



Inhibitory effects of kaempferol-3-O-rhamnoside on ovalbumin-induced lung inflammation in a mouse model of allergic asthma

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ABSTRACT

The modification of natural flavonoid by glycosylation alters their physicochemical and pharmacokinetic properties, such as increased water solubility and stability, reduced toxicity, and sometimes enhanced or even new pharmacological activities. Kaempferol (KF), a plant flavonoid, and its glycosylated derivative, kaempferol-3-O-rhamnoside (K-3-rh), were evaluated and compared for their anti-inflammatory, anti-oxidant, and anti-asthmatic effects in an asthma model mouse. The results showed that K-3-rh fully maintained its anti-inflammatory and anti-asthmatic effects compared with KF in an asthma model mouse. Both KF and K-3-rh significantly reduced the elevated inflammatory cell numbers in the bronchoalveolar lavage fluid (BALF). KF and K-3-rh also significantly inhibited the increase in Th2 cytokines (IL-4, IL-5, and IL-13) and TNF- α protein levels through inhibition of the phosphorylation Akt and effectively suppressed eosinophilia in a mouse model of allergic asthma. The total immunoglobulin (Ig) E levels in the serum and BALF were also blocked by KF and K-3-rh to similar extents. K-3-rh exerts similar or even slightly higher inhibitory effects on Th2 cytokines and IgE production compared with KF, whereas K-3-rh was less effective at DPPH radical scavenging and the inhibition of ROS generation in inflammatory cells compared with KF. These results suggested that the K-3-rh, as well as KF, may also be a promising candidate for the development of health beneficial foods or therapeutic agents that can prevent or treat allergic asthma.

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1. Introduction

Allergic asthma is characterized by chronic inflammation of the airways, bronchial hyperreactivity, overexpression of allergen-reactive T helper type-2 (Th2) cells and proinflammatory cytokines, and an infiltration of lymphocytes and eosinophils into the airway submucosa [1–3]. Eosinophil recruitment is induced in ovalbumin-induced asthmatic mouse models [4,5], and allergic asthma also increased the immunoglobulin E (IgE) levels [6]. The activation of T cells is mediated by the production of Th2 cytokines, such as interleukin-4 (IL-4), IL-5, and IL-13. Asthmatic animals are reported to have high concentrations of Th2 cytokines and proinflammatory cytokine tumor necrosis factor (TNF- α) in the bronchoalveolar lavage fluid (BALF), lung and serum [1,2,7]. Airway eosinophilia and Th2 cytokines may ultimately contribute to airway hyperresponsiveness in asthma [2,3].

Kaempferol (KF) is a natural flavonoid that can be isolated from citrus fruits, brussels sprouts, broccoli, apples, and other plant sources [4]. Flavonoids, which are ubiquitously found in plants, are the most

common group of polyphenolic compounds in the human diet and are sub-grouped into flavones, flavonols, flavanones, flavanols, chalcones, anthocyanins, and isoflavones [7,8]. Previous studies have reported that KF has anti-proliferation activities in many cancer cell lines [9], results in a reduced risk of cardiovascular diseases and asthma [4] and exhibits several pharmacological activities, including antioxidant, anti-inflammatory, and antiallergic activities [10].

Most flavonoids are restricted to pharmacological applications because they lack water solubility and stability. The glycosylation, acylation, and methylation of flavonoids are common modifications that diversify flavonols, and glycosylation renders them more water-soluble and less toxic. Previous studies have shown that the genistin glycosides exhibit a marked 1000-to-10,000-fold increase in water solubility compared with genistin [11]. Thus, an increase in water solubility by the glycosylation of flavonoids may diversify and/or enhance their industrial application and biological functions. Additionally, glycosylation alters the pharmacokinetic properties and selectivities of compounds [12–14].

Kaempferol-3-O-rhamnoside (K-3-rh), a polyphenolic glycoside flavone, has been reported for its anti-cancer activity and neuroprotective effects on Alzheimer's disease [15,16]. Simkhada et al. [17] recently reported the development of techniques for the large-scale production of K-3-rh from *Escherichia coli* using a genetic engineering approach. Therefore, it would be worth evaluating K-3-rh for its pharmacological

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activities to diversify and enhance its industrial applicability for the development of new and/or improved therapeutic agents. KF is effective at ameliorating allergic and inflammatory airway diseases through the inhibition of the ovalbumin (OVA) challenge-elevated expression of eotaxin-1 and eosinophil major basic protein by blocking NF- κ B transactivation [4]. However, whether glycosylated KF (K-3-rh) has protective effects on airway allergic inflammation has not yet been examined. In this study, KF and its glycosylated derivative, K-3-rh, were evaluated and compared for their inhibitory effects on OVA-induced airway allergic inflammation *in vivo* by measuring the asthma-related cytokines (IL-4, IL-5, IL-13 and TNF- α) and phosphorylation of Akt and mitogen-activated protein kinase (MAPK) such as Erk1/2 and p38 in BALF, lung tissue and serum and through blunting eosinophil accumulation in the airway and lung tissue of an OVA-sensitized/challenged mouse asthma model.

2. Materials and methods

2.1. Preparation of kaempferol and kaempferol-3-O-rhamnoside

Kaempferol (molecular weight: 284.24 Da, KF) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and its glycosylated derivative, kaempferol-3-O-rhamnoside (molecular weight: 432.37 Da, K-3-rh), was prepared by the biotransformation of KF using genetically engineered *E. coli* cells. Previously constructed *E. coli* BL21(DE3)/ Δ pgi strain harboring thymidine diphosphate (dTDP)-L-rhamnose biosynthetic pathway genes along with a glycosyltransferase from *Arabidopsis thaliana* (ArGT3) was used for the biotransformation [17]. Briefly, for the large scale production of K-3-rh, a glass autoclavable self-controlled fermentor system (Biotron, Korea) with a 3 l capacity was used. When the optical density at 600 nm was above 5.0, the culture was induced by 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and temperature was lowered to 30 °C. After 5 h of culture induction, KF (200 μ M final concentration dissolved in DMSO) was supplemented and incubation was continued until 60 h. After completion of fermentation, the culture broth was extracted with an equal volume of ethyl acetate.

