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β -eudesmol suppresses allergic reactions via inhibiting mast cell degranulation

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SUMMARY

The regulatory effect of β -eudesmol, which is an active constituent of Pyeongwee-San (KMP6), is evaluated for allergic reactions induced by mast cell degranulation. Phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187-stimulated human mast cell line, HMC-1 cells, and compound 48/80-stimulated rat peritoneal mast cells (RPMCs) are used as the *in vitro* models; mice models of systemic anaphylaxis, ear swelling, and IgE-dependent passive cutaneous anaphylaxis (PCA) are used as the *in vivo* allergic models. The results demonstrate that β -eudesmol suppressed the histamine and tryptase releases from the PMA plus calcium ionophore A23187-stimulated HMC-1 cells. β -eudesmol inhibits the expression and activity of histidine decarboxylase in the activated HMC-1 cells. In addition, β -eudesmol inhibits the levels of histamine and tryptase released from the compound 48/80-stimulated RPMCs. Furthermore, β -eudesmol decreases the intracellular calcium level in the activated RPMCs. β -eudesmol also decreases the compound 48/80-induced mortality and ear swelling response. β -eudesmol suppresses the serum levels of histamine, IgE, IL-1 β , IL-4, IL-5, IL-6, IL-13, and VEGF under PCA mice as well as PCA reactions. Therefore, the results from this study indicate the potential of β -eudesmol as an anti-allergic drug with respect to its pharmacological properties against mast cell-mediated allergic reactions.

Key words: β -eudesmol, mast cells, histamine, degranulation, anaphylaxis, ear swelling.

INTRODUCTION

Allergic disorders such as anaphylaxis affect approximately 25% of the population in the developed world.¹ Mast cells have a significant effect in inflammatory and immediate allergic reactions and contribute to several features of allergic inflammation.² Exposure to allergens cross-links the cell-bound IgE and triggers releases of preformed histamines and newly generated cytokines in the mast cells.³ Histamine is synthesized via histidine decarboxylase (HDC).⁴ The increased circulating histamine enhances vascular permeability with subsequent overproduction of pro-inflammatory mediators.⁵ Tryptase functions as a signal for mast cell activation⁶ and a calcium signal is essential for degranulation and production of cytokines in the mast cells.⁷

It has been reported that allergic disorders and gastrointestinal symptoms may be correlated.^{1,8} Allergic diseases can be induced by a disorder in the digestive system.⁹ Minor gastrointestinal abnormalities are frequent in individuals with allergic diseases, such as atopic eczema¹⁰ and asthma.¹¹

It has been reported that Pyeongwee-San (KMP6), which has been used to treat digestive diseases in traditional Korean medicine, has a beneficial effect on allergic reactions¹² and atopic dermatitis.¹³ *Atractylodes japonica* is the primary component of KMP6 and β -eudesmol is a significant constituent of *Atractylodes japonica*.¹⁴ *Atractylodes japonica* also has pharmaceutical activities, such as an anti-inflammatory effect.¹⁵ β -eudesmol also inhibits inflammatory reactions¹⁶ and tumor development.¹⁷ However, the fundamental

therapy approaches of β -eudesmol for allergic diseases have not yet been established. In this study, mouse models of systemic anaphylaxis, ear swelling, and IgE-dependent passive cutaneous anaphylaxis (PCA) are used to investigate the effects of β -eudesmol on the activation of mast cells, particularly degranulation of the mast cells. Thus, in this study, how β -eudesmol regulates several features of these allergic reactions in mast cells is investigated using the human mast cell line HMC-1 and rat peritoneal mast cells (RPMCs).

RESULTS

Effect of β -eudesmol on degranulation of HMC-1 cells

First, β -eudesmol was pretreated in phorbol 12-myristate 13-acetate plus calcium ionophore A23187 (PMACI)-stimulated HMC-1 cells in order to determine whether β -eudesmol would regulate the degranulation of the mast cells. Ketotifen and dexamethasone (Dex) were used as positive controls. Ketotifen has been reported to reduce the mast cell degranulation and inflammatory mediator release.¹⁸ Dex suppresses histamine synthesis through inhibiting the HDC activity.¹⁹ β -eudesmol significantly reduced the levels of histamine, HDC, and tryptase in the activated HMC-1 cells ($P < 0.05$; Fig. 1a-e). The inhibition rates of β -eudesmol (20 μ M) for the histamine, HDC activity, and tryptase were approximately 78%, 32%, and 68%, respectively. The vehicle control did not significantly regulate the histamine level in the inactivated HMC-1 cells and PMACI-stimulated HMC-1 cells (Fig. 1a). Furthermore, β -eudesmol did not affect these levels in the inactivated HMC-1 cells (Fig. 1a-e). Thus, the subsequent *in vitro* experiments were conducted, except for the vehicle control group. β -eudesmol significantly decreased the level of intracellular calcium similar to 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-AM (BAPTA-AM; intracellular calcium

chelator) on the level of intracellular calcium in the activated HMC-1 cells ($P < 0.05$; Fig. 1f). KMP6 significantly inhibited the levels of histamine, HDC, tryptase, and intracellular calcium ($P < 0.05$; Fig. 1). Also, ketotifen significantly inhibited the levels of histamine, tryptase, and intracellular calcium ($P < 0.05$; Fig. 1a,e,f). Dex decreased the expression and activity of HDC in the activated HMC-1 cells (Fig. 1b-d).

