



Antiallergic effect of KOB03, a polyherbal medicine, on mast cell-mediated allergic responses in ovalbumin-induced allergic rhinitis mouse and human mast cells

Hyo Won Jung^a, Jin-Ki Jung^b, Cheong Weon Cho^c, Jong-Seong Kang^c, Yong-Ki Park^{a,b,*}

^a Oriental Medicine R&D Center, Dongguk University, Gyeongju 780-714, Republic of Korea

^b Department of Herbology, College of Oriental Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea

^c College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea

ARTICLE INFO

Article history:

Received 6 March 2012

Received in revised form

22 May 2012

Accepted 23 May 2012

Available online 9 June 2012

Keywords:

Allergic rhinitis

Anti-allergic effect

KOB03

Mast cells

Ovalbumin

Polyherbal medicine

ABSTRACT

Ethnopharmacological relevance: KOB03 is a polyherbal medicine consisting of five different herbs and has commonly been used for the treatment of various allergic diseases. However, its precise anti-allergic effect and mechanism remain unknown.

Aim of the study: The aim of this study was to investigate the effect of KOB03 on allergic responses through the regulation of mast-cell mediated allergic inflammation.

Materials and methods: To determine the effect of KOB03 on mast cell-mediated allergic reactions, we investigated the parameter changes of in vivo models such as compound 48/80-induced systemic anaphylaxis and ovalbumin (OVA)-induced allergic rhinitis, and the release of allergic inflammatory mediators such as histamine, immunoglobulin (Ig) E, and inflammatory cytokines via the MAPKs and NF-kappaB pathways.

Results: The oral administration of KOB03 at doses of 100 and 200 mg/kg inhibited histamine release and mortality in compound 48/80-induced anaphylactic rats. KOB03 also improved rhinitis symptoms, inhibited the histopathological changes of nasal mucosa, and decreased the serum levels of histamine, OVA-specific IgE and TNF- α in OVA-induced allergic rhinitis in mice. In vitro, KOB03 suppressed compound 48/80-induced histamine release by blocking mast cell degranulation. In addition, KOB03 inhibited the production of inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-8 in PMA/A23187-stimulated HMC-1 mast cells by suppressing their gene expression and blocking the ERK1/2 and p38 MAPK and NF- κ B pathways.

Conclusions: These results suggest that KOB03 has an anti-allergic effect by modulating mast cell-mediated allergic responses in allergic rhinitis.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Allergic rhinitis (AR) is characterized by inflammation of the nasal mucosa with hypersensitivity resulting from seasonal or perennial responses to specific environmental allergens and by symptoms like nasal rubbing, sneezing, rhinorrhea, lacrimation, nasal congestion and obstruction, and less frequently cough (Lee and Arriola, 1999). AR is frequently involved in acute and chronic airway diseases such as bronchial asthma, which is also caused by hypersensitivity to antigens, resulting in greater local inflammation as well as bronchoconstriction, vasomotor change, and mucus hypersecretion (Smolensky et al., 2007). Among immune cells, mast cells play critical roles in the pathogenesis of allergic responses in AR.

Thus, mast cells express the immunoglobulin Fc epsilon receptor 1 (Fc ϵ RI) that triggers specific IgE responses to antigen. After IgE-dependent stimulation, mast cells release allergic mediators such as histamine, α -hexosaminidase, cytokines, chemokines and arachidonic acid derivatives, mediating acute and chronic inflammation (Church and Levi-Schaffer, 1997). Therefore, treatment options for AR consist of allergen avoidance, symptomatic treatment and allergen-specific immunotherapy to further improve the control of allergic responses (Nouri-Aria, 2008). Although therapeutic agents are greatly required, the current treatment of AR is limited to anti-histamine, nasal corticosteroid, anti-leukotriene and anti-allergen immunotherapy, which are still not ideal, and it is important to continue to study the pharmacology of this disease in the search for better drugs (Leung and Hon, 2008).

Recently, increasing interest in various medicinal plants and their bioactive compounds that show potential in the treatment of allergic diseases has led to increased attention on their safety and efficacy. KOB03 is a polyherbal medicine prescribed in oriental

* Corresponding author at: Department of Herbology, College of Oriental Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea.

Tel.: +82 54 770 2661; fax: +82 54 770 2647.

E-mail address: yongki@dongguk.ac.kr (Y.-K. Park).

clinics for the treatment of hyperhidrosis (Heo, 1985) and allergic diseases such as allergic asthma and rhinitis (Kim et al., 1991; Na et al., 2004; Shin, 2005). However, no studies of KOB03 have been conducted to date. In this study, we evaluated the effect of KOB03 on allergic responses in ovalbumin-induced allergic rhinitis mice and on mast cell-mediated allergic inflammatory reactions in mast cells. We further identified the mechanism of action of KOB03 in the mitogen-activated protein kinases (MAPKs) and NF- κ B (NF- κ B) signaling pathways in activated mast cells.

2. Materials and methods

2.1. Animals

Male Balb/c mice (20 ± 2 g) and male Sprague-Dawley (SD) rats (200 ± 10 g) were purchased from Koatech Co., Ltd. (Gyeonggi-do, Republic of Korea). The animals were housed under controlled environmental conditions at a temperature of 22 ± 3 °C with a relative humidity of $55 \pm 5\%$ and 12 h light/dark cycle throughout the study. The care and treatment of animals were in accordance with the guidelines established by the Korean National Institute of Health at the Korean Academy of Medical Sciences for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Dongguk University.

2.2. Preparation of KOB03

Herbs used in KOB03 preparation (Table 1) were purchased from the Medicinal Materials Company (Kwangmyungdang Medicinal Herbs, Ulsan, Republic of Korea). All herbs were authenticated by Professor J.-H. Lee, a medical botanist in the Department of Herbology, College of Oriental Medicine, Dongguk University (DUCOM), Republic of Korea. Voucher specimens were deposited in the Herbarium of DUCOM under registration number 10-KOB06-01-03. One kilogram of KOB03 was extracted with boiling water for 3 h and filtered through Whatman no. 1 paper; the resulting product was freeze-dried (yield = 36.31%).

2.3. Induction of compound 48/80-induced systemic anaphylaxis in rats

Compound 48/80-induced systemic reaction was carried out as described previously (Shin, 2005). Briefly, the rats ($n = 10$ per group) were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. KOB03 or disodium cromoglycate (DSCG), a calcium channel blocker, was dissolved in saline and orally administered at doses of 100 and 200 mg/kg BW for KOB03 or 50 mg/kg for DSCG one hour before the compound 48/80 injection. Mortality was monitored for 1 h after induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each rat to measure serum histamine content. Blood was clotted for 2 h at room temperature and centrifuged for 20 min at $2000 \times g$ at 4 °C to obtain serum.

Table 1
Herbal constituents of KOB03.