The purification of K-3-rh was carried out by preparative- high performance liquid chromatography with a C₁₈ column (YMC-Pack ODS-AQ (150 $\times$ 20 mm I.D., 10 μ m)) connected to an ultraviolet detector (290nm) using a 36 min binary program with acetonitrile 20% (0–5 min), 40% (5–10 min), 40% (10–15 min), 90% (15–25 min), 90% (25–30 min), and 10% (30–35 min) at a flow rate of 10 ml/min. The structure of the purified product was confirmed by high resolution quantitative time-of-flight electrospray ionization mass spectrometry carried out in positive ion mode on ACQUITY (UPLC; Waters, Milford, MA, USA) coupled with SYNAPT G2-S (Waters). The structure was further elucidated by various nuclear magnetic resonance analyses including ¹H NMR, ¹³C NMR, nuclear Overhauser effect spectroscopy (NOESY), rotating-frame NOE spectroscopy (ROESY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple-bond correlation (HMBC) with a 900 MHz Avance II 900 Bruker BioSpin Spectrometer (Germany) using a Cryogenic TCI Probe (5 mm) (data not shown). The purified compound (K-3-rh) was used for the subsequent experiments.

2.2. Animals, diets and experimental protocol

The female BALB/c mice (age 6 weeks; KOATECH, Gyeonggi-do, Korea) were housed in an air-conditioned (21–25 °C) and humidity-controlled room with a 12 h on/12 h off light. The mice were maintained under standard laboratory conditions for one week prior to the experiments and were then divided into three groups of 8–10 mice. Animal care and handling were performed under the protocols approved by the Committee on Animal Experimentation of the Catholic University of Korea. A schematic diagram of the treatment schedule is shown in Fig. 1. A total of 500 μ g/ml of OVA was complexed with 500 μ g/ml of

alum (Sigma-Aldrich, USA), and the final volume ratio of alum (500 μ g/ml) to OVA (500 μ g/ml) was 1:3. Each mouse was immunized by the intraperitoneal (IP) injection of 14.7 ml/kg body weight of OVA, complexed with alum on days 0 and 14 (Fig. 1). The control group received 14.7 ml/kg body weight of phosphate-buffered saline (PBS) with alum by IP injection. In brief, 0.2 ml of the KF solution (0.34 mg KF/0.2 ml/mouse/day = 17 mg KF/10 ml/50 mice/day = 6 mM/kg body weight/day = 17 mg/kg body weight/day) and 0.2 ml of the K-3-rh solution (0.52 mg K-3-rh/0.2 ml/mouse/day = 26 mg K-3-rh/10 ml/50 mice/day = 6 mM/kg body weight/day = 26 mg/kg body weight/day) were orally administered every day from days 18 to 24.

The OVA-challenged mice were exposed to 2% OVA (w/v) in PBS for 20 min (over a period of 5 min) by inhalation using a Compressor Nebulizer (0.4 ml/min, NE-C28, Omrom, Tokyo, Japan) on days 21, 22, 23 and 24 after KF or K-3-rh feeding, but asthma control mice (positive control) were orally administered with water instead of KF and K-3-rh under the same conditions. The normal mice were exposed to PBS without OVA and orally administered with water instead of KF and K-3-rh under the same condition.

After feeding, the mice were fasted overnight (16–19 h) and sacrificed on day 26. The mice were killed with an IP injection of a Zoletil 50 (Virbac S.A., France) and Rompun (Bayer, Germany) mixture (3:2). The blood samples were collected into tubes. The lungs were lavaged with ice-cold PBS (0.5 ml), and the BALF was obtained with three lavages (a total volume of 1.5 ml). The BALF was centrifuged, and the supernatant was stored at –80 °C until the cytokine assays were performed. The cell pellets were used for the measurement of intracellular reactive oxygen species (ROS) and cell numbers. Part of lung tissue was fixed in 4% paraformaldehyde for lung histology, and the other lung tissues were rapidly frozen on liquid nitrogen and stored at –80 °C for protein extraction.

2.3. RNA extract and RT-PCR analysis of cytokine mRNA production

The total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. The RNA was reverse-transcribed using a Power cDNA synthesis kit (iNtRON Biotech, Seongnam, Korea) according to the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed with a Maxime PCR PreMix kit (iNtRON Biotech, Seongnam, Korea) in 20 μ l of each primer (forward and reverse, 10 pmol/ μ l). The following primers were used: IL-13, F, 5'-TTGCTTGCTTGCTGGTC-3' and R, 5'-GGCTGGAGACCGTAGTGG-3'; TNF- α , F, 5'-GGCAGGTCTACTTTGGAGTCATTGC-3' and R, 5'-ACATTCGAGGCTCCAGTGAATTCGG-3'; and β -actin, F, 5'-TGCTGTCCCTGTATGCCTCT-3' and R, 5'-AGGTCTTTACGGATGTCAACG.

The PCRs with the IL-13, TNF- α and β -actin primers were performed with an initial cycle of 2 min at 94 °C, followed by 38 cycles (IL-13 and TNF- α) or 25 cycles (β -actin) for 20 s at 94 °C, 20 s at 60 °C, and 40 s at 72 °C, and a final extension for 5 min at 72 °C; the PCR using the β -actin transcripts served as internal controls for the *in vivo* experiments. The PCR products were analyzed by 2% agarose gel electrophoresis.