Effect of β -eudesmol on the degranulation of RPMCs

It was examined whether β -eudesmol regulates the degranulation of mast cells using compound 48/80, which induces the degranulation of mast cells as a basic secretagogue.²⁰

Compound 48/80 significantly increased the release of histamine from the RPMCs ($P < 0.05$;

Fig. 2a) however, β -eudesmol dose-dependently reduced the histamine level released from the compound 48/80-stimulated RPMCs ($P < 0.05$; Fig. 2a, 20 μ M of β -eudesmol:

approximately 24% inhibition). Furthermore, β -eudesmol significantly reduced the tryptase level in the activated RPMCs ($P < 0.05$; Fig. 2b, 20 μ M of β -eudesmol: approximately 61% inhibition). However, β -eudesmol did not affect the levels of histamine and tryptase in the

inactivated RPMCs (Figs. 2a,b). β -eudesmol also significantly suppressed the intracellular calcium level in the compound 48/80-stimulated RPMCs ($P < 0.05$; Fig. 2c). BAPTA-AM

significantly inhibited the level of intracellular calcium in the activated RPMCs ($P < 0.05$;

Fig. 2c). KMP6 and ketotifen significantly reduced the levels of histamine, tryptase, and

intracellular calcium in the compound 48/80-stimulated RPMCs ($P < 0.05$; Fig. 2). In order to

confirm these results for β -eudesmol on the degranulation, the morphology was further

observed in the activated RPMCs. The compound 48/80-stimulated RPMCs were swollen and

had disrupted boundaries and multiple granules. However, β -eudesmol, KMP6, and ketotifen

retained the basal RPMCs with a circular shape compared with the activated RPMCs. β -

eudesmol, KMP6, and ketotifen significantly decreased the number of degranulated RPMCs ($P < 0.05$; Fig. 3).

Effect of β -eudesmol on systemic anaphylaxis

Because β -eudesmol had inhibitory activity on the degranulation of the mast cells, it was investigated whether β -eudesmol would regulate systemic anaphylaxis. The mortality was measured through intraperitoneally injecting compound 48/80 into mice. As described in Table 1, β -eudesmol decreased the compound 48/80-induced mortality. KMP6 and ketotifen also inhibited the mortality (Table 1). The vehicle control group did not affect the mortality (Table 1). Thus, subsequent *in vivo* experiments were conducted, excluding the vehicle control group.

Effect of β -eudesmol on the ear swelling response

Next, it was investigated whether β -eudesmol would regulate the ear swelling response. As expected, β -eudesmol significantly decreased the compound 48/80-induced ear swelling response ($P < 0.05$; Fig. 4, 20 μ M of β -eudesmol: approximately 67% inhibition). KMP6 and ketotifen also significantly decreased the ear swelling response that was increased by compound 48/80 ($P < 0.05$; Fig. 4).

Effect of β -eudesmol on the IgE-dependent PCA

Finally, it was investigated whether β -eudesmol would regulate a local anaphylaxis (PCA). The mice sensitized with an intradermal injection of anti-dinitrophenyl (DNP)-IgE antibody were pretreated with β -eudesmol, KMP6, or ketotifen, and then injected with a mixture of DNP-human serum albumin (HSA) and Evans blue. The PCA reaction was significantly inhibited by β -eudesmol ($P < 0.05$; Fig. 5, 20 μ M of β -eudesmol: approximately 90% inhibition). KMP6 and ketotifen significantly inhibited the PCA reaction ($P < 0.05$; Fig. 5). Furthermore, the results from the PCA reaction demonstrated that the interaction between the anti-DNP IgE antibody and DNP-HSA markedly increased the levels of histamine, IgE, and cytokines in the serum ($P < 0.05$; Table 2). However, β -eudesmol and KMP6 significantly suppressed the serum levels of histamine, IgE, IL-1 β , IL-4, IL-5, IL-6, IL-13, and vascular endothelial growth factor (VEGF) ($P < 0.05$; Table 2). Ketotifen significantly decreased the serum levels of histamine, IgE, IL-5, IL-6, IL-13, and VEGF ($P < 0.05$; Table 2). However, β -eudesmol, KMP6, and ketotifen did not regulate the level of serum tumor necrosis factor (TNF)- α . β -eudesmol did not influence the levels of histamine, IgE, and cytokines in the serum of unchallenged mice.

