucosa tissue. TNF- $\alpha$ , IL-1 $\beta$ , MIP-2, and ICAM-1 measured in the tissue homogenate were presented as a ratio to the total protein level in tissue. (E) COX-2 protein expression was evaluated by using Western blot analysis. (F) The protein levels were quantitated by densitometry. Results are representative of three independent experiments. \* $P$  < 0.05: significantly different from the OVA-sensitized mice. \* $P$  < 0.05: significantly different from the OVA-unsensitized mice. KMP6, Pyeongwee-San; HS-PS, Pyeongwee-San from Han Kook Shin Yak pharmaceutical Co.; HES, hesperidin; DEX, dexamethasone.  $N$  = 5.

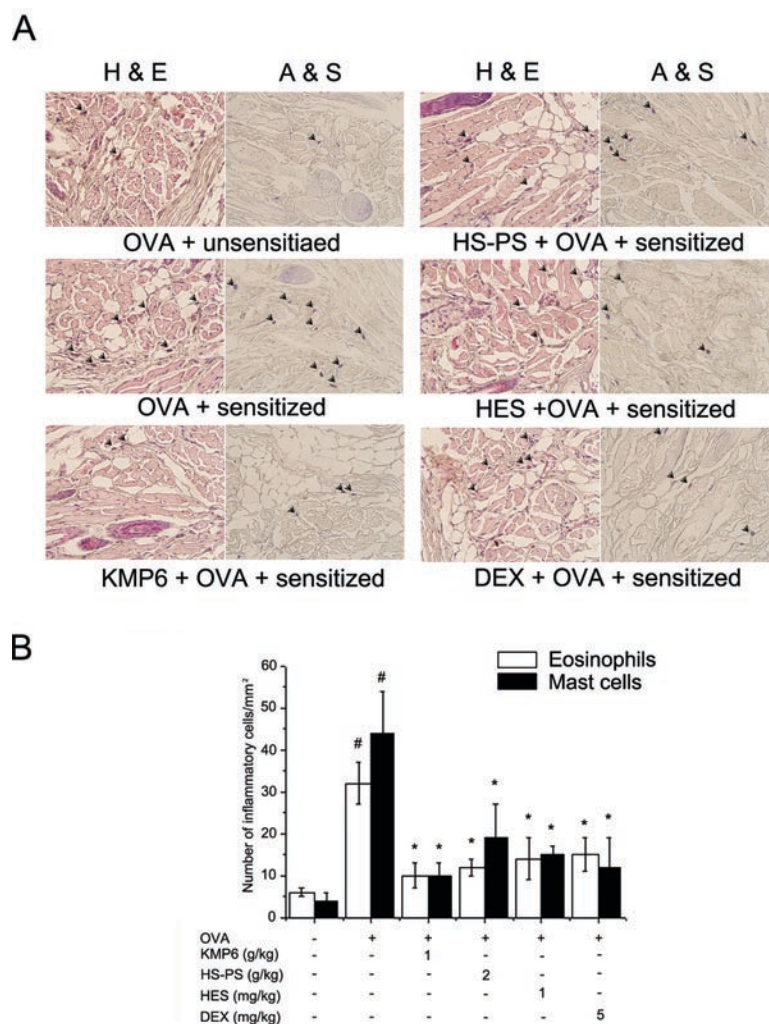


Figure 4. Effect of KMP6 and HES on eosinophils and mast cells infiltration in the nasal mucosa tissue. (A) Nasal mucosa stained with H&E (for eosinophils), alcian blue and safranin O (for mast cells). Eosinophils and mast cells were indicated by arrows. (B) Eosinophils and mast cells were counted by two individuals. After five randomly selected tissue sections per mouse were counted. The absolute number of cells was counted as the mean  $\pm$  SEM. <sup>#</sup> $P < 0.05$ : significantly different from the OVA-unsensitized mice. <sup>\*</sup> $P < 0.05$ : significantly different from the OVA-sensitized mice. KMP6, Pyeongwee-San; HS-PS, Pyeongwee-San from Han Kook Shin Yak pharmaceutical Co.; HES, hesperidin; DEX, dexamethasone (original magnification  $\times 400$ ).

phytochemicals including rosmarinic acid have been shown to inhibit IgE response<sup>(22,23)</sup> and inflammation characterized by polymorphonuclear leukocytes (eosinophils and neutrophils) infiltration.<sup>(24,25)</sup> TER is the most effective drug for AR. TER is an antihistamine with selective peripheral H<sub>1</sub>-receptor antagonist activity.<sup>(26)</sup> OVA-induced nasal symptoms and nasal vascular permeability in sensitized rats were clearly inhibited by TER.<sup>(27)</sup> Glucocorticosteroid (GC) is the most effective drug for AR. GC inhibits the function of infiltrating inflammatory cells and their recruitment into the nasal mucosa. GC inhibits the maturation, cytokine production, COX-2 expression, Fc $\epsilon$ RI expression, and mediator release of mast cells.<sup>(28,29)</sup> We observed that KMP6, HS-PS, and HES reduced the IgE and inflammatory related protein level in the AR model. Dexamethasone also reduced the allergic and inflammatory reaction. Therefore, our results suggest that the effects of KMP6 and HS-PS are similar to the mechanism of rosmarinic acid or GC. We can

also deduce that KMP6 and HS-PS have an anti-allergic effect.

KMP6 is a traditional Korean medicine which has been using as a prescription for digestive system. As is already described in the materials and methods section, KMP6 consists of 6 different herbs. HES, an active component of KMP6, is a major component of *C. Sunki*. Some studies have suggested that HES attenuated the production of VEGF, IL-1 $\beta$ , IL-8, TNF- $\alpha$ , and HIF-1 $\alpha$  induced by PMA plus A23187 in mast cells.<sup>(30)</sup> In addition, HES reduced the production of IL-6 in SW982 synovial cells by inhibiting c-Jun N-terminal kinase.<sup>(31)</sup> Beta-eudesmol, component of the *A. japonica* inhibited the TNF- $\alpha$  production and expression in activated mast cells.<sup>(32)</sup> Magnolol, component of the *M. thunbergii* inhibited the inflammatory reaction via inhibiting NF- $\kappa$ B activation.<sup>(33)</sup> Glycyrrhizic acid, component of the *G. uralensis* has the effect of attenuation in asthma and chronic obstructive pulmonary disease.<sup>(34)</sup> Therefore, we suggest that KMP6 has an

anti-inflammatory effect by preventing the expression of inflammatory protein.

A focused point of oriental medicine is to achieve well-balanced efficacy with several ingredients. Deletion of some herbs like one or two from this prescription may show probably similar activity but no doubt less activity. However, we need to carry out the isolation of active compounds and mechanism study of active compound. The experiments were proved to be active compound HES for KMP6. Active components of other herbs need further research.

In conclusion, this study suggests that KMP6 can inhibit the induction of allergic and inflammatory reaction. In addition, this study suggests that the effect of these actions by KMP6 may be direct or indirect through blockade of inflammatory cell infiltration and cytokine activation in the nasal mucosa. From this, our results provide for the therapeutic potential of the KMP6 for AR.

## Declaration of interest

This study was supported by a grant of the Traditional Korean Medicine R&D Project, Ministry for Health & Welfare & Family Affairs, Republic of Korea (No. B100037).

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