2.4. Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

For the ELISAs and Western blot analysis, the lung tissues were homogenized at 4 °C in PBS containing a protease inhibitor cocktail (Sigma-Aldrich, USA) or RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, pH to 7.8), containing protease and phosphatase inhibitor cocktails (Roche, Germany). The lysate was clarified by centrifugation at 13,000 rpm and 4 °C for 20 min. The protein concentration was determined with a Bio-Rad protein kit (Hercules, CA, USA) using bovine serum albumin (BSA, Sigma-Aldrich, USA) as the standard. The protein levels of IL-4, IL-5, IL-13 and TNF- α in the BALF and lung tissues were also measured by ELISA kits (IL-4,

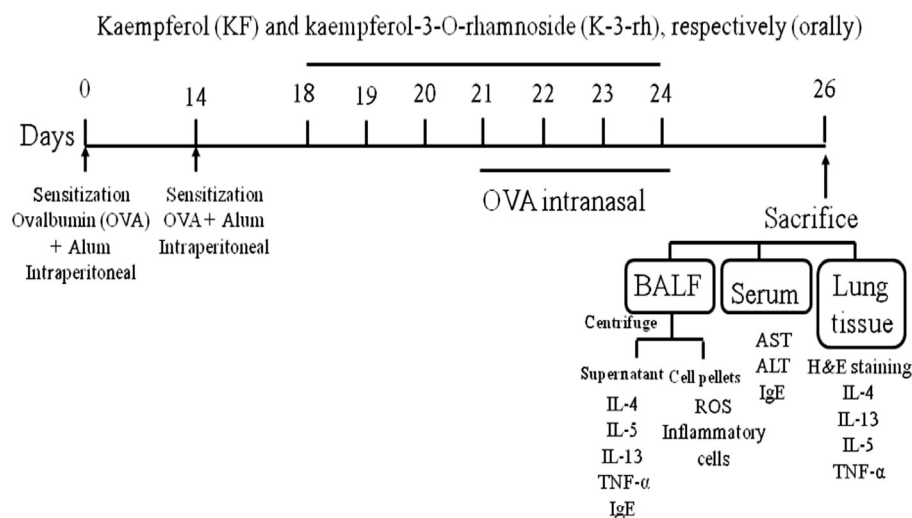


Fig. 1. Mouse model of airway inflammation induced by ovalbumin (OVA) and effects of treatment with kaempferol (KF) and kaempferol-3-O-rhamnoside (K-3-rh). BALF, bronchoalveolar lavage fluid.

IL-5 and IL-13, eBioscience, San Diego, CA, USA; TNF- α , BD Biosciences Heidelberg, Germany) according to the manufacturers' instructions. The IgE concentrations in the BALF and serum were determined through a mouse IgE ELISA kit (eBioscience, San Diego, CA, USA) using the manufacturer's instructions.

For the Western blot analysis, the protein (30 μ g) was loaded onto 12% SDS-PAGE gels, and then transferred to nitrocellulose membranes. The membranes were incubated with the indicated primary antibodies, overnight at 4 °C. The membranes were washed three times, incubated with horseradish peroxidase-conjugated antibodies for 1.5 h, and then visualized, using the enhanced chemiluminescence method (AbClon, Seoul, Korea). The primary antibodies such as Akt, phospho-Akt (p-Akt), phospho-p44/42 MAPK (p-Erk1/2), p44/42 MAPK (Erk1/2), phospho-p38 MAPK (p-p38), p38 MAPK (p-38), and β -actin (Cell Signaling Technology, Beverly, MA) were used at 1:1000 dilution, and horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG antibodies (Cell Signaling Technology, Beverly, MA) at 1:2000 were used as the secondary antibody. The membrane was exposed to X-ray film. Equal protein loading was confirmed by the β -actin antibody (Cell Signaling Technology, Beverly, MA).

2.5. Measurement of serum alanine transaminase (ALT) and aspartate transaminase (AST)

The ALT and AST levels in the serum of OVA-induced asthma mice were measured using commercial kits (Asan Chemical, Seoul, Korea), according to the manufacturer's instructions, after the mice were fed with KF or K-3-rh (6 mM/kg body weight/day) for seven days.

2.6. Inflammatory cell counts in the bronchoalveolar lavage fluid (BALF)

The total inflammatory cell number was assessed according to the previously described method [18]. The BALF was centrifuged, the cells from the BALF were washed three times with PBS, and the pellet was re-suspended in 100 μ l of PBS. The total cell number was counted using a hemocytometer, and a cytospin preparation of BALF cells was fixed and stained using a Diff-Quik Staining reagent (B4132-1A, Dade Behring Inc., Deerfield, IL, USA). The different cell types were enumerated based on their morphology and staining profile.

2.7. Measurement of intracellular ROS and DPPH radical scavenging activities

The intracellular ROS was measured by the 2,7-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, OR, USA) method [7]. After the incubation of the cells from the BALF with 50 μ M DCF-DA for 45 min and the removal of PBS with the DCF-DA, the cells were washed twice with 1 $\times$ PBS (pH 7.4), and DCF fluorescence was measured using a fluorometric microplate reader (BioTek, Winooski, VT, USA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

To direct the antioxidant activity, the DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma-Aldrich, USA) radical-scavenging assay was performed as described previously with slight modifications [7]. Various concentrations (0, 1, 2.5, 5, 10, 25 and 50 μ M) of KF, K-3-rh or α -tocopherol (Sigma-Aldrich, USA) were mixed with 120 μ l of DPPH radical solution (1.5×10^{-4} M), and the decrease in absorbance at 517 nm was monitored. The DPPH solution plus methanol was used as a control, and α -tocopherol was used as a standard antioxidant and positive control. The inhibition percentage was calculated using the following equation:

$$\text{scavenging activity(\%)} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100,$$

where A_{control} is the absorbance of the control and A_{sample} is the absorbance of the flavonoids or standard.

2.8. Lung tissue histopathology

The lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h. The tissues were then embedded in paraffin, cut into sections with 4- μ m thickness, and stained with a H&E solution (hematoxylin and eosin, Sigma-Aldrich, USA) to confirm cellular penetration.

2.9. Statistical analysis

The data from the three independent experiments were expressed as the mean $\pm$ S.D. The data were analyzed using the SPSS package (Version 10.0, SPSS, SPSS Inc., Chicago, IL, USA), and one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests was used to compare the results from different treatments. The data were considered to have statistical significances at $p < 0.05$. The correlation

coefficient was used for comparisons between the DPPH radical scavenging activity and the inhibitory effect on ROS generation, and the statistical significance and correlation coefficient of the data were evaluated using the GraphPad InStat software (Version 3.00, GraphPad InStat, Inc., San Diego, CA, USA).