DISCUSSION

It has been demonstrated that β -eudesmol and KMP6 improve the systemic anaphylaxis, ear swelling response, and PCA reactions through down-regulating the mast cell degranulation. KMP6 has been used to treat disorders of the digestive system in traditional Korean medicine for centuries. The anti-ulcer effect of *Atractylodes japonica*, which is a primary component of KMP6, was established via its anti-inflammatory properties and gastroprotective effects.²¹ β -

eudesmol, which is an active constituent of *Atractylodes japonica*, also exerted an inhibitory effect on the gastric secretion stimulated by histamines and prevented gastric ulceration.²² In addition, Koonrungsesomboon *et al.* reported that β -eudesmol has anti-inflammatory, anti-microbial, and anti-pyretic effects.²³ Furthermore, a new beneficial effect of β -eudesmol was determined for mast cell-mediated allergic reactions in the present study.

Mast cells are a principal effector cell of immediate hypersensitivity reactions.²⁴ The various mediators released shortly after mast cell degranulation cause an immediate hypersensitivity reaction.²⁵ Histamine and tryptase are the significant markers of mast cell degranulation and are prominent contributors to allergic diseases.²⁶ In anaphylaxis, histamine mediates vascular permeability, smooth muscle contraction, and vasodilation.²⁷ HDC-deficient mice exhibited significant decreases in histamine concentration and numbers of mast cells, which exhibited reduced granular content.²⁸ Thus, HDC is a potential target in anti-allergic inflammation therapies.²⁹ The tryptase levels are increased in the serum of most individuals with anaphylaxis.³⁰ The morphological studies of specimens exhibited higher levels of degranulation of the mast cells in patients with fatal asthma.³¹ Kempuraj *et al.* revealed that flavonoids were suitable for the treatment of allergic diseases through inhibiting the levels of histamine and tryptase as well as IL-6 and TNF- α from mast cells.³² Dex has also been reported to suppress histamine synthesis through inhibiting HDC activity and ameliorate allergic reactions.¹⁹ Similar to the inhibitory effect of Dex, β -eudesmol decreased the expression and activity of HDC in the activated HMC-1 cells in the present study. Ketotifen, which is a mast cell stabilizer, prevents mast cell degranulation through reducing the histamine secretion and release of inflammatory mediators. Thus, ketotifen is commonly used in the treatment of allergic disorders.¹⁸ Similar to the inhibitory effect of ketotifen, β -eudesmol diminished the levels of histamine and tryptase in the activated HMC-1 cells and

RPMCs in this study. In addition, β -eudesmol prevented the compound 48/80-induced systemic anaphylaxis and ear swelling response. Thus, β -eudesmol might inhibit mast cell-mediated allergic reactions via mast cell stabilization and a decrease in histamine synthesis.

Intracellular calcium is critical for mast cell degranulation.³³ Compound 48/80 stimulates G proteins, phospholipase C, and phospholipase D (PLD), which provide a significant signal for secretion in mast cells.³⁴ The calcium release from the endoplasmic reticulum can activate protein kinase C (PKC), which stimulates PLD activity.³⁵ PMA is a potent PKC activator and A23187 is a calcium ionophore. In this study, β -eudesmol decreased the intracellular calcium level that had been increased by compound 48/80 and PMACI in the mast cells. Thus, it is considered that β -eudesmol would regulate mast cell degranulation through blocking the intracellular calcium signaling. However, Ohara *et al.* reported that β -eudesmol activated a calcium-permeable non-selective cation channel and increased the intracellular calcium level in kidney-derived cells.³⁶ Kimura *et al.* reported that β -eudesmol had a blocking effect on intracellular calcium in the muscles of mice.³⁷ Thus, it is postulated that the regulatory effect of β -eudesmol on intracellular calcium would be dependent on each experiment model. More intensive studies are required for accurate understanding of the regulatory effect of β -eudesmol on the intracellular calcium levels in mast cells.

The IgE-dependent mast cell activation via Fc ϵ RI induced immediate release of inflammatory mediators, such as histamine, IL-5, IL-13, and TNF- α .³⁸ Cytokines have a primary function in orchestrating the allergic inflammatory responses.³⁹ The increased vascular permeability is induced from several mediators released from the activated mast cells and contributes to anaphylaxis through decreasing the intravascular volume.⁴⁰ IL-6