Herbal name	Species	Ratio
<i>Atractylodis Rhizoma Alba</i>	<i>Atractylodes japonica</i> Koidz	2
<i>Astragali Radix</i>	<i>Astragalus membranaceus</i> Bunge	1.5
<i>Saposhnikoviae Radix</i>	<i>Saposhnikovia divaricata</i> Schischkin	1
<i>Osterici Radix</i>	<i>Ostericum koreanum</i> maximowicz	1
<i>Scutellariae Radix</i>	<i>Scutellaria baicalensis</i> Georgi	1

2.4. Preparation of OVA-induced allergic rhinitis mouse model

Male Balb/c mice (10 weeks old) were divided into four groups and given free access to a standard laboratory diet and water during the experimental period. OVA solution (0.5 mg/mL in saline) and $\text{Al}(\text{OH})_3$ (20 mg/mL in saline) were mixed in a 1:1 ratio, and mice were then sensitized with an intraperitoneal injection at a dosage of 0.1 mL/mouse (Fig. 1). For the second immunization, OVA solution (0.4 mg/mL in saline; antigen challenge) was injected into a footpad (0.02 mL/mouse) on days 7 and 14. In addition, local challenge was performed twice daily from day 21 to day 28 by instilling 0.1% OVA solution into the bilateral nasal cavities using a micropipette. KOB03 was administrated orally to the OVA-sensitized mice at a dose of 100 or 200 mg/kg body weight once daily for 7 consecutive days during OVA challenge until the assay date. Normal and OVA-sensitized control mice were given saline alone on the same schedule. The thickness of the foot pad was measured with a dial thickness gage (Ozaki Mfg Co., Ltd., Tokyo, Japan) 15 min after antigen challenge. Blood samples were taken from each mouse by cardiac puncture under isoflurane anesthesia 24 h after the oral administration of OVA. Serum was prepared and frozen at -70 °C prior to analysis. The nasal tissues were removed from the body and histopathological changes were assessed.

2.5. Serological analysis

Serum levels of histamine (BioNovus Pty Ltd, SPIbio, Cherrybrook, Australia), anti-OVA specific IgE (Chondrex, Inc., Redmond, WA, USA) and TNF- α (R&D Systems Inc., Minneapolis, MN, USA) in animals were measured using commercially available enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay (EIA) kits according to the manufacturer's recommendations. The concentrations of histamine, anti-OVA specific IgE and TNF- α were calculated from the equations obtained from standard curve plots for the standard solutions in the kits.

2.6. Evaluation of nasal symptoms

On day 29 of treatment, mice were placed in an observation cage for about 10 min for acclimatization before the experiments. After nasal instillation of 20 μ L of OVA (4 mg/mL in saline) into bilateral nasal cavities, the animals were placed in observation cages (one animal/cage), and the numbers of sneezes and nasal rubbing movements were counted for 30 min.

2.7. Histopathological evaluation of nasal cavity

Mice were euthanized with a high dose of pentobarbital and exsanguination after final OVA challenge. The head was removed and the lower jaw was discarded. The nasal cavity was separated from the skin, muscle and soft tissue and immersed in freshly prepared 10% neutral buffered formalin for 48 h. The tissue was then rinsed in running tap water and decalcified in 10% nitric acid solution for 5 days. After rinsing in tap water, the tissue was processed for dehydration through graded alcohol and embedded in paraffin. Serial sections (5- μ m) were cut at the level of the incisive papillae and first and second palatal ridges and then stained with hematoxylin and eosin (H&E). Sections were examined under a light microscope.

2.8. Cell culture

The human mast cell line, HMC-1 cells, was cultured in Iscove's Modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 μ g/mL of streptomycin at 37 °C in a humidified incubator with an atmosphere containing 5% CO_2 .

2.9. Cytotoxicity assay

Cell viability was assessed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Inc., St. Louis, MO, USA) reduction assay to measure the cytotoxic response to KOB03. The cells were seeded at 1×10^5 cells/well in 96-well flat-bottom culture plates. After 24-h incubation with KOB03 at various concentrations (0.1, 0.2, 0.5, 1 and 2 mg/mL), 20 μ L of MTT (5 mg/mL) was added per well and incubated for 4 h. The formazan crystals were dissolved in acidic isopropanol and the absorbance was read at 570 nm using a PowerWavex Microplate spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT, USA).

2.10. Measurement of histamine release from mast cells

HMC-1 cells were pretreated with various concentrations of KOB03 at 37 °C for 1 h and then incubated with compound 48/80 (final concentration; 0.5 μ g/mL) at 37 °C for 30 min. The culture supernatants were harvested by centrifugation at 400 g for 5 min at 4 °C. The concentration of histamine was measured by the EIA kit according to the manufacturer's protocol. After incubation, the cells were fixed in 4% paraformaldehyde and stained with toluidine blue (0.1% w/v, pH 3.0); mast cell degranulation was then observed under a high-power microscope.

2.11. Measurement of cytokines from the mast cells

HMC-1 cells were pretreated with various concentrations of KOB03 at 37 °C for 1 h and then incubated with PMA (20 nmol/L)/A23187 (1 mmol/L) at 37 °C for 24 h. The culture supernatants were harvested by centrifugation at 400 g for 5 min at 4 °C. The concentrations of TNF- α , IL-1 β , IL-6 and IL-8 were measured by ELISA according to the manufacturer's recommendations (R&D Systems). The amount of cytokine released was calculated from the equation obtained from a standard curve plot for each cytokine standard solution in the kit.

2.12. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was isolated from HMC-1 cells (1×10^6 cells/mL) using TRIzol reagent (Life Technologies™, Carlsbad, CA, USA) after stimulation with PMA/A23187 with or without KOB03 for 6 h. The first-strand cDNA was synthesized using Superscript II reverse transcriptase (Promega, Madison, WI, USA). A reverse-transcription polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF- α , IL-6 and GAPDH (internal control). PCR was carried out with following primers: TNF- α (sense, 5'-cac cag ctgc gtt atc tct ca-3'; antisense, 5'-cgg gac gtg gag ctg gcc gag gag-3'), IL-1 β (sense, 5'-aaa cag atg aag tgc tcc tt-3'; antisense, 5'-tgg aga aca cca ctt gtt gc-3'), IL-6 (sense, 5'-gat gga tgc ttc caa tct gga t-3'; antisense, 5'-agt tct cca tag aga aca ta-3'), IL-8 (sense, 5'-cga tgt cag tgc ata aag aca-3'; antisense, 5'-tga att ctc agc cct ctt caa aaa-3') and GAPDH (sense, 5'-gga ctt cga gca aga gat gg-3'; antisense, 5'-agc act gtg ttg gcg tac ag-3'). The cycle number was optimized to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 1% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera, and digitized with UN-SCAN-IT software (Silk Scientific, Orem, UT, USA). The band intensity was normalized to that of GAPDH in the same sample.

2.13. Western blot analysis

The cell pellets were harvested, resuspended in an appropriate volume of lysis buffer (1% Triton X-100, 1% deoxycholate and 0.1% sodium azide) and then incubated for 30 min on ice. Lysates were centrifuged at $14,000 \times g$ for 10 min at 4 °C and the supernatants were used as the total cell lysates. For nuclear and cytosolic fractions, cells were washed twice with ice-cold PBS and lysed with ice-cold NE-PER™ 205 nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). After centrifugation at $14,000 \times g$ for 10 min, the supernatant was stored at –80 °C until use. The protein concentration of each sample was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). 50 μ g of protein were separated on 7–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). Each membrane was incubated for 1 h with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBS-T) buffer (0.1 M Tris-HCl; pH 7.4, 0.9% sodium chloride, 0.1% Tween-20) to block non-specific binding and then incubated with primary antibodies that recognized β -actin (1:5000, Sigma), the phosphorylated or total forms of extracellular signal-related kinases (ERK) 1/2, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)- κ B p65, and I- κ B α (1:1000, Cell Signaling Technology, Boston, MA, USA). Each protein was detected using a chemiluminescence detection system according to the manufacturer's protocol (ECL, Amersham, Berkshire, UK). The band intensity was quantified by NIH ImageJ densitometric analysis.