3. Results

3.1. Effects of KF and K-3-rh on OVA-induced total and different inflammatory cells in BALF

The treatment of the animals used for the OVA-induced airway inflammation mouse model of asthma and the diets are summarized in Fig. 1, and the changes in the total number of different inflammatory cells in the BALF following OVA immunization and challenge was examined. The induction of allergic airway disease by sensitization and challenge with OVA (the asthma group as the asthma positive control group) resulted in an increase in the numbers of eosinophils, other inflammatory cells and total cells in the BALF compared with the PBS-challenged control [negative control (NC) group] (Fig. 2B). The mice in the K-3-rh group had a lower number of eosinophils, other inflammatory cells, and total cells in the BALF compared with the asthma group (Fig. 2B). KF also reduced the numbers of these cells in the BALF (Fig. 2B). There were no significant differences between KF and K-3-rh in the numbers of inflammatory cells in the BALF of mice (Fig. 2B).

3.2. Effects of KF and K-3-rh on OVA-induced intracellular ROS generation in the BALF and DPPH radical scavenging activity

Oxidative stress occurs in many allergic and immunological disorders. Many studies have shown an increased production of ROS in an OVA-induced mouse allergic asthma model [7,19]. The effect of KF and K-3-rh on ROS generation is shown in Fig. 2C. The levels of ROS in the BALF of the mice in the OVA-induced asthma group were increased significantly compared with the levels observed in the PBS-challenged control group (NC group). ROS generation was decreased by KF or K-3-rh administration compared with that observed in the OVA-induced asthma group (Fig. 2C). KF was a more effective inhibitor of intracellular ROS generation than K-3-rh. These results suggest that KF and K-3-rh exerted an inhibitory effect on ROS generation. ROS are heavily implicated in the mechanism of chronic inflammation of the airways [19]. Thus, the inhibition of ROS generation is an important therapeutic goal in airway inflammation allergic asthma. To investigate whether KF and K-3-rh have antioxidative activity, the scavenging activity of KF and K-3-rh on the DPPH radical was measured. The DPPH radical scavenging activity of KF was found to be approximately 100% at KF concentrations of 50, 25, 10 and 5 mM (Fig. 2D). The DPPH radical scavenger activity of KF ($89.3 \pm 1.9\%$) was higher than that of K-3-rh ($16.1 \pm 2.3\%$) at a concentration of 5 mM. α -Tocopherol was used as a positive control, and α -tocopherol and KF exerted a similar effect on the DPPH radical scavenger activity.

3.3. Effects of KF and K-3-rh on OVA-induced histopathological changes in the lung

To evaluate airway inflammation, lung sections were stained with H&E. It was shown that OVA treatment (asthma group) induced the infiltration of inflammatory cells into the lung tissue compared with that observed in the negative control group (NC group), whereas the administration of KF and K-3-rh markedly attenuated the accumulation of inflammatory cells compared with that observed in the OVA-sensitized/challenged mice (asthma group, Fig. 3). These results suggest that KF and K-3-rh inhibited the OVA-induced inflammatory infiltration in a mouse model of asthma.

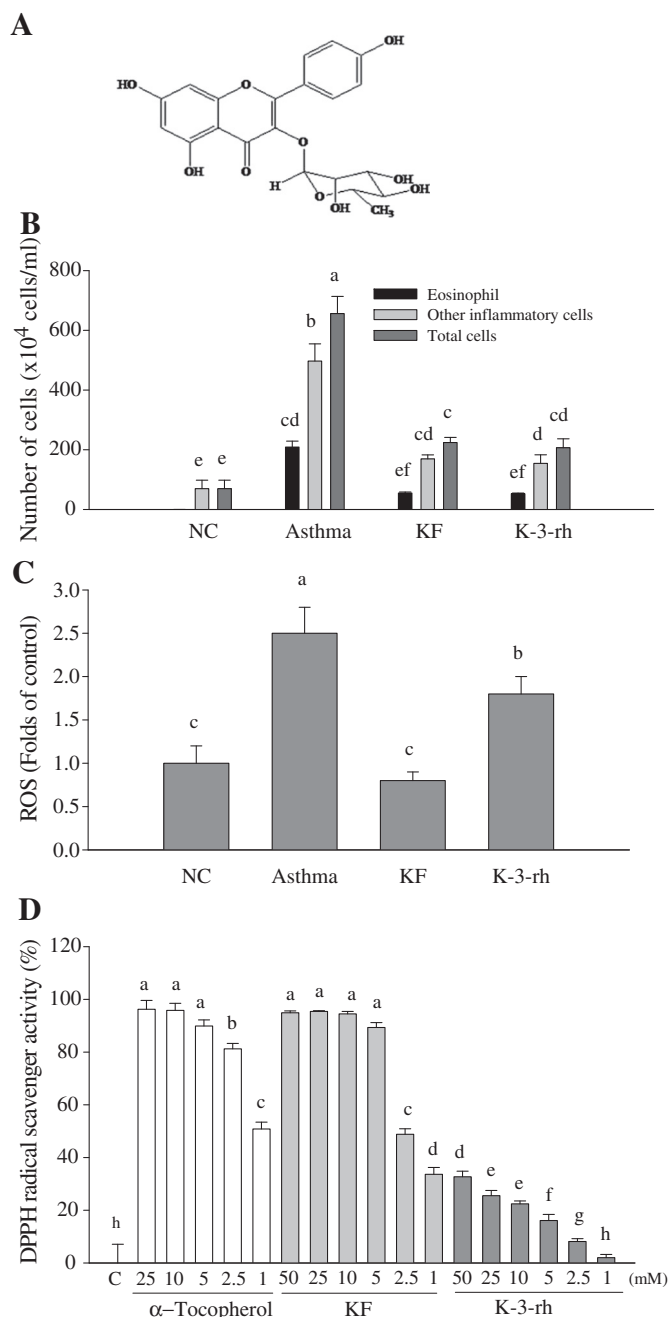


Fig. 2. Effect of KF and K-3-rh on the recruitment of inflammatory cells and reactive oxygen species (ROS) in the bronchoalveolar lavage fluid (BALF) of mice and on DPPH radical scavenger activity. (A) Structures of K-3-rh [27]. (B) The cells were isolated by centrifugation and stained with the Diff-Quik Stain reagent. (C) The intracellular ROS in the BALF were quantified based on DCF fluorescence. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice; and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. (D) The DPPH radical-scavenging activities of KF and K-3-rh were measured at the indicated concentrations. The values are expressed as the mean $\pm$ SD ($n = 8-10$), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test.