increased the vasopermeability at the PCA site and aggravated the PCA reaction, which is an IgE-mediated and mast cell-dependent reaction.⁴¹ IL-4 induced FcεRI expression in the mast cells, which is associated with the generation of IL-5, IL-13, and other cytokines in mast cells after FcεRI cross-linkage.^{42,43} Excessive IL-4 production was also associated with elevated IgE production and allergy.⁴⁴ The VEGF released from the mast cells was critical for the initiation and development of the allergic response.⁴⁵ The mast cell activation sites were associated with allergic reaction and IL-1β production.⁴⁶ Anti-histamine drugs reduced the pro-inflammatory markers in the allergic reactions and improved the symptoms.⁴⁷ In this study, β-eudesmol inhibited the PCA reaction and levels of histamine, IgE, IL-1β, IL-4, IL-5, IL-6, IL-13, and VEGF in the serum of mice under PCA. In addition, Seo *et al.* proposed that β-eudesmol could be used in therapy in mast cell-mediated inflammatory diseases through regulating IL-6 levels via blocking the caspase-1/NF-κB signaling in activated HMC-1 cells.¹⁶ Thus, β-eudesmol might inhibit mast cell-mediated and IgE-dependent allergic reactions through down-regulating the production of inflammatory cytokines. However, β-eudesmol did not regulate the level of serum TNF-α in mice under PCA. Kim *et al.* reported that β-eudesmol inhibited the TNF-α production and expression in activated HMC-1 cells.⁴⁸ Thus, further investigation is required in order to elucidate the regulatory effect of β-eudesmol on TNF-α levels in various experimental models.

KMP6 includes various active constituents. Atractylone, which is an active constituent of *Atractylodes japonica* and comprises 0.72 mg in 1 g of KMP6, regulated the mast cell-mediated allergic reactions and allergic rhinitis symptoms.^{49,50} Furthermore, hesperidin, which is an active constituent of *Citrus sunki* and comprises 5.26 mg in 1 g of KMP6, ameliorated the mast cell-mediated allergic reactions and allergic rhinitis

symptoms.^{12,51} Glycyrrhizic acid, which is an active constituent of *Glycyrrhiza uralensis* and comprises 2.60 mg in 1 g of KMP6, inhibited atopic dermatitis-like skin lesions.¹³ However, the regulatory effects of β -eudesmol, which comprises 40.66 mg in 1 g of KMP6, on mast cell-mediated allergic reactions have not been previously clarified. Here, it was determined that β -eudesmol, which is an active constituent of KMP6, regulates the mast cell-mediated allergic reactions.

Numerous studies have proposed that allergic disorders and gastrointestinal disorders might be correlated.^{1,8-11} In this study, it was demonstrated that β -eudesmol, which is a constituent of KMP6 that has been used to treat gastrointestinal disorders, ameliorates mast cell-mediated allergic reactions through inhibiting the degranulation of mast cells. However, further investigation is required in order to fully elucidate the anti-allergic effect of β -eudesmol in allergy models with digestive disorders because it is known that the consequences of the allergic reaction might be dependent on the digestive disorder.

METHODS

Chemicals and reagents

PMA, A23187 (Calcimycin; $C_{29}H_{37}N_3O_6$), ketotifen, Dex, BAPTA-AM, Fura-2/AM, compound 48/80, anti-DNP IgE antibody, DNP-HSA, and Evans blue were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA); β -eudesmol, anti-HDC, and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA); anti-IgE, IL-4, IL-6, and TNF- α antibodies were obtained from BD Pharmingen (San Diego, CA, USA); and anti-IL-1 β , IL-5, IL-13, and VEGF antibodies were obtained from R&D Systems (Minneapolis, MN, USA).

Preparation of drugs

β -eudesmol was dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions (Santa Cruz Biotechnology, USA). According to the manufacturer's instructions, β -eudesmol is soluble in water, but partly miscible. In order to assess the effect of β -eudesmol with a clear liquid for the *in vitro* experiments, β -eudesmol was dissolved in DMSO. DMSO has been used to dissolve β -eudesmol in numerous *in vitro* studies.⁵²⁻⁵⁴ The concentration of DMSO in the incubation medium was 0.1% (V/V). Thus, 0.1% DMSO was used as a vehicle control. The β -eudesmol dosage was determined according to a previous report^{16,48} and it was diluted with distilled water (DW) (0.2, 2, and 20 μ M). KMP6 extract (Korea Bio Medical Science Institute, Seoul, Republic of Korea) was obtained through decocting 13.3 g of *Atractylodes japonica* Koidzumi, 10 g of *Magnolia officinale* Rehder et Wils, 10 g of *Citrus sunki* Hort. ex Tanaka, 3.3 g of *Zingiber officinale* Roscoe, 3.3 g of *Glycyrrhiza uralensis* Fisch, and 6.7 g of *Zizyphus jujuba* var. *inermis* (Bunge) Rehder with DW for 3 h. Then, the extract was filtered and lyophilized. The KMP6 extract yield was approximately 21% (w/w). The lyophilized powder of KMP6 was dissolved with DW and filtered through a 0.22 μ m syringe filter. Ketotifen was dissolved with DW and filtered through a 0.22 μ m syringe filter. It has been previously reported that KMP6 suppresses allergic reactions at various concentrations ranging from 0.01 to 1 g/kg.¹² In this study, the focus was the effect of β -eudesmol; thus, the effects of β -eudesmol were assessed at various doses and KMP6 was assessed at one dose (0.1 g/kg). The dosage of KMP6 (0.1 g/kg) and ketotifen (2 mg/kg) was determined according to previous reports.^{12,55,56} β -eudesmol, KMP6, and ketotifen were orally administered in a volume of 0.1 ml/10 g for mice.⁵⁷