2.14. HPLC analysis

HPLC analysis for identification of the standard compounds was performed on a HPLC system (Shimadzu, Tokyo, Japan) equipped with LC-10AD pump, CBM-10 A Control bus, and SPD-M10AVP UV/Vis detector. 5 mg/mL of KOB03 or 0.05 mg/mL baicalin were dissolved in 1 mL of 100% methanol for pattern analysis using HPLC with a photodiode array detector. Chromatographic separation was carried out using a Phenomenex Luna 5 μ C18(2) 100 A (250 $\times$ 4.60 mm) (Torrance, CA, USA) heated to room temperature. The injection volume was 10 μ L and the following solvent ratios were used for the mobile phase with a flow rate of 0.8 mL/min, where solvent A is 0.5% acetic acid in water and solvent B is 0.5% acetic acid in methanol: 0 min, 10% B; 10 min, 20% B; 25 min, 40% B and 65 min, 80% B. The detection wavelength (277 nm) was scanned at a flow rate of 1 mL/min at 30 °C. The peak analysis and assignment were performed using the standard compound, baicalin, which was identified in accordance with its UV spectra and retention time in the HPLC chromatogram.

2.15. Statistical analysis

Data signify the means $\pm$ the standard error (means $\pm$ S.E.) of three samples and are representative of three independent experiments. Statistical analysis was carried out by one-way ANOVA with the Post-Hoc test using GraphPad Prism 5.0 statistical analysis software (GraphPad Software, Inc., San Diego, CA). Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of KOB03 on compound 48/80-induced anaphylactic reactions

Initial experiments examined the effect of KOB03 on compound 48/80-induced systemic anaphylactic reaction. When mice were administered KOB03 at doses of 100 and 200 mg/kg for 1 h,

compound 48/80-induced mortality was dose-dependently reduced (Table 2). Next, to confirm that the effect of KOB03 on the anaphylactic reaction was due to inhibited histamine release, we investigated the serum level of histamine after 48/80-induced anaphylactic shock. At doses of 100 and 200 mg/kg, KOB03 significantly decreased histamine levels in the sera of compound 48/80-induced anaphylactic rats. The results of the mortality test correlated with the measured serum histamine contents. These

Table 2

Effects of KOB03 in compound 48/80-induced systemic anaphylactic reaction in rats.

Group	Dose (mg/kg)	Compound 48/80 (8 mg/kg)	Mortality (%)	Serum histamine (μ M)
Normal	–	–	0	267.9 $\pm$ 30.26
Control	–	+	100	1181.1 $\pm$ 19.66 ^{a***}
KOB03	100	+	60 $\pm$ 10 ^{b*}	452.0 $\pm$ 29.66 ^{b***}
–	200	+	40 ^{b*}	358.2 $\pm$ 25.33 ^{b***}
DSCG	50	+	50 ^{b*}	323.6 $\pm$ 42.06 ^{b***}

The rats ($n=10$ per group) were intraperitoneally pretreated with 0.2 mL saline or various doses of drugs 1 h before the injection of 8 mg/kg compound 48/80. Mortality (%) was monitored for 1 h after induction of anaphylactic reaction as no. of dead mice $\times$ 100/ no. of total experimental rats. The level of histamine was measured in the serum by enzyme-immunoassay. Each datum represents the mean $\pm$ S.E. of three independent experiments. * $P<0.05$ and *** $P<0.001$ significantly different from the ^anormal group or ^bOVA-control group; KOB03, KOB03-treated group; and DSCG, disodium cromoglycate-treated group.

data indicate that there was potential protection from mortality by KOB03 via the inhibition of histamine release from systemic anaphylactic reaction.

3.2. Effect of KOB03 on serological marker levels in OVA-sensitized mice

To demonstrate the effect of KOB03 on allergic responses in vivo, the levels of histamine, OVA-specific IgE and TNF- α were measured by ELISA of the sera of OVA-sensitized mice. The administration of KOB03 at doses of 100 and 200 mg/kg significantly decreased histamine levels in the sera of OVA-sensitized mice compared to that in control mice given OVA sensitization alone (Fig. 2A). Enhanced OVA-specific IgE level by the sensitization was also significantly reduced by KOB03 administration (Fig. 2B). In addition, the level of the inflammatory cytokine TNF- α was significantly decreased in the KOB03-treated group (Fig. 2C). This data indicate that KOB03 might have anti-allergic activity via suppression of allergic mediator production.

3.3. Effect of KOB03 on the nasal symptoms in OVA-sensitized/challenged mice

We evaluated the effect of KOB03 on nasal symptoms by scoring the numbers of sneezes and nasal rubbing movements for 30 min in OVA-sensitized mice. At doses of 100 and 200 mg/

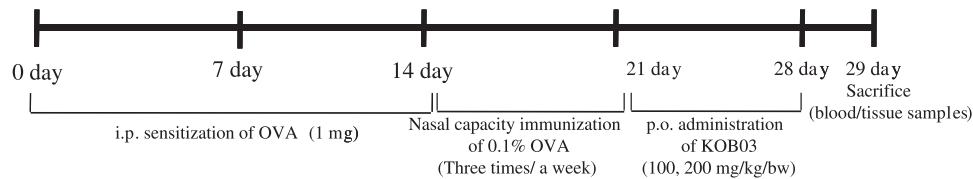


Fig. 1. Study plan for the development of the experimental in vivo model.

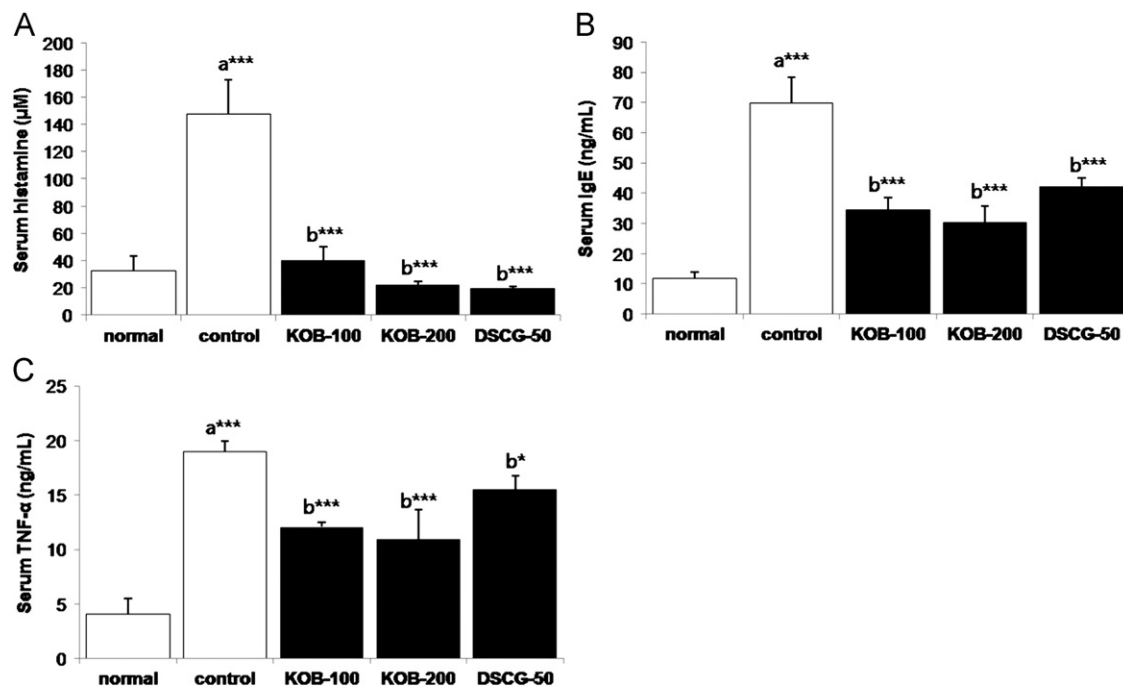


Fig. 2. Effects of KOB03 on the serum levels of histamine, IgE and TNF- α in OVA-sensitized/challenged mice. The levels of (A) histamine, (B) OVA-specific IgE and (C) TNF- α were measured in mouse sera by ELISA. Independent experiments were performed ($n=10$ per group), and the data shown indicate the means $\pm$ SDs. A difference was considered statistically significant when * $P<0.05$ and *** $P<0.001$ vs. normal (a) or OVA-control mice (b).

kg, KOB03 significantly inhibited the nasal symptoms (Fig. 3A). Similar results were obtained with sneezing. We next investigated the effect of KOB03 on histological changes of nasal mucosa by H&E staining. In the OVA-sensitized control mice, histological observation revealed epithelial disruption, mucosa edema, goblet

cell hyperplasia and infiltration of numerous inflammatory cells such as eosinophils in the nasal mucosa (Fig. 3B). However, at doses of 100 and 200 mg/kg, KOB03 protected the nasal mucosa against damages and greatly reduced infiltration of eosinophils, especially at a dose of 200 mg/kg.