3.4. Effects of KF and K-3-rh on OVA-induced phosphorylation of Akt and MAPK pathways in the lung tissue

To determine the effects of KF and K-3-rh on the Akt and MAPK signaling pathways, we examined the effects of these KF and K-3-rh on the phosphorylation of Akt, Erk1/2, and p38 in the OVA-sensitized/

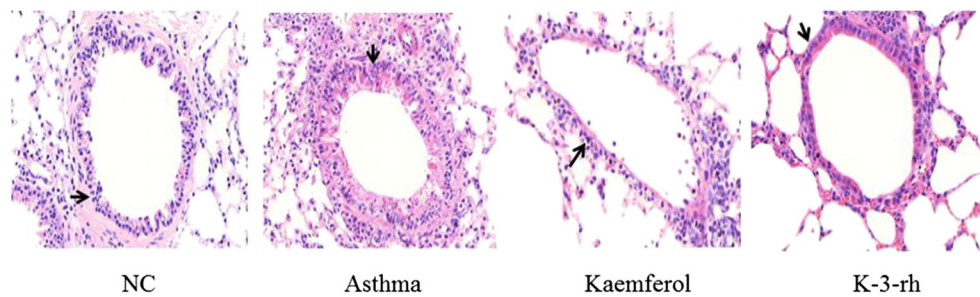


Fig. 3. Effects of KF and K-3-rh on airway inflammation caused by cell infiltration in lung tissue. Pathological changes in lung tissues were confirmed by hematoxylin and eosin staining (magnification, 200 $\times$). NC, negative control (PBS only); Asthma, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice; and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. The values are expressed as the mean $\pm$ SD ($n = 8-10$), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test. Arrow heads indicate the infiltrated inflammatory cells in the lung tissue.

challenged mice because of their role in production of TNF- α , IL-4 and IL-13 [7].

The phosphorylation of Akt [an indicator of the activation of phosphatidylinositol 3-kinase (PI3-kinase)] and MAPK pathways including Erk1/2 and p38 were induced in lung tissue obtained from an OVA-sensitized/challenged mouse asthma model. The phosphorylation of Akt, p38 and Erk1/2 was significantly suppressed by KF and K-3-rh and the inhibitory effects on the phosphorylation of the Akt and MAPK pathways by K-3-rh were considerably higher compared to that of KF (Fig. 4).

3.5. Effects of KF and K-3-rh on OVA-induced cytokine levels in the BALF and lung tissue

The protein levels of IL-4, IL-5, IL-13, and TNF- α were significantly increased in the BALF and lung tissue obtained from an OVA-

sensitized/challenged mouse asthma model (Figs. 5 and 6). The levels of Th2 cytokines, such as IL-4, IL-5, and IL-13, in the BALF and lung tissue were significantly decreased in the mice treated with KF or K-3-rh compared with those observed in the OVA-sensitized/challenged mice (asthma group). In addition, the TNF- α levels in the BALF and lung tissue were markedly decreased in the mice group treated with KF or K-3-rh compared with the OVA-sensitized/challenged asthma group. The protein levels of IL-4, IL-13 and TNF- α in the BALF (Fig. 5) and the levels of IL-4, IL-5 and TNF- α in the lung tissue (Fig. 6C–F) induced by the administration of KF were not significantly different compared with those observed in the group administered K-3-rh. In contrast, the levels of IL-5 in the BALF and IL-13 in the lung tissue of KF-treated allergic asthma mice were higher than those observed in the K-3-rh-treated allergic asthma mice.

The effects of KF and K-3-rh on the production of the IL-13 and TNF- α mRNA levels were also assessed in the lung tissue of OVA-sensitized

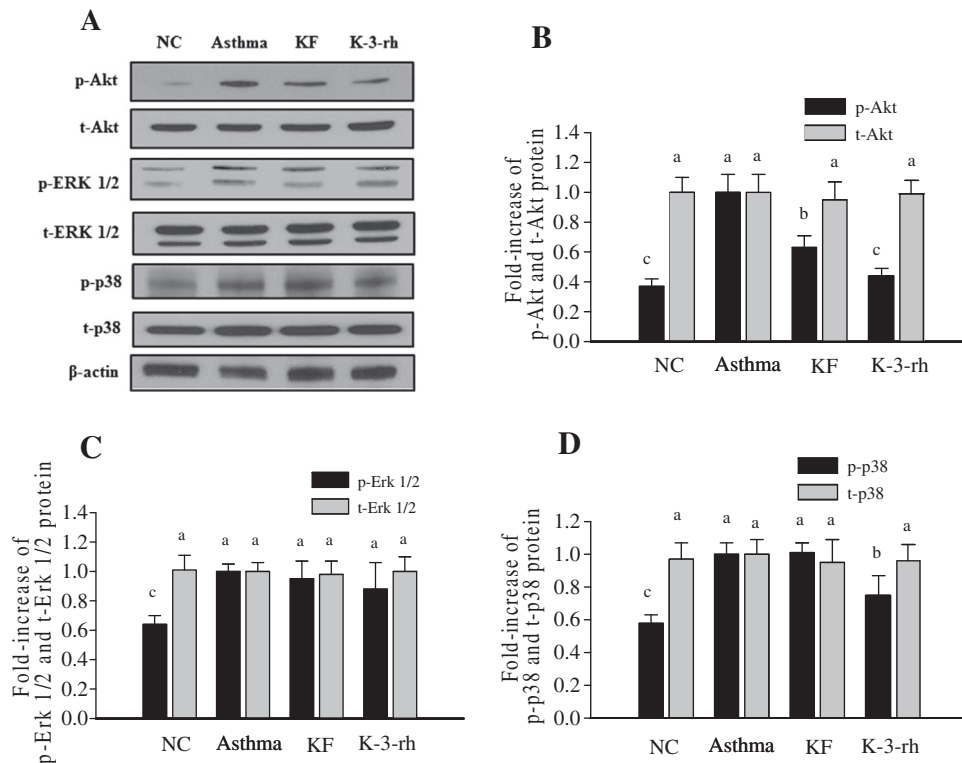


Fig. 4. Effects of KF and K-3-rh on phosphorylation of Akt and MAPKs (Erk1/2 and p38) in the lung tissue of mice. NC, negative control (PBS only); Asthma, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. The p-Akt, t-Akt, p-Erk1/2, t-Erk1/2, p-p38 and t-p38 levels in each sample were normalized to the β -actin levels. The density of each protein band was quantified using SigmaGel software (Jandel Scientific, San Rafael, CA). The values are expressed as the mean $\pm$ SD ($n = 8-10$), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test.