Analysis of β -eudesmol in KMP6

The concentration of β -eudesmol in KMP6 was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS; LC: 1290 Infinity, Agilent Technologies, USA; MS/MS: API 4000, Applied Biosystems, USA). KMP6 was extracted with 0.4 ml acetonitrile and filtered. Aliquots of 5 μ l were injected into Kromasil 100.3.5.-C18 column (100 $\times$ 2.1 mm; particle size: 5 μ m). Separation was achieved using a flow rate of 200 μ l/min. The mobile phase 65/35 (v/v) was acetonitrile with 0.1% formic acid. The concentration was analyzed using Analyst software (version 1.4.2, Applied Biosystems, USA). The content of β -eudesmol in KMP6 was approximately 40.66 mg/g. The concentration of β -eudesmol contained in 0.1 mg/ml of KMP6 extract was approximately 4 μ g/ml ($\sim$ 20 μ M, data not shown).

Mast cell culture

HMC-1 cells were cultured in Isocove's Modified Dulbecco's Medium (GibcoTM, Grand Island, NY, USA) with 10% fetal bovine serum (GibcoTM), 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in 5% CO₂ with 95% humidity. The RPMCs were prepared according to the method described by Kim *et al.*⁵⁸

Histamine assay

The histamine levels from the HMC-1 cells, RPMCs, and serum of mice under PCA were measured using *o*-phthalaldehyde spectrofluorometric analyses.⁴⁸ The fluorescent intensity was determined at 440 nm (an excitation wavelength of 360 nm) in a spectrofluorometer.

Western blot analysis

HMC-1 cells (3×10^6 cells) were preincubated with β -eudesmol, KMP6, or Dex for 2 h and then stimulated with PMACI for 6 h. The Western blot analysis was performed as previously reported.¹⁶

HDC assay

HMC-1 cells (3×10^6 cells) were preincubated with β -eudesmol, KMP6, or Dex for 2 h and then stimulated with PMACI for 4 h. The HDC activity was determined using histamine assays, which were conducted as previously reported.⁵⁹ The HMC-1 cells were lysed in a potassium phosphate buffer (0.1 M HDC reaction buffer, pH 6.8) containing 0.2 mM dithiothreitol, 0.01 mM pyridoxal-phosphate (5%), polyethylene glycol ($M_r = 300$, 1%), and 100 μ g/ml phenylmethylsulphonyl fluoride. The supernatant was incubated with a HDC reaction buffer including 0.25 mM L-histidine for 2 h at 37 °C after centrifugation.

Tryptase assay

HMC-1 cells (1×10^6 cells) were preincubated with β -eudesmol, KMP6, or ketotifen for 2 h and then stimulated with PMACI for 6 h. Purified RPMCs (2×10^5 cells) were treated with β -eudesmol, KMP6, or ketotifen for 40 min and then stimulated with compound 48/80 (6 μ g/ml) for 15 min. The tryptase level was analyzed using a mast cell degranulation assay kit (Millipore Co., Billerica, MA, USA).

Fluorescent measurement of intracellular calcium levels

HMC-1 cells or purified RPMC suspensions were treated with Fura-2/AM for 30 min and then harvested. The cell suspension (1×10^5 cells) was placed in a 96-well plate after washing twice with a medium containing 0.5 mM extracellular calcium chelator (EGTA) and treated with β -eudesmol, KMP6, ketotifen, or BAPTA-AM for 20 min. The HMC-1 cells were stimulated with PMACI and the RPMCs were stimulated with compound 48/80 (6 μ g/ml). The intracellular calcium levels were evaluated at excitation wavelengths of 360 and 440 nm in a spectrofluorometer.

RPMC morphology

Purified RPMCs were preincubated with β -eudesmol, KMP6, or ketotifen for 40 min and then stimulated with compound 48/80 (6 μ g/ml). The morphology was observed under a microscope at 400 $\times$ magnification. The number (%) of degranulated RPMCs was counted at five random sections using the method reported by Penissi *et al.*⁶⁰

Animals

Male 4-week-old ICR mice and male 7-week-old Sprague Dawley rats were obtained from Dae-Han Experimental Animal Center (Eumsung, Republic of Korea). All procedures involving the experimental animals conformed to the ethical guidelines for animal care at Kyung Hee University (No. KHUASP(SE)-15-118). The mice were acclimatized to the standard laboratory conditions for at least one week prior to experimentation.

Compound 48/80-induced systemic anaphylaxis

The systemic anaphylactic reactions were conducted using the method reported previously.⁶¹ Mice (5 mice per group) were orally treated with β -eudesmol, KMP6, or ketotifen for 1 h and then injected with compound 48/80 (8 mg/kg, i.p.). The untreated control mice were treated with DW or 0.1% DMSO. The mortality was evaluated based on survival time (approximately 13 min) of the control mice after the injection of compound 48/80.