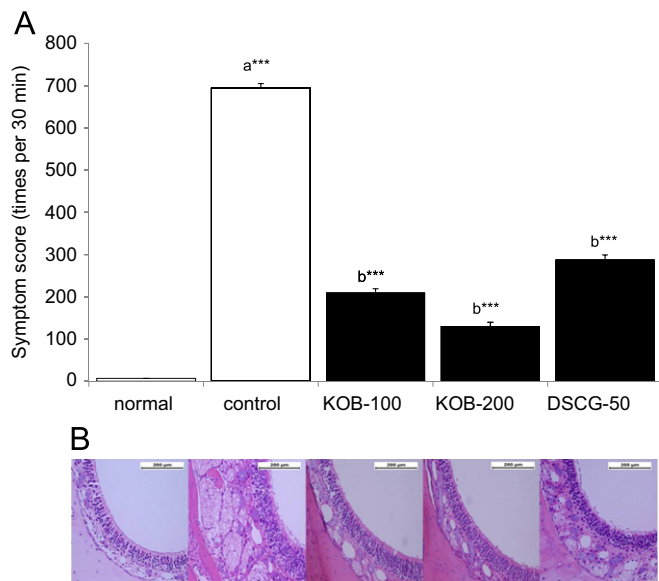


Fig. 3. Effects of KOB03 on clinical scores and histological changes of nasal mucosa in OVA-sensitized/challenged mice. (A) Nasal symptoms were measured by clinical scoring. Independent experiments were performed ($n=10$ per group), and the data shown indicate the means $\pm$ SDs. A difference was considered statistically significant when *** $P < 0.001$ vs. normal (a) or OVA-control mice (b). (B) The histological changes of nasal mucosa were observed by microscope after H&E staining (100X).

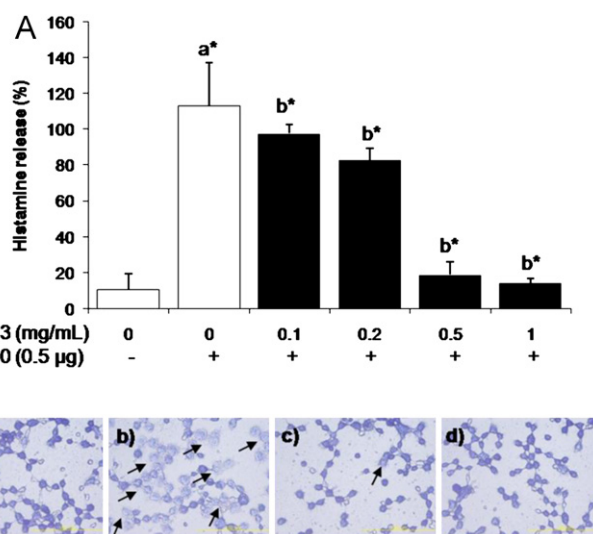


Fig. 4. Effects of KOB03 on compound 48/80-induced histamine release and cell degranulation in HMC-1 cells. (A) Histamine concentrations were measured in culture medium by ELISA. Three independent experiments were performed, and the data shown indicate the means $\pm$ SDs. A difference was considered statistically significant when * $P < 0.05$ vs. cells alone (a) or compound 48/80-treated cells (b). Mast cell degranulation was observed by microscope 15 min after compound 48/80 treatment. Cells were stained with toluidine blue (40X). (a) Cells alone; (b) compound 48/80-treated cells; (c) KOB03 0.5 mg/mL-treated cells and (d) KOB03 1 mg/mL-treated cells.

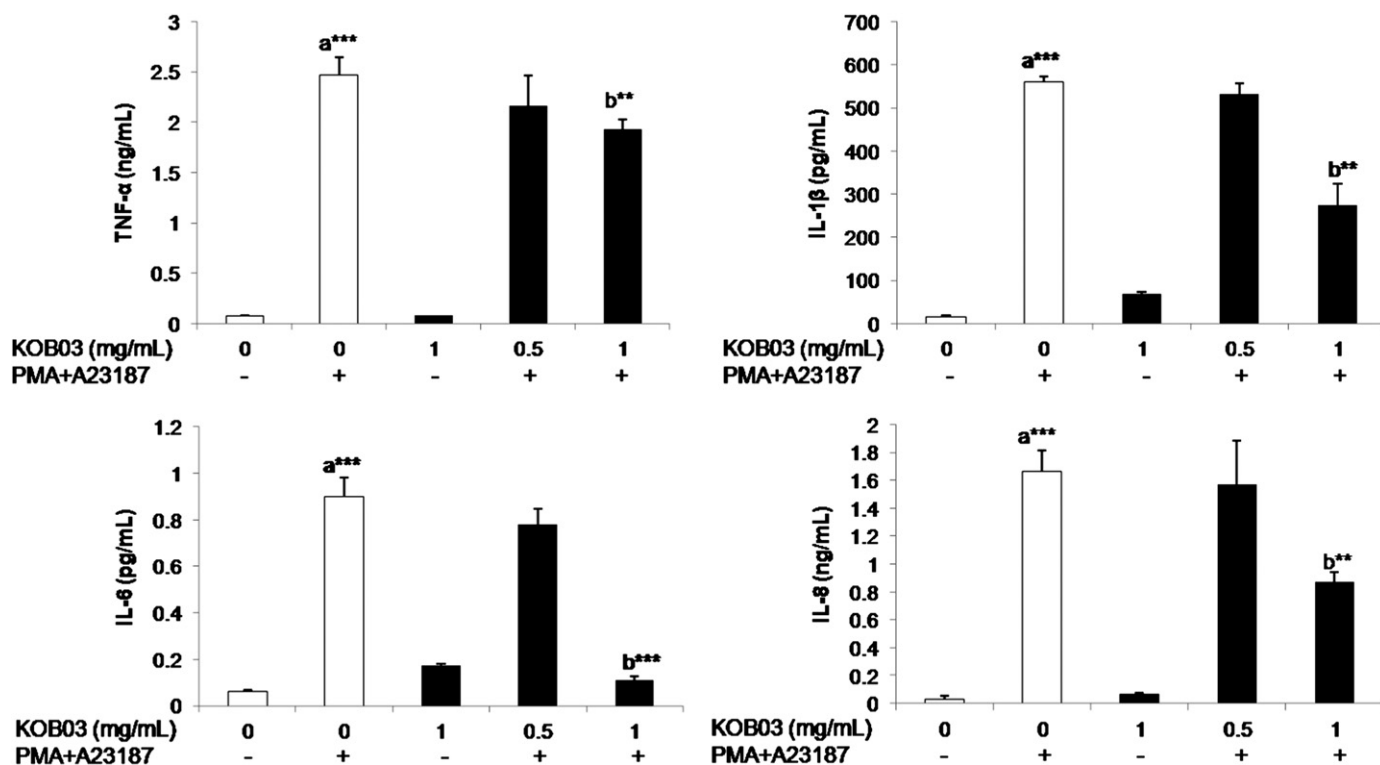


Fig. 5. Effects of KOB03 on PMA/A23187-induced production of inflammatory cytokines in HMC-1 cells. The cells were pretreated with KOB03 (0.5 and 1 mg/mL) for 1 h and then stimulated with PMA (20 nmol/L)/A23187 (1 mmol/L) for 24 h. TNF- α , IL-1 β , IL-6 and IL-8 levels were measured in culture medium by ELISA. Three independent experiments were performed, and the data shown indicate the means $\pm$ SDs. A difference was considered statistically significant when ** $P < 0.01$ and *** $P < 0.001$ vs. cells alone (a) or compared with PMA/A23187-treated cells (b).