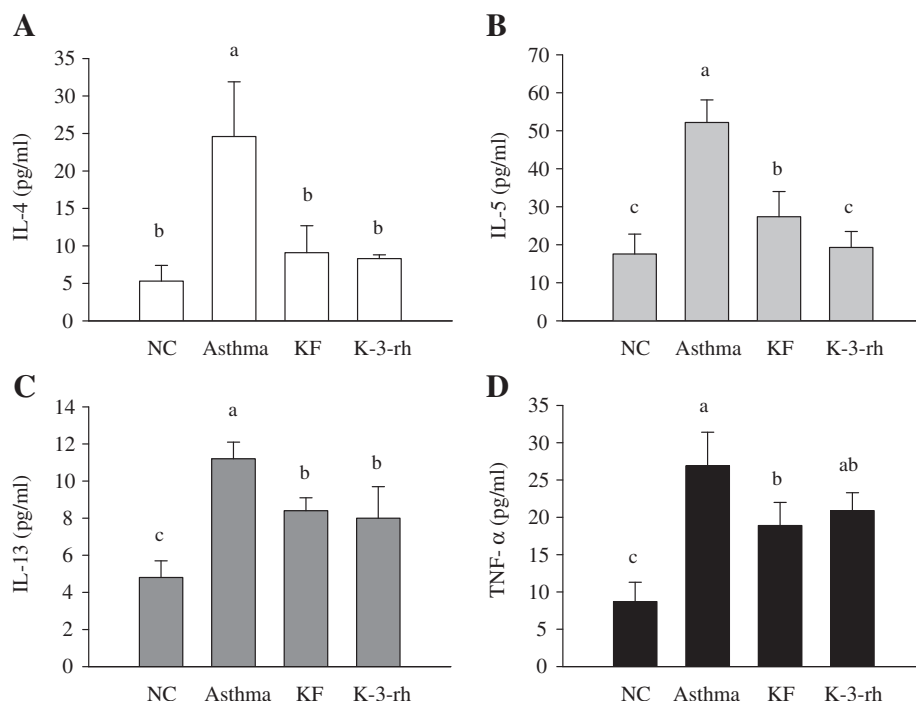


Fig. 5. Effects of KF and K-3-rh on cytokine levels in the bronchoalveolar lavage fluid (BALF) of mice. NC, negative control (PBS only); Asthma, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice; and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. The values are expressed as the mean $\pm$ SD ($n = 8-10$), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test.

asthma mice. The mRNA levels of IL-13 and TNF- α in OVA-sensitized asthma mice were significantly higher than those in the PBS-challenged control mice (Fig. 6A, B). Treatment of these mice with KF and K-3-rh, respectively resulted in a marked reduction in the levels of both cytokines in the lung tissues in a manner similar to that shown on the production of inflammatory cytokine protein in the lung and BALF fluid.

3.6. Effects of KF and K-3-rh on the total IgE levels in the BALF and serum

The total IgE levels in the BALF and serum were measured by ELISA. The development of OVA-induced allergic airway disease (asthma group) resulted in an increase in the total serum IgE and BALF IgE levels compared with those found in the negative control (NC group, Fig. 7A, B). There was a significant decrease in the groups treated with KF and K-3-rh, respectively, compared with the OVA-induced asthma group. The consumption of K-3-rh further decreased the total serum IgE levels more than the consumption of KF (Fig. 7B). No differences in the BALF total IgE levels were observed after the oral administration of KF and K-3-rh.

3.7. Effects of KF and K-3-rh on the serum ALT and AST levels in mice with OVA-induced asthma

To confirm the absence of liver toxicity induced by KF and K-3-rh, the effects of KF and K-3-rh on the serum ALT and AST levels in the OVA-induced asthma mice were determined. The KF- or K-3-rh-treated asthma group (KF and K-3-rh groups) did not exhibit altered serum ALT and AST levels (Fig. 7C), demonstrating that KF and K-3-rh exhibited no detectable levels of liver toxicity.

4. Discussion

Allergic asthma is a common chronic inflammatory disease of the airways that is characterized by airway eosinophilia and elevated IgE and inflammatory cytokine levels [1,4]. Gong et al. [4] reported that KF

has anti-inflammatory effects in an OVA-induced asthma mouse model. However, the effect of the glycosylated derivative of KF, kaempferol-3-O-rhamnoside (K-3-rh), on airway inflammation induced by allergic asthma has not been studied. This study provided the first demonstration of these properties using an OVA-induced airway inflammation mouse model of asthma, and we showed that both KF and K-3-rh significantly reduced the characteristics of airway inflammation, including the infiltration of inflammatory cells and the production of inflammatory cytokines and IgE. In addition, KF and K-3-rh decreased ROS generation in the OVA-induced airway inflammation reaction.

Oxidative stress stimulates inflammatory responses that can lead to allergic asthma [20]. Asthmatic patients are exposed to additional endogenous oxidative stress [21]. Antioxidative activities, such as DPPH radical scavenging activity, are one mechanism that explains the anti-inflammatory actions of phytoconstituents, such as flavonoids [22].

Consistent with these findings, our present results show that ROS production is increased in bronchial inflammatory cells in the OVA-induced asthma mouse model of allergic airway inflammation and that the administration of KF and K-3-rh significantly attenuates ROS generation. In addition, the results of this study showed that KF and K-3-rh possess dose-dependent DPPH radical scavenging activities, with KF showing higher antioxidant efficiency than K-3-rh. The DPPH radical activity of KF and K-3-rh was positively correlated with the inhibitory effects of intracellular ROS formation ($r = 0.9971$; $p = 0.0484$). K-3-rh exhibited a lower capacity to inhibit intercellular ROS and DPPH radical generation than KF. This finding indicated that glycosylation of the hydroxyl groups diminished the antiradical capacity of the flavonoid, perhaps because this reduces the number of free hydroxyl groups or destroys the ortho-hydroxyl structure and because the linkage of sugars may hinder access to the free radical scavenger of the radical center [23].