Ear swelling response

The ear swelling responses were conducted as previously reported.⁶¹ Mice (5 mice per group) were orally treated with β -eudesmol, KMP6, or ketotifen for 1 h. Then, compound 48/80 (100 μ g/site) was intradermally injected into each ear. The increase in the ear thickness was measured using a digimatic micrometer (Mitutoyo, Japan) 40 min after the compound 48/80 injection.

PCA reaction

Mice skin (5 mice per group) was sensitized with an intradermal injection of anti-DNP IgE antibody (100 ng/site) followed 48 h later with oral administration of β -eudesmol, KMP6, or ketotifen. Each mouse received an injection of 200 μ l of a 1:1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue via the tail vein. Blood from the heart and dorsal skin were obtained 40 min after the intravenous injection. The amount of dye was measured as previously described.⁶¹

Enzyme-linked immunosorbent assay (ELISA)

The levels of IgE, IL-1 β , IL-4, IL-5, IL-6, IL-13, VEGF, and TNF- α in the serum of mice under PCA were measured using the ELISA method according to the manufacturer's instructions (R&D Systems and BD Pharmingen).

Statistical analysis

The experiment results were analyzed using one-way analysis of variance with a Tukey *post hoc* test and an independent *t*-test in order to determine the significance of the differences between the groups. The data is presented as mean $\pm$ standard error of the mean. A value of *P* < 0.05 is considered statistically significant.

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DISCLOSURE

The authors declare no conflicts of interest.

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Figure legends

Fig. 1. Effect of β -eudesmol on degranulation of HMC-1 cells. HMC-1 cells were pretreated with 0.1% DMSO (vehicle control), β -eudesmol, KMP6, ketotifen, dexamethasone (Dex), or BAPTA-AM and then stimulated with PMA plus A23187 (PMACI). (a) The histamine intensity was analyzed using *o*-phthalaldehyde spectrofluorometric assay. $^{\$}P < 0.05$; significantly different from the vehicle control group of inactivated HMC-1 cells. $^{\#}P < 0.05$; significantly different from the inactivated HMC-1 cells. $^{*}P < 0.05$; significantly different from the PMACI-stimulated HMC-1 cells. (b) The expression of HDC was analyzed using Western blot analysis. (c) The relative expression of HDC was quantified via densitometry. (d) The HDC activity was measured using HDC assay as described in the Method section. (e) The tryptase level was measured using a mast cell degranulation assay kit. (f) The intensity of the intracellular calcium was measured using a fluorometer. $^{\#}P < 0.05$; significantly different from the inactivated HMC-1 cells. $^{*}P < 0.05$; significantly different from the PMACI-stimulated HMC-1 cells.

Fig. 2. Effect of β -eudesmol on the degranulation of RPMCs. RPMCs were pretreated with β -eudesmol, KMP6, ketotifen, or BAPTA-AM, and then stimulated with compound 48/80 (COM 48/80). (a) The histamine intensity was analyzed using an *o*-phthalaldehyde spectrofluorometric assay. (b) The tryptase level was measured using a mast cell degranulation assay kit. (c) The intensity of the intracellular calcium was measured using a fluorometer. $^{\#}P < 0.05$; significantly different from the inactivated RPMCs. $^{*}P < 0.05$; significantly different from the compound 48/80-stimulated RPMCs.

Fig. 3. Effect of β -eudesmol on the morphology in activated RPMCs. The number of degranulated RPMCs is presented as a percentage of the degranulated RPMCs. The images are representative of degranulated RPMCs. $^{\#}P < 0.05$; significantly different from the inactivated RPMCs. $^{*}P < 0.05$; significantly different from the compound 48/80 (COM 48/80)-stimulated RPMCs.

Fig. 4. Effect of β -eudesmol on the ear swelling response. Mice were orally treated with β -eudesmol, KMP6, or ketotifen for 1 h. Then, compound 48/80 (COM 48/80) was intradermally injected into each ear. $^{\#}P < 0.05$; significantly different from the untreated control mice. $^{*}P < 0.05$; significantly different from the ear thickness increased by compound 48/80.

Fig. 5. Effect of β -eudesmol on IgE-dependent PCA. Mice sensitized with anti-DNP IgE antibody intradermally were orally treated with β -eudesmol, KMP6, or ketotifen for 1 h. Then, the mice were injected with DNP-HSA in the tail vein. Each amount of dye was measured at 620 nm in an ELISA reader. KMP6, Pyeongwee-San. $^{\#}P < 0.05$; significantly different from the untreated control mice. $^{*}P < 0.05$; significantly different from the IgE + DNP-HSA-challenged mice.