3.4. Effect of KOB03 on histamine release from mast cells

To investigate whether KOB03 inhibits histamine release from mast cells, we measured compound 48/80-induced histamine release in HMC-1 cells. As shown in Fig. 4A, KOB03 significantly decreased the compound 48/80-induced histamine levels in the cells. We next observed the degranulation in HMC-1 cells by microscopic analysis. Fig. 4B shows that the cytoplasm of normal mast cells is dominated by closely packed secretory granules, while the cells stimulated with compound 48/80 clearly showed extensive degranulation processes and the presence of multiple extruding granules. However, KOB03 treatment at doses of 0.5 and 1 mg/ml for 15 min prior to stimulation with compound 48/80 inhibited this degranulation, and the treated cell morphology was similar to that of normal cells. In the MTT assay, KOB03 at doses of 0.1, 0.2, 0.5 and 1 mg/mL had no cytotoxic effects with almost 100% viable HMC-1 cells (data not shown).

3.5. Effect of KOB03 on cytokine release from mast cells

We also examined the effects of KOB03 on the secretion and mRNA expression of the inflammatory cytokines, TNF- α , IL-1 β , IL-8 and IL-6, in HMC-1 cells using ELISA and RT-PCR analysis. PMA/A23187-induced release of TNF- α , IL-1 β , IL-8 and IL-6 from the mast cells was significantly decreased by KOB03 treatment at

concentration of 1 mg/mL (Fig. 5). In addition, KOB03 treatment suppressed the PMA/A23187-induced expression of the cytokine mRNAs (Fig. 6). These results suggest that KOB03 had anti-inflammatory effects on mast cells by inhibiting the mRNA expression and production of inflammatory cytokines related to allergic reactions.

3.6. Effect of KOB03 on MAPK/NF- κ B pathways

The signaling pathways of the MAPK molecules and NF- κ B, a transcription factor, are important in the control of the synthesis and release of inflammatory mediators by activated mast cells during allergic inflammation (Wershil et al., 1991; Howarth et al., 1993). Therefore, to investigate the effect of KOB03 on signaling pathways in mast cells, the phosphorylation of three MAPK molecules ERK1/2, p38 MAPK and JNK and the nuclear translocation of NF- κ B p65 subunit were analyzed in HMC-1 cells by Western blot analysis. Briefly, PMA/A23187 stimulation rapidly induced the phosphorylation of ERK 1/2, p38 MAPK and JNK within 15 min in the mast cells (Fig. 7). However, at a dose of 1 mg/mL, KOB03 significantly suppressed PMA/A23187-induced phosphorylation of ERK1/2 and p38 MAPK, but not the phosphorylation of JNK MAPK, in the mast cells. The non-phosphorylated levels of the MAPK molecules remained the same. KOB03 also attenuated the PMA/A23187-induced phosphorylation of I κ -B α in

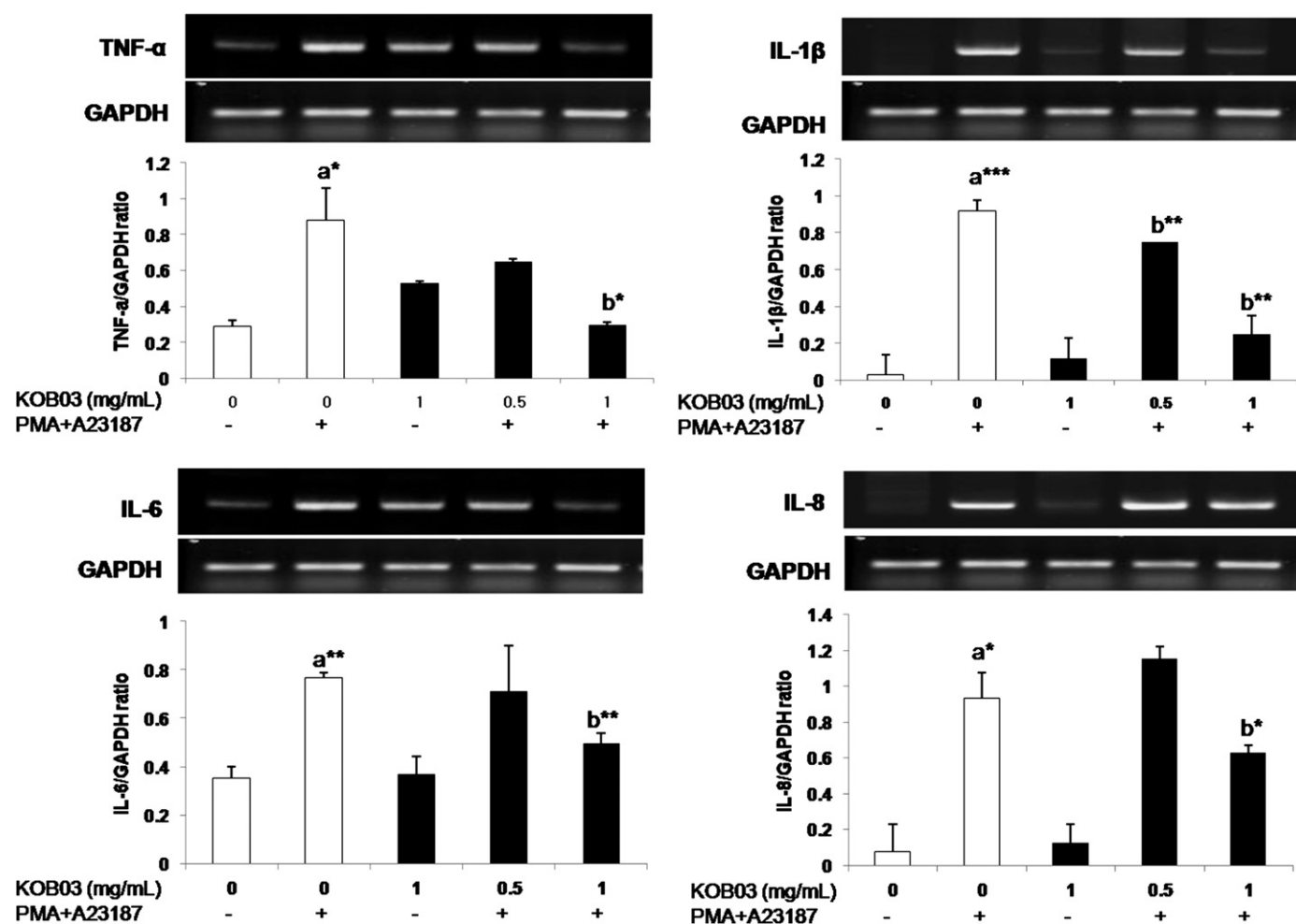


Fig. 6. Effects of KOB03 on PMA/A23187-induced expression of inflammatory cytokines mRNA in HMC-1 cells. The cells were pretreated with KOB03 (0.5 and 1 mg/mL) for 1 h, and then stimulated with PMA (20 nmol/L)/A23187 (1 mmol/L) for 6 h. The mRNA levels of each cytokine were measured by RT-PCR. GAPDH expression was used as an internal control. The figures show representative results of three independent experiments. The ratio of mean densitometry values of each cytokine was quantified by densitometry analysis and expressed in bar charts. A difference was considered statistically significant when * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. cells alone (a) or PMA/A23187-treated cells (b).

