Pistacia weinmannifolia root exerts a protective role in ovalbumin-induced lung inflammation in a mouse allergic asthma model

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Abstract. *Pistacia weinmannifolia* (Anacardiaceae) has been used in herbal medicine for the treatment of influenza, dysentery and enteritis in China. It was recently observed that *P. weinmannifolia* root extract (PWRE) exerts anti-inflammatory effects both in *in vitro* and *in vivo* models. Based on the results from previous studies, the present study investigated the protective effect of PWRE on airway inflammation and mucus hypersecretion. Treatment with PWRE significantly decreased the number of eosinophils and the levels of Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13, in the bronchoalveolar lavage fluid (BALF) of OVA-exposed mice. PWRE decreased the high serum levels of total and OVA-specific immunoglobulin E. PWRE also effectively inhibited the influx of inflammatory cells into the lung, as well as airway mucus hypersecretion. In addition, the increased level of monocyte chemoattractant protein-1 was significantly decreased with the PWRE treatment in the BALF of OVA-exposed mice and in lipopolysaccharide-stimulated RAW264.7 macrophages. These protective effects of PWRE on OVA-induced pulmonary inflammation were accompanied by the downregulation of mitogen associated protein kinases and nuclear factor-κB activation. Thus, the results from the present study indicate that PWRE could be valuable adjuvant for the treatment of asthma.

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Abbreviations: OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; IL-4, interleukin-4; IL-5, interleukin-5, IL-13, interleukin-13; IgE, immunoglobulin E; MCP-1, monocyte chemoattractant protein-1; MAPKs, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; p38, ERK, extracellular-signal-regulated kinase; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB

Key words: Pistacia weinmannifolia root, allergic asthma, airway inflammation, mucus hypersecretion, NF-κB

Introduction

Allergic asthma is a chronic inflammatory disease and a major health issue, and its prevalence is increasing worldwide (1). The major features of asthma pathophysiology include airway inflammation and mucus hypersecretion (2,3). It is well known that the increased levels of eosinophil recruitment and T helper lymphocytes 2 (Th2) cytokines, such as interleukin-4 (IL-4), IL-5 and IL-13, are closely associated with sustained airway inflammation (4). Macrophages-derived chemokines such as monocyte chemoattractant protein-1 (MCP-1) increased the recruitment of inflammatory cells including eosinophils in asthma pathogenesis (5,6) The increased concentration of immunoglobulin E (IgE) has a pivotal role in allergic reactions and is much higher in asthmatic patients (7). Changes in the number of goblet cells and production of mucus are key to airway inflammation and obstruction (8). The mitogen-activated protein kinase (MAPK) signaling pathways have an important role in the inflammatory processes of allergic asthma (9). The activation of c-Jun N-terminal kinase (JNK) has been implicated in IgE class switching (10). Extracellular signal-regulated kinase (ERK) and p38 have been reported to play a role in the production of cytokines, including IL-5 (11). Nuclear factor (NF)-κB plays an important role in inflammatory cell influx, Th2 cytokine levels and inflammatory molecules in allergic asthma (12,13).

In recent years, the approaches to improve the side effects of medicine have focused on research into allergic asthma (14) and natural herbal extracts are attracting increased attention due to their prominent biological activities and minimal side

effects (15). *Pistacia weinmannifolia* (PW) is used as a herbal medicine in China (16,17) and its major metabolites possess biological activities, such as inhibitory activities against histamine release (16,18,19). In a previous study, it was confirmed that the anti-inflammatory activities of *P. weinmannifolia* root extract (PWRE) in PMA/tumour necrosis factor-α-stimulated airway epithelial cells and in pulmonary inflammatory response induced by cigarette smoke and lipopolysaccharide (LPS) (20). Based on these results and those of other studies (16-20), which reflect the anti-inflammatory activities of PWRE on pulmonary inflammation, it was hypothesized that PWRE could exert a protective effect against ovalbumin (OVA)-induced lung inflammation. Therefore, the aim of the present study was to evaluate the regulatory effects of PWRE against eosinophil recruitment and Th2 cytokines, IgE and mucus overproduction, which are the major characteristics of allergic asthma.

Materials and methods

Preparation of PWRE. PWRE was prepared as previously described (20). *P. weinmannifolia* roots (PWRs) were collected from the Yunnan province of China. A voucher specimen recorded as D180305001 was deposited at the International Biological Material Research Center, Korea Research Institute of Bioscience and Biotechnology. The active substance of PWR was extracted by the processing method described in the International Conference on Harmonisation and Ministry of Food and Drug Safety guidelines (20). The collected roots were dried immediately following sampling and then ground to a powder. The raw materials were then packed in laminated bags and delivered to Korea. The PWREs were provided by the BTC Corporation. The powdered samples were extracted with 50% ethanol at 80˚C and the product was dried in a freeze dryer (-70˚C) to produce dried extracts (~19%) [Korea Good Manufacturing Practice (KGMP), lot no. BTC-PWE-180118].