Table 1 Effect of β -eudesmol on compound 48/80-induced systemic anaphylaxis

Treatment	Number of mice (n)	Dose	Compound 48/80	Mortality (%)
None (DW)	10	–	+	100 $\pm$ 0.00
Vehicle control	10	0.1% DMSO	+	100 $\pm$ 0.00
β -eudesmol	10	0.2 μ M	+	60 $\pm$ 0.00
β -eudesmol	10	2 μ M	+	30 $\pm$ 10.00*
β -eudesmol	10	20 μ M	+	20 $\pm$ 0.00*
KMP6	10	0.1 g/kg	+	30 $\pm$ 10.00*
Ketotifen	10	2 mg/kg	+	0 $\pm$ 0.00*

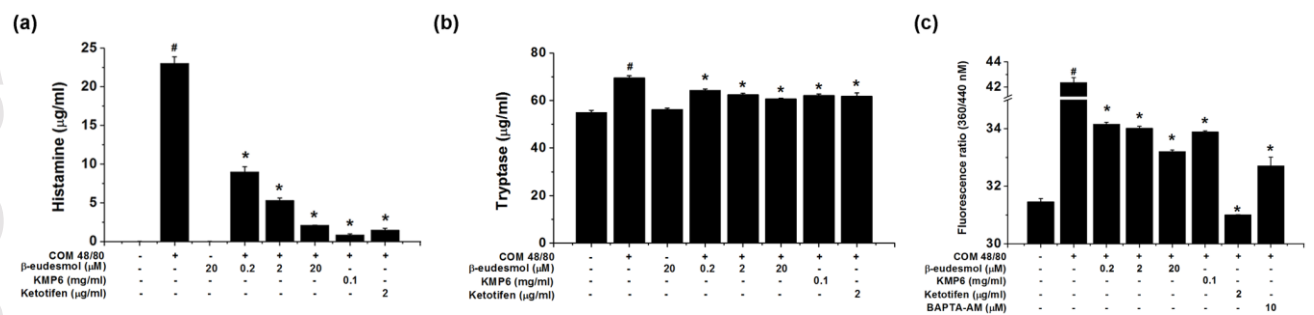
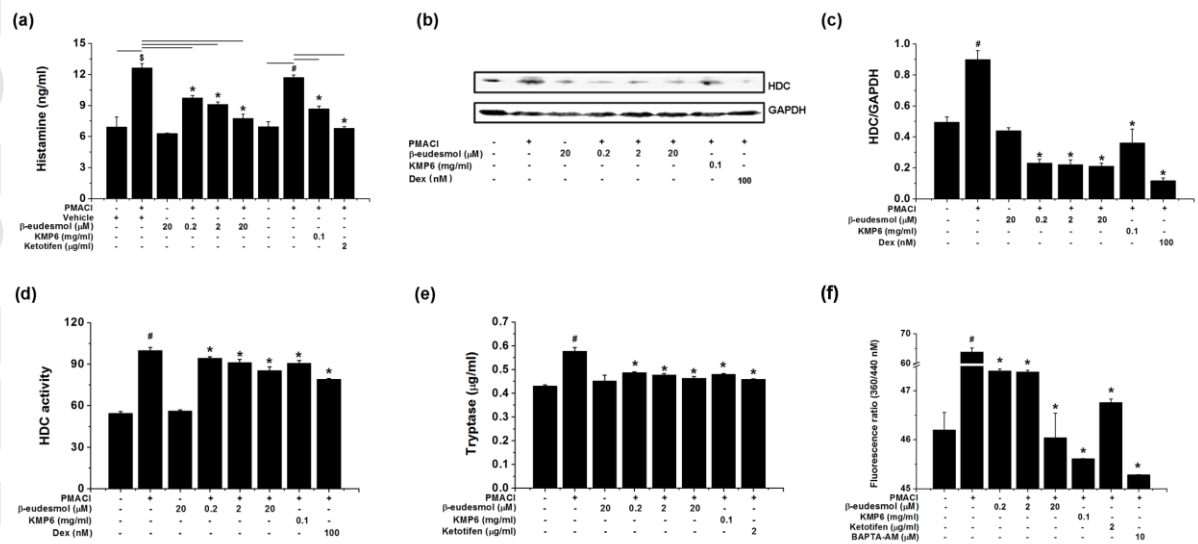
The groups of mice were orally treated with DW, vehicle control, β -eudesmol, KMP6, or ketotifen for 1 h and then intraperitoneally injected with compound 48/80 (8 mg/kg).

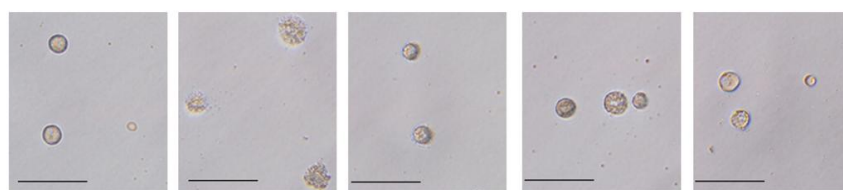
Mortality (%) is expressed as the ‘number of deceased mice $\times$ 100 / total number of mice’. * P < 0.05; significantly different from the mortality by compound 48/80. KMP6, Pyeongwee-San.