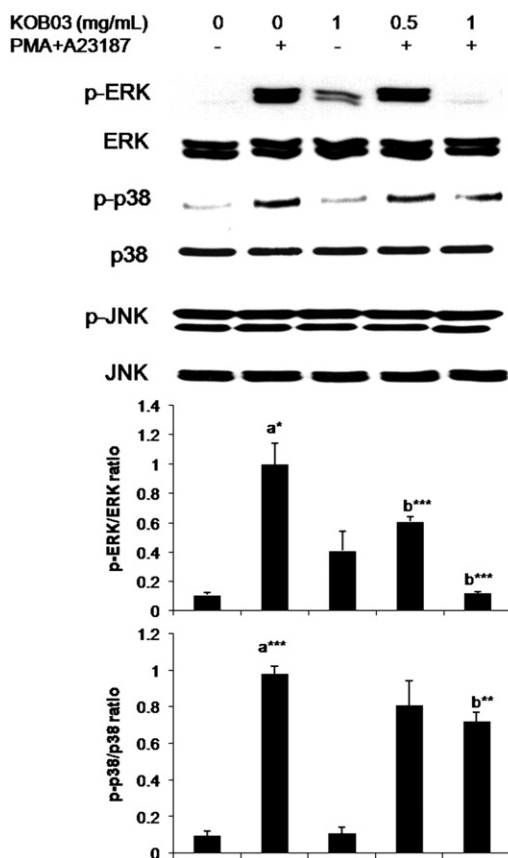


Fig. 7. Effects of KOB03 on PMA/A23187-induced phosphorylation of MAPKs in HMC-1 cells. The cells were pretreated with KOB03 (0.5 and 1 mg/mL) for 1 h and then stimulated with PMA (20 nmol/L)/A23187 (1 mmol/L) for 15 min. The total cellular proteins were used for the detection of phosphorylated or total forms of three MAPK molecules: ERK1/2, p38 MAPK and JNK. The figures show representative results of three independent experiments. The ratio of mean densitometry values of each MAPK molecule was quantified by densitometry analysis and expressed in bar charts. A difference was considered statistically significant when * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. cells alone (a) or PMA/A23187-treated cells (b).

cytosol and the nuclear translocation of NF- κ B p65 in the mast cells (Fig. 8). These results indicated the potential role of ERK1/2 MAPK and NF- κ B pathways in the suppression of inflammatory mediators by KOB03 in mast cell-mediated allergic inflammation.

3.7. HPLC analysis

In HPLC analysis of KOB03, the peak assignment was based on the standard compound, baicalin, in the HPLC chromatogram (Fig. 9).

4. Discussion

Treatment strategies for allergic rhinitis aim either to reduce the effect of local release of allergic mediators from activated mast cells and eosinophils or to end organ effects of release mediators (Bahekar et al., 2008). Although various novel drugs such as H1 receptor antagonists, corticosteroids, anti-leukotrienes, mast cell stabilizers, decongestants and intranasal anticholinergic agents are under development for pharmacotherapy of allergic diseases such as allergic rhinitis, problems such as side effects and low clinical efficacy remain because many drugs used to manage rhinitis must be administered over a period of a few weeks to months (Al Suleimani and Walker, 2007; Leung and Hon, 2008).

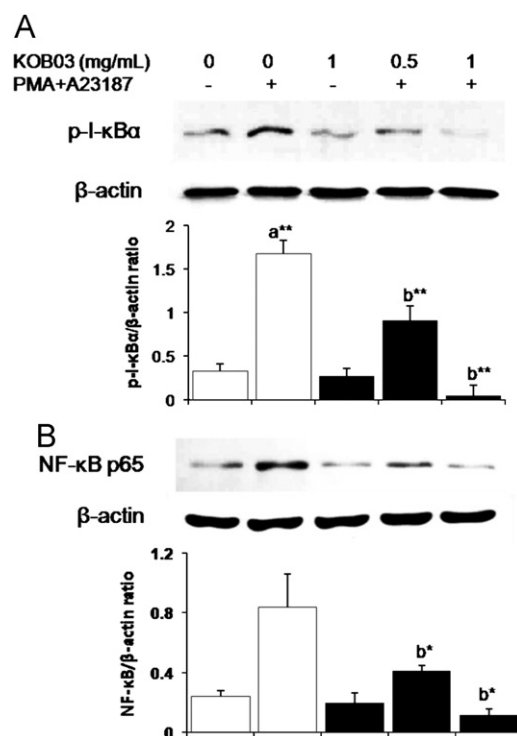


Fig. 8. Effects of KOB03 on PMA/A23187-induced phosphorylation of I- κ B α and the activation of NF- κ B in HMC-1 cells. The cells were pretreated with KOB03 (0.5 and 1 mg/mL) for 1 h and then stimulated with PMA (20 nmol/L)/A23187 (1 mmol/L) for 1 h. Cytosolic extract was prepared for the detection of phosphorylated I- κ B α (A) and nuclear extracts for the detection of NF- κ B p65 subunit (B) by Western blot. The figures show representative results of three independent experiments. The ratio of mean densitometry values of each protein were quantified by densitometry analysis and expressed in bar charts. A difference was considered statistically significant when * $P < 0.05$ and ** $P < 0.01$ vs. cells alone (a) or PMA/A23187-treated cells (b).

Nutritional supplementation with complementary and alternative medicines, including herbal medicines, improves multifactorial diseases such as allergic diseases and is considered a good treatment paradigm for allergic rhinitis (Resnick et al., 2008). It is of great interest to the general public, medical and nutritional experts, and health and food researchers to know the bioactive potential and constituents of the foods and medicines we consume. Among various herbal medicines, including the prescriptions produced from Traditional Korean Medicine, KOB03 is one of the most promising candidates for anti-allergic and anti-inflammatory effects in acute and chronic allergic diseases (Kim et al., 1991; Na et al., 2004). In the present study, we evaluated that KOB03 has a good therapeutic effect on allergic responses in OVA-induced allergic rhinitis in mice and can control mast cell activation by suppressing the release of allergic mediators such as histamine and by blocking the ERK/p38 MAPKs and NF- κ B pathways.

OVA sensitization and challenge in the in vivo model led to an increase in the levels of histamine and antigen-specific IgE in serum and the infiltration of inflammatory cells in epithelium and subepithelium of the nasal mucosa (Bahekar et al., 2008). Hence, this model presents the characteristic pathology of rhinitis such as sneezing, nasal rubbing, congestion, and edema. It is believed that nasal symptoms are mediated not only by histamine but also by prostaglandins, leukotrienes and trypase from activated mast cells, eosinophils and basophils (Martynova et al., 2005; Shinmei et al., 2009). In the present study, KOB03 remarkably inhibited rhinitis symptoms including the clinical scores, the morphological changes of nasal mucosa, and the histamine release in OVA-sensitized mice.