The inflammatory response involves the recruitment and activation of inflammatory cells. An important cell type in the inflammation of allergic asthma is eosinophils in the lung, and a rise in the number of eosinophils in the BALF and eosinophilic infiltrates in the lung are characteristic of asthma [4]. In this study, we demonstrated that the

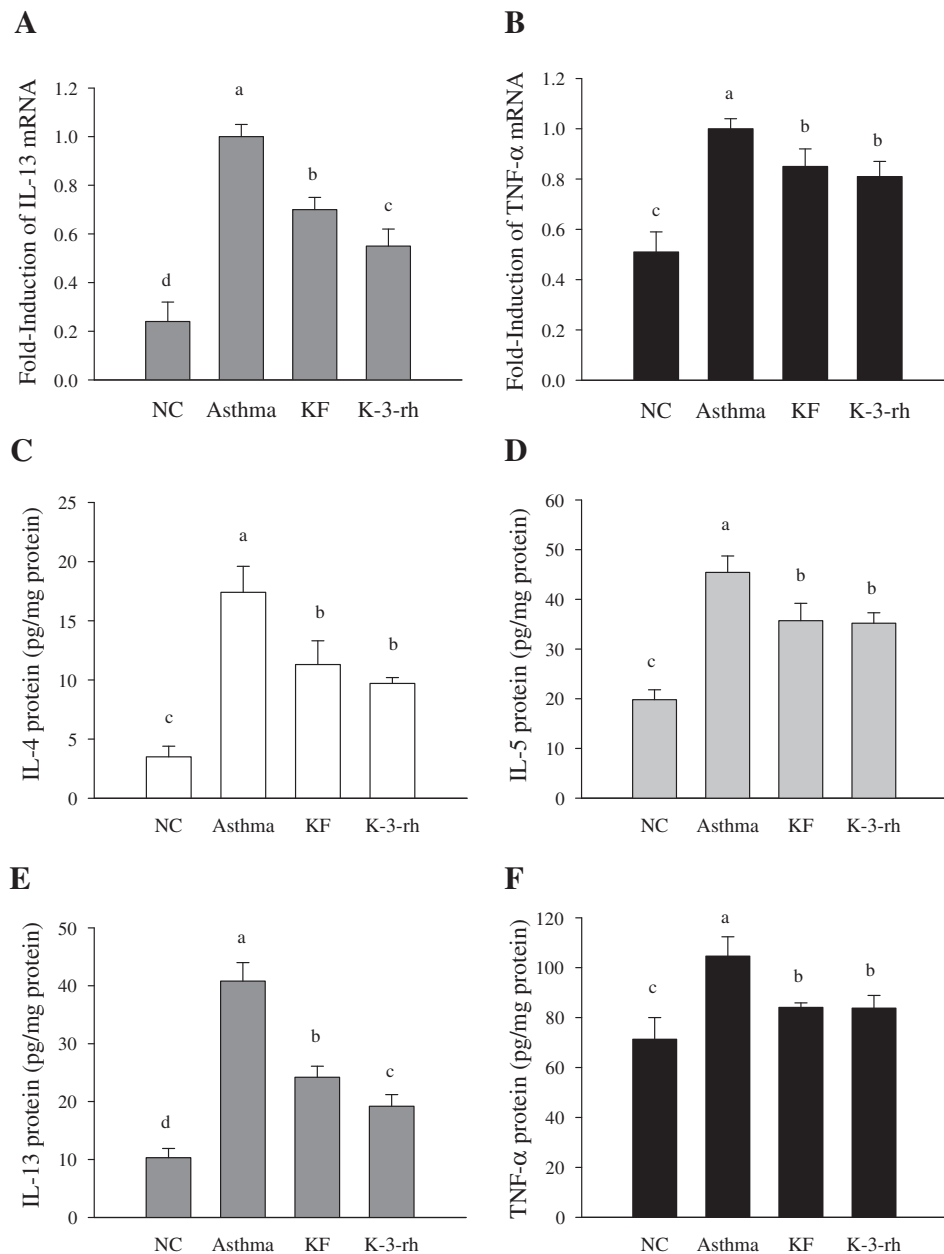


Fig. 6. Effects of KF and K-3-rh on cytokine levels in the lung tissue of mice. NC, negative control (PBS only); Asthma, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice; and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. The levels of A) IL-13 and B) TNF-α mRNA in each sample were normalized to the β-actin levels. The density of each mRNA was quantified by using SigmaGel software (Jandel Scientific, San Rafael, CA). The production of C) IL-4, D) IL-5, E) IL-13, and F) TNF-α protein was determined in lung tissue using the commercial ELISA kit. The values are expressed as the mean ± SD (n = 8–10), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test.

administration of KF and K-3-rh decreases eosinophilic infiltration in the lung and the eosinophil numbers in the BALF, respectively, and the results suggest that KF and K-3-rh have an anti-inflammatory allergic asthma effect. The effects of K-3-rh on the decrease in the eosinophil infiltrates in the lung and the eosinophil numbers in the BALF were similar to those of KF.

The inhibition of Akt and p38 phosphorylation is one of the important signaling pathways in the immune response because it plays an important role in regulating the transcription activity of pro-inflammatory cytokine genes such as IL-4, IL-13 and TNF-α [7]. In this study, the phosphorylation of Akt and p38 was inhibited by K-3-rh and Akt phosphorylation was inhibited by KF. K-3-rh inhibited Akt and p38 phosphorylation more potently than did the KF.