Table 2 Effect of β -eudesmol on levels of serum histamine, IgE, and cytokines under PCA

	DW	DW	β -eudesmol (20 μ M)	β -eudesmol (0.2 μ M)	β -eudesmol (2 μ M)	β -eudesmol (20 μ M)	KMP6 (0.1 g/kg)	Ketotifen (2 mg/kg)
	-	IgE + DNP-HSA	-	IgE + DNP-HSA	IgE + DNP-HSA	IgE + DNP-HSA	IgE + DNP-HSA	IgE + DNP-HSA
Histamine intensity	55.61 $\pm$ 1.11	67.59 $\pm$ 0.74 [#]	57.93 $\pm$ 1.63	67.85 $\pm$ 1.49	62.57 $\pm$ 2.20	60.42 $\pm$ 0.67 [*]	59.90 $\pm$ 1.04 [*]	61.43 $\pm$ 1.01 [*]
IgE (ng/ml)	11.96 $\pm$ 1.46	19.58 $\pm$ 0.97 [#]	13.43 $\pm$ 1.99	15.21 $\pm$ 1.29	11.01 $\pm$ 0.25 [*]	10.51 $\pm$ 0.93 [*]	11.68 $\pm$ 1.97 [*]	12.42 $\pm$ 1.42 [*]
IL-1β (ng/ml)	1.38 $\pm$ 0.10	6.52 $\pm$ 0.32 [#]	1.47 $\pm$ 0.09	1.71 $\pm$ 0.05 [*]	1.45 $\pm$ 0.10 [*]	1.41 $\pm$ 0.11 [*]	3.51 $\pm$ 0.31 [*]	5.57 $\pm$ 0.33
IL-4 (ng/ml)	3.45 $\pm$ 0.08	5.77 $\pm$ 0.39 [#]	3.77 $\pm$ 0.17	6.24 $\pm$ 0.21	4.50 $\pm$ 0.24 [*]	4.28 $\pm$ 0.07 [*]	4.48 $\pm$ 0.25 [*]	5.26 $\pm$ 0.24
IL-5 (ng/ml)	0.56 $\pm$ 0.02	0.73 $\pm$ 0.06 [#]	0.54 $\pm$ 0.01	0.59 $\pm$ 0.02 [*]	0.58 $\pm$ 0.01 [*]	0.55 $\pm$ 0.01 [*]	0.55 $\pm$ 0.01 [*]	0.60 $\pm$ 0.02 [*]
IL-6 (ng/ml)	5.16 $\pm$ 0.08	7.17 $\pm$ 0.10 [#]	5.40 $\pm$ 0.19	6.59 $\pm$ 0.18	6.29 $\pm$ 0.22	6.05 $\pm$ 0.23 [*]	5.78 $\pm$ 0.13 [*]	6.06 $\pm$ 0.06 [*]
IL-13 (ng/ml)	3.91 $\pm$ 0.05	4.60 $\pm$ 0.14 [#]	3.81 $\pm$ 0.08	4.25 $\pm$ 0.25	3.98 $\pm$ 0.06 [*]	3.56 $\pm$ 0.03 [*]	3.70 $\pm$ 0.06 [*]	3.72 $\pm$ 0.13 [*]
VEGF (ng/ml)	1.17 $\pm$ 0.07	1.40 $\pm$ 0.08 [#]	0.97 $\pm$ 0.10	1.14 $\pm$ 0.10	0.90 $\pm$ 0.03 [*]	0.71 $\pm$ 0.02 [*]	0.78 $\pm$ 0.03 [*]	0.93 $\pm$ 0.08 [*]
TNF-α (ng/ml)	3.21 $\pm$ 0.04	4.10 $\pm$ 0.05 [#]	3.53 $\pm$ 0.16	3.97 $\pm$ 0.08	3.74 $\pm$ 0.24	3.73 $\pm$ 0.02	3.85 $\pm$ 0.26	4.12 $\pm$ 0.11

Mice were orally pretreated with β -eudesmol, KMP6, or ketotifen for 1 h and then challenged with DNP-HSA. Serum was prepared from blood. The levels of histamine, IgE, or cytokines were analyzed with o-phthalaldehyde spectrofluorometric assay or ELISA method. Each result shows the mean $\pm$ SEM (5 mice per group). KMP6, Pyeongwee-San. [#]*P* < 0.05; significantly different from unchallenged mice. ^{*}*P* < 0.05; significantly different from IgE + DNP-HSA-challenged mice.





COM 48/80	-	+	+	+	+
β -eudesmol (μ M)	-	-	20	-	-
KMP6 (mg/ml)	-	-	-	0.1	-
Ketotifen (μ g/ml)	-	-	-	-	2