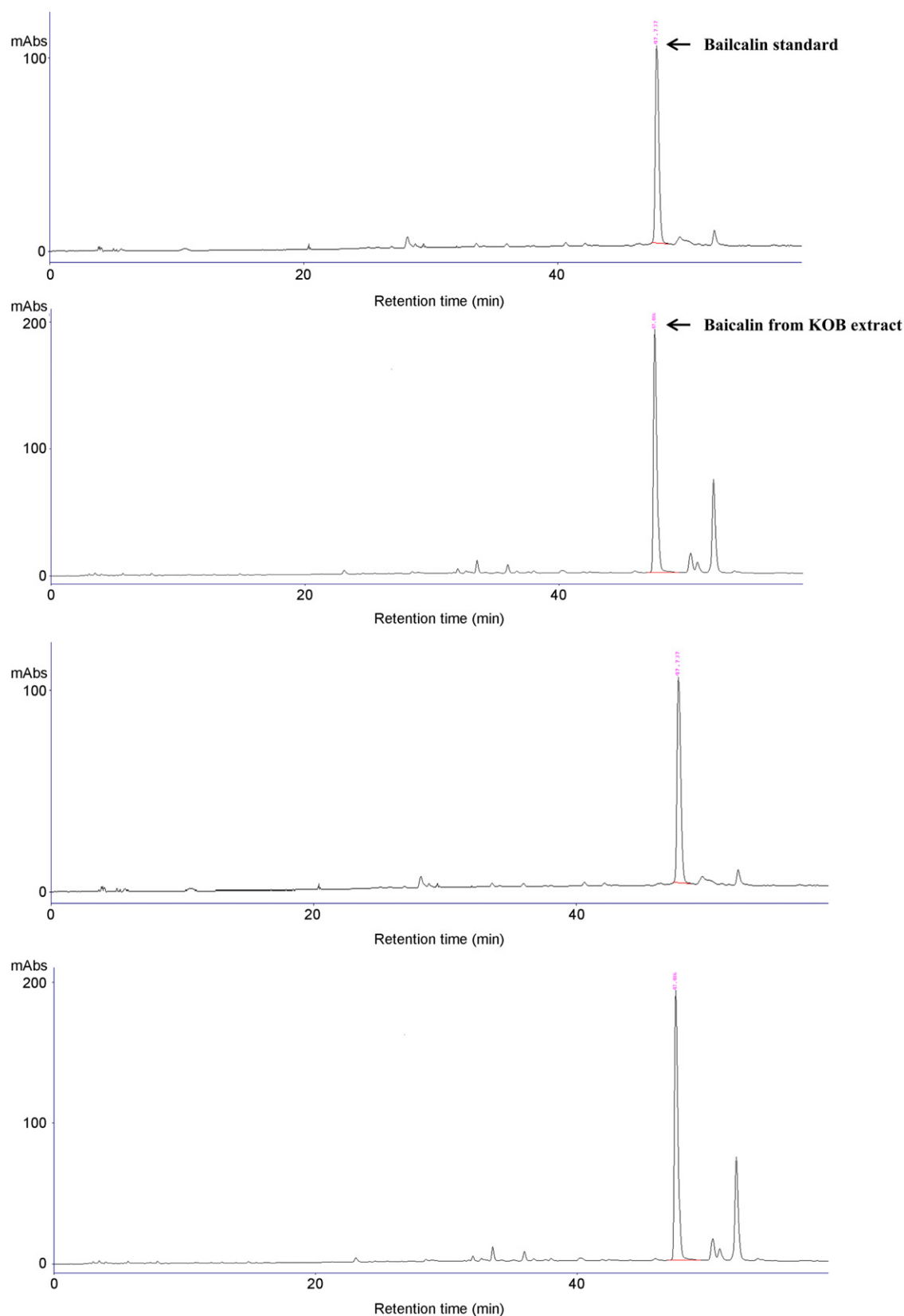


Fig. 9. HPLC pattern analysis of KOB03.

These results demonstrated that, similar to sodium cromoglycate (DSCG), KOB03 can improve rhinitis symptoms. DSCG is used as an anti-inflammatory agent in the treatment of asthma; it also alleviates rhinitis symptoms (Sur and Scandale, 2010). DSCG acts

by preventing extracellular calcium influx into the cytoplasm of mast cells, maintaining their physiological integrity and thereby preventing release of various mediators including histamine. The present study is in agreement with previous studies showing that

DSCG has a weak effect on compound 48/80-induced scratching behavior in mice or antigen-induced rhinitis symptoms.

Mast cells play a central role in immediate allergic responses through the release of numerous mediators such as histamine and cytokines via degranulation (Church and Levi-Schaffer, 1997). Therefore, mast cell degranulation has been considered a major target for effective anti-allergic drugs. Mast cell degranulation can be elicited by the synthetic compound 48/80, which has therefore been used as a direct reagent to study the mechanism of anaphylactic shock (Sur and Scandale, 2010; Choi et al., 2010). In the present study, KOB03 attenuated compound 48/80-induced release of histamine from mast cells by inhibiting their degranulation. These results demonstrated that KOB03 induced the mast cell stabilization, and had antihistamine activity against the compound 48/80-induced allergic response.

Allergic mucosal inflammation is characterized by tissue infiltration by eosinophils and associated activation of mast cells and T lymphocytes. Pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 and IL-8 are potent multifunctional cytokines that play an important role in the pathogenesis of many inflammatory diseases such as allergic rhinitis and asthma (Grzelewska-Rzymowska and Pietrkowicz, 2004). These cytokines are produced by a variety of cells in the airways and are released via the IgE-dependent activation of mast cells. Among them, TNF- α is related to the pathogenesis of these events through its capacity to upregulate the expression of endothelial cell adhesion molecules, mediate granulocyte chemotraction, and activate eosinophils, mast cells and T cells (Bradding et al., 1993). Recent research shows that TNF- α may be associated with acquired airway hyperresponsiveness, a pathophysiological hallmark of allergic and inflammatory diseases, and can also induce transepithelial migration of neutrophils through production of IL-8 and the upregulation of adhesion molecules (Bradding et al., 1995; Wilson et al., 1998). In this study, KOB03 treatment significantly inhibited the production of TNF- α , IL-1 β , IL-6 and IL-8 in PMA/A23187-stimulated mast cells. These results demonstrate that KOB03 can inhibit IgE production by downregulating pro-inflammatory cytokine transcription in allergic inflammation. KOB03 has been reported to inhibit the production of Th2 cytokines such as IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid of OVA-induced allergic asthmatic mice (Kim et al., 2000).

MAPKs such as ERK1/2, JNK and p38 MAPK play a central role in regulating the cell growth/differentiation as mediators of cellular responses to cytokines (Johnson and Lapadat, 2002). Thus, the synthesis and expression of pro-inflammatory cytokines are regulated upon activation of normal T cells, and the expression and secretion of monocyte chemoattractant protein-1 and eotaxin by mast cells can influence eosinophil function via signaling pathways such as MAPKs and NF- κ B (Shakoory et al., 2004). In this study, KOB03 significantly inhibited PMA/A23187-induced phosphorylation of ERK1/2 and p38 MAPK, which is involved in the production of inflammatory mediators in activated mast cells. These results indicate that KOB03 has an anti-inflammatory effect by blocking the ERK1/2 and p38 MAPK signal to subsequently inhibit the inflammatory cytokine production in PMA/A23187-stimulated mast cells. The activation of the NF- κ B pathway results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of I κ B, followed by the nuclear translocation of NF- κ B (Karin, 1999). Therefore, blocking NF- κ B transcriptional activity in the mast cell nucleus can suppress the expression of inflammatory mediators, including cytokines and adhesion molecules. In this study, KOB03 inhibited the degradation of I- κ B α in cytosol and the subsequent nuclear translocation of NF- κ B in PMA/A23187-stimulated mast cells. These results indicate that KOB03 could prevent nuclear localization of NF- κ B, which is a mechanism for controlling the activation of the NF- κ B pathway in mast cells and thereby inhibiting the expression of the inflammatory cytokines,

IL-1, TNF- α , IL-6 and IL-8. KOB03 is a polyherbal medicine prescribed in oriental clinics for the treatment of several allergic diseases (Kim et al., 2000; Shin, 2005; Kil et al., 2006).