One pathway of allergic reaction in the airways is an increase in the IL-5-dependent eosinophil recruitment to the lung by eosinophil

activation [24], and the other pathway involves the induction of IgE by B cells via IL-4 released from Th2 cells and mast cells. Thus, allergic asthma is recognized as a Th2-mediated immune system disease. Asthma and inflammation are associated with enhanced production of Th2 cytokines (IL-4, IL-5 and IL-13), and IL-4, IL-5, IL-13 and TNF-α are abnormally expressed in a mouse model of allergic asthma [1]. In this study, we found that the levels of IL-4, IL-5, IL-13 and TNF-α in the BALF and lung were significantly elevated by airway challenge with OVA, but the levels of IL-4, IL-5, IL-13 and TNF-α were significantly inhibited after KF and K-3-rh administration compared with those found in the OVA-sensitized/challenged positive control group (asthma group). K-3-rh was shown to be more effective for the inhibition of IL-5 production in the BALF and for IL-13 production in the lung tissue than KF in a mouse model of allergic asthma. The results suggest that K-3-rh plays a key role in blocking the

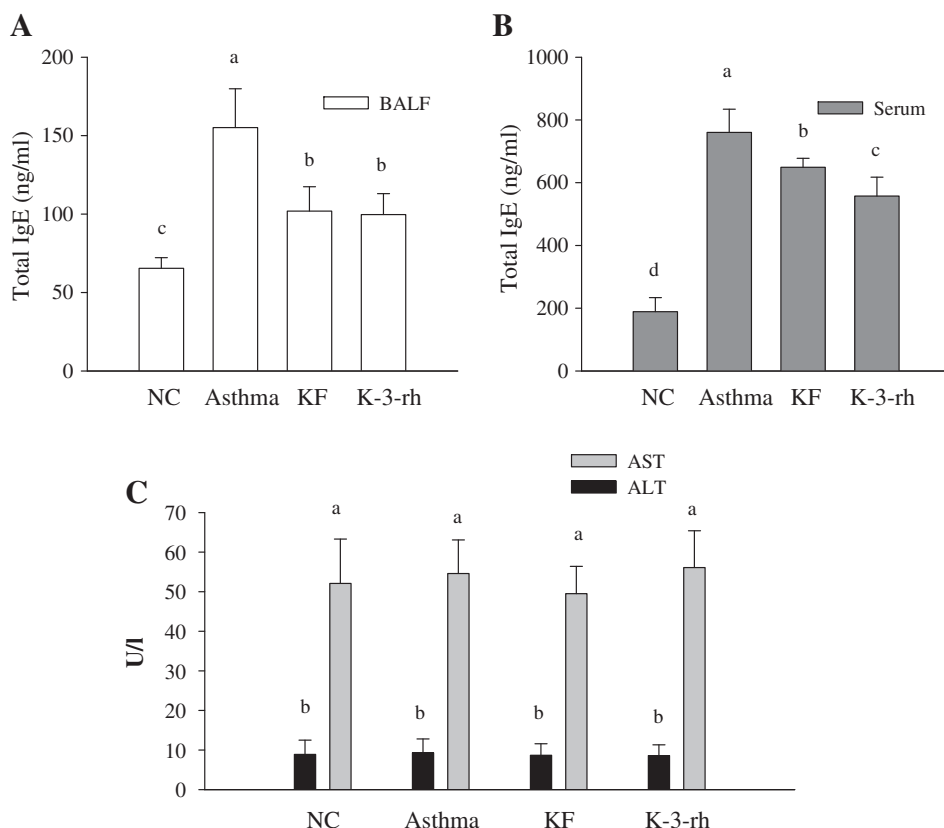


Fig. 7. Effects of KF and K-3-rh on the total IgE, ALT and AST levels in the bronchoalveolar lavage fluid (BALF) and/or serum of mice. NC, negative control (PBS only); OVA, OVA-sensitized/challenged mice; KF, KF + OVA-sensitized/challenged mice; and K-3-rh, K-3-rh + OVA-sensitized/challenged mice. KF or K-3-rh treatment was performed 1 h before challenge on days 21 to 24. The values are expressed as the mean $\pm$ SD ($n = 8-10$), and the means with different letters are significantly different from each other ($p < 0.05$), as determined by Duncan's multiple range test.

recruitment of eosinophils to the lung due to inhibition of IL-4-, IL-5- and IL-13-dependent pathways.

IL-4 can induce IgE production, and IgE promotes the inflammation progress by binding to and activating the Fc ϵ R in macrophages and mast cells [25]. IgE is a key target for the development of anti-allergic asthma functional foods and drugs, including natural compounds with anti-allergic and anti-asthmatic effects, which are mainly attributed to a reduction of free IgE secondary to the formation of IgE-anti-IgE immune complexes, thereby preventing the progression of allergic and asthmatic reactions [26]. In our results, the total IgE levels in the serum and BALF were significantly elevated by airway challenge with OVA, but the levels of total IgE in the BALF and serum were significantly inhibited after the administration of KF or K-3-rh compared with those observed in the OVA-induced asthma group. K-3-rh was shown to have a higher inhibitory effect on serum IgE production than KF in a mouse model of allergic asthma. ALT and AST levels, known indicators of hepatocellular damage, were not significantly altered by the oral administration of KF or K-3-rh and the results showed that the dose of KF and K-3-rh used in the present study had no cytotoxic effects. The KF showed lower cytotoxicity than other flavonoids such as fisetin, resveratrol, biochanin A, morin and phloretin in rat basophilic leukemia RBL-2H3 cells and it did not show cytotoxicity up to 200 μ M (data not shown). In addition, K-3-rh (cell viability: 100.2%) was less cytotoxic than kaempferol (cell viability: 85.0%) at 400 μ M (data not shown). Further studies would be needed to further evaluate safety on toxicity of KF and K-3-rh in animal model system.

KF exhibited inhibitory effects on OVA-induced lung inflammation by inhibition of the production of inflammatory-related cytokines, including Th2 cytokines, through the inhibition of intracellular ROS production and other pathways. In contrast, K-3-rh showed only a slight effect on the inhibition of intracellular ROS production and exerted a

higher and similar inhibitory effect on the production of Th2 cytokines (IL-4, IL-5 and IL-13) and TNF- α , Akt and p38 phosphorylation, and IgE production. Thus, the anti-inflammatory and anti-asthmatic effects of K-3-rh may be achieved by multiple mechanisms, including a reduction of the levels of Th2 cytokines (IL-4, IL-5 and IL-13), TNF- α through the inhibition of Akt and p38 phosphorylation, and IgE. The glycosylated derivative of KF, K-3-rh, may be effective as a versatile biomaterial with increased water solubility and stability and reduced toxicity and bioactivity and could be used to develop functional foods and/or therapeutic agents that prevent or/and treat allergic asthma.

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