This study is the first to report the efficacy of KOB03 in mast cell-mediated allergic responses. In general, herbal medicines possess many components with different biological properties. KOB03 is a decoction of five herbs with different pharmacological actions, and the main compound, baicalin, has been identified by HPLC analysis with a standard compound. Baicalin, a flavonoid extracted from various herbal plants, is reported to have anti-allergic properties in atopic dermatitis (Yun et al., 2010), type IV allergic reaction (Taniguchi et al., 2000), IgE-mediated allergic reaction (Kimata et al., 2000) and anaphylaxis (Koda et al., 1970).

In conclusion, our observations suggest that KOB03 has an anti-allergic effect by improving rhinitis symptoms, inhibiting the release of allergic mediators in OVA-induced allergic rhinitis mice, and inhibiting mast cell activation. Furthermore, KOB03 has anti-inflammatory activity by suppressing the production of inflammatory cytokines, TNF- α , IL-1 β , IL-6 and IL-8, via the ERK1/2 and p38 MAPK and NF- κ B pathways in activated mast cells. These results suggest that KOB03 might be useful for the treatment of allergic inflammatory diseases such as allergic rhinitis.

Acknowledgments

This work was supported by grants from the Ministry of Health & Welfare, Republic of Korea, for the development of herbal medicine drugs for allergic rhinitis (F090002, to Y.-K. Park).

References

- Al Suleimani, Y.M., Walker, M.J., 2007. Allergic rhinitis and its pharmacology. *Pharmacology & Therapeutics* 114, 233–260.
- Bahekar, P.C., Shah, J.H., Ayer, U.B., Mandhane, S.N., Thennati, R., 2008. Validation of guinea pig model of allergic rhinitis by oral and topical drugs. *International Immunopharmacology* 8, 1540–1551.
- Bradding, P., Feather, I.H., Wilson, S., Bardin, P.G., Heusser, C.H., Holgate, S.T., Howarth, P.H., 1993. Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. *The Journal of Immunology* 151, 3853–3865.
- Bradding, P., Mediawake, R., Feather, I.H., Madden, J., Church, M.K., Holgate, S.T., Howarth, P.H., 1995. TNF alpha is localized to nasal mucosal mast cells and is released in acute allergic rhinitis. *Clinical & Experimental Allergy* 25, 406–415.
- Choi, Y.H., Yan, G.H., Chai, O.H., Song, C.H., 2010. Inhibitory effects of curcumin on passive cutaneous anaphylactoid response and compound 48/80-induced mast cell activation. *Anatomy & Cell Biology* 43, 36–43.
- Church, M.K., Levi-Schaffer, F., 1997. The Human Mast Cell. *The Journal of Allergy Clinical Immunology* 99, 155–160.
- Grzelewska-Rzymowska, I., Pietrkowicz, M., 2004. Role of tumor necrosis factor-alpha in allergic inflammation and airway hyperresponsiveness. *Pol Merkur Lekarski* 16, 173–178.
- Heo, J., 1985. Dongui Bogam. Dae Sung Publisher Co., Seoul, Republic of Korea 123.
- Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911–1912.
- Karin, M., 1999. The beginning of the end: I κ B kinase (IKK) and NF- κ B activation. *The Journal of Biological Chemistry* 274, 27339–27342.
- Kil, K.J., Lee, Y.J., Kim, S.H., 2006. A study on a morphological identification of *Ostericum* root. *The Korea Journal of Herbology* 21, 181–188.
- Kim, C.M., Heo, M.Y., Kim, H.P., Sin, K.S., Pachaly, P., 1991. Pharmacological activities of water extracts of Umbelliferae plants. *Archives of Pharmacol Research* 14, 87–92.
- Kim, H.M., Yoon, G.S., Seo, J.U., Moon, G., Kim, H.R., Shin, T.Y., 2000. Inhibition of mast cell-mediated anaphylaxis by sochungryong-tang. *The American Journal of Chinese Medicine* 28, 69–76.
- Kimata, M., Inagaki, N., Nagai, H., 2000. Effects of luteolin and other flavonoids on IgE-mediated allergic reactions. *Planta Medica* 66, 25–29.
- Koda, A., Nagai, H., Wada, H., 1970. Pharmacological actions of baicalin and baicalein. 2. On passive anaphylaxis. *Nihon Yakurigaku Zasshi Folia pharmacologica Japonica* 66, 237–247.
- Lee, N.P., Arriola, E.R., 1999. How to treat allergic rhinitis. *Best Practice* 171, 31–34.
- Leung, A.K., Hon, K.L., 2008. Seasonal allergic rhinitis. *Recent Patents on Inflammation & Allergy Drug Discovery* 2, 175–185.

- Martynova, M.G., Bystrova, O.A., Moiseeva, O.M., Evdonin, A.L., Kondratov, K.A., Medvedeva, N.D., 2005. The presence of ANP in rat peritoneal mast cells. *Cell Research* 15, 811–816.
- Na, H.J., Jeong, H.J., Hong, S.H., Hwang, C.Y., Cha, W.S., Kim, H.M., 2004. Effect of Boo Yong-Tang on mast cell-mediated allergic reaction. *Immunopharmacology and Immunotoxicology* 26, 445–454.
- Nouri-Aria, K.T., 2008. Recent progress in allergen immunotherapy. *Iranian Journal of Immunology* 5, 1–24.
- Resnick, E.S., Bielory, B.P., Bielory, L., 2008. Complementary therapy in allergic rhinitis. *Current Allergy and Asthma Reports* 8, 118–125.
- Smolensky, M.H., Lemmer, B., Reinberg, A.E., 2007. Chronobiology and chronotherapy of allergic rhinitis and bronchial asthma. *Advanced Drug Delivery Reviews* 59, 8528–8882.
- Shakoory, B., Fitzgerald, S.M., Lee, S.A., Chi, D.S., Krishnaswamy, G., 2004. The role of human mast cell-derived cytokines in eosinophil biology. *Journal of Interferon & Cytokine Research* 24, 271–281.
- Shin, S., 2005. In vitro effects of essential oils from *Ostericum koreanum* against antibiotic-resistant *Salmonella* sp. *Archives of Pharmacol Research* 28, 765–769.
- Shinmei, Y., Yano, H., Kagawa, Y., Izawa, K., Akagi, M., Inoue, T., Kamei, C., 2009. Effect of Brazilian propolis on sneezing and nasal rubbing in experimental allergic rhinitis of mice. *Immunopharmacology and Immunotoxicology* 31, 688–693.
- Sur, D.K., Scandale, S., 2010. Treatment of allergic rhinitis. *South African Family Practice* 81, 1440–1446.
- Taniguchi, C., Homma, M., Takano, O., Hirano, T., Oka, K., Aoyagi, Y., Niitsuma, T., Hayashi, T., 2000. Pharmacological effects of urinary products obtained after treatment with saiboku-to, a herbal medicine for bronchial asthma, on type IV allergic reaction. *Planta Medica* 66, 607–611.
- Wilson, S.J., Lau, L., Howarth, P.H., 1998. Inflammatory mediators in naturally occurring rhinitis. *Clinical & Experimental Allergy* 28, 220–227.
- Yun, M.Y., Yang, J.H., Kim, D.K., Cheong, K.J., Song, H.H., Kim, D.H., Cheong, K.J., Kim, Y.I., Shin, S.C., 2010. Therapeutic effects of Baicalein on atopic dermatitis-like skin lesions of NC/Nga mice induced by *dermatophagoides pteronyssinus*. *International Immunopharmacology* 10, 1142–1148.