Induction of ovalbumin (OVA) and alum-induced lung inflammation in murine models. Healthy female BALB/c mice (n=30, 6 weeks old; body weight, 16-18 g) were purchased from Koatech Co., Ltd., and used after 1 week of acclimatization with free access to food and water in specific pathogen-free conditions (22-23˚C; 55-60% humidity; 12-h light/dark cycle). The experimental procedure was performed according to the methods described by Park *et al* (21). Briefly, the mice were sensitized twice intraperitoneally on day 0 and 14 with 30 μg OVA and 3 mg Alums (Thermo Fisher Scientific, Inc.) dissolved in a solution of 0.2 ml PBS. On days 21-23, the mice were aerosol challenged with 1% OVA (alum-free saline solution, 60 min/day) with a nebulizer (NE-U12; OMRON Corp.). The PWRE or montelukast (MON) was given by oral gavage for 6 consecutive days (from day 18 to 23). The mice were sacrificed on day 25. The mice were randomly divided into 4 groups (n=6 per subgroup) as follows: i) The normal control (NC) group; ii) the OVA group (intraperitoneally sensitized with OVA-Alum); iii) the MON group (intraperitoneally sensitized with OVA-Alum) + MON (30 mg/kg, per os); and iv) the PW group (intraperitoneally sensitized with OVA-Alum) + PWRE (7.5 or 15.0 mg/kg, per os). MON was used as a positive control. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and performed in compliance with the National Institutes of Health Guidelines for the care and use of laboratory animals and Korean national laws for animal welfare. The humane endpoints are the condition of rapid loss of weight (>20% of normal body weight) and/or rapid or labored breathing.

Counting the inflammatory cells. BALF collection was performed in order to count the inflammatory cells and evaluate the levels of inflammatory cytokines as previously described (22). The mice were anesthetized with Zoletil 50® (30-50 mg/kg IP; Virbac) and Xylazine (5-10 mg/kg IP; Bayer Korea) on day 25 based on prior anesthesia condition (20). Briefly, on day 25, the trachea was cannulated and infused with 0.7 ml PBS for the collection of BALF (infusion was performed twice with a total volume of 1.4 ml) and blood was collected for the detection of IgE. Mice were sacrificed under Zoletil/Xylazine anaesthesia and exsanguinated. In order to distinguish the different cells, 0.1 ml of BALF was centrifuged at 246 x g for 5 min at room temperature to transfer the cells to the glass slide and then the glass slide was stained with Diff-Quik® solution (IMEB, Inc.) according to the manufacturer's protocol.

Measuring the Th2 cytokines and IgE production. The levels of IL-4, IL-5 and IL-13 in the BALF were determined using ELISA kits (R&D Systems, Inc.; IL-4, cat. no. M4000B; IL-5, cat. no. M5000; IL-13, cat. no. M1300CB) according to the manufacturer's protocol. Blood (0.4 ml) was collected in order to determine the serum IgE levels. The concentration of the total or OVA-specific IgE in the serum was determined using an ELISA (Biolegend, Inc.; Total IgE, cat. no. 432404; R&D Systems, Inc., OVA-specific IgE, cat. no. 439807). The absorbance was measured at 450 nm with a Spark™ 10 M multimode microplate reader (Tecan System Inc.).

Western blot analysis. Lung tissues were removed 48 h after the final OVA inhalation and incubated in CelLytic™ MT Cell Lysis reagent (cat. no. c3228; Sigma-Aldrich; Merck KGaA) containing protease and phosphatase inhibitors (cat. nos. 11836153001 and 04906837001; Roche Diagnostics) in order to obtain the proteins. The protein concentration was measured with the Pierce bicinchoninic acid Protein assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The proteins (50 μg/lane) were separated via SDS-PAGE (10-12% gels) and then transferred to PVDF membranes (EMD Millipore). The membranes were blocked in 5% skimmed milk dissolved in TBS and 0.05% Tween-20 (TBST) for 1 h at room temperature and probed overnight with primary antibodies at 4˚C. The primary antibodies used were as follows: Anti-phosphorylated (p)-extracellular signal-regulated kinase (ERK; 1:1,000; cat. no. 9101; 1:1,000; Cell Signaling Technology, Inc.), anti-p-p38 (cat. no. 9211; 1:1,000; Cell Signaling Technology, Inc.), anti-p-NF-κB p65 (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), anti-p-inhibitor of NF-κB (p-IκBα; cat. no. 2859; 1:1,000; Cell Signaling Technology, Inc.), anti-β-actin (1:2,500; cat. no. 4967; 1:1,000; Cell Signaling Technology, Inc.), anti-ERK (cat. no. sc-154; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-p-c-Jun N-terminal kinase (JNK; cat. no. sc-6254; 1:1,000; Santa Cruz

Biotechnology, Inc.), anti-JNK (cat. no. sc-474; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-p38 (cat. no. sc-7149; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-MCP-1 (cat. no. sc-28879; 1:1,000; Santa Cruz Biotechnology, Inc.), anti-NF-κB p65 (cat. no. sc-372; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-IκBα (cat. no. MA5-15132; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were washed five times with TBST for 10 min and developed with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse & anti-rabbit; 1:2,000; cat. nos. 115-035-003 and 111-035-003; Jackson ImmunoResearch Laboratories, Inc.) at room temperature (RT) for 1 h. The membranes were developed with an ECL kit (Thermo Fisher Scientific, Inc.). All bands were visualized using a LAS-4000 luminescent image analyzer (Fujifilm) and quantified by densitometry using Fuji Multi Gauge software version 3.0 (Fujifilm).

Histological analysis of lung tissue. A total of 24 h after the final administration of PWRE and MON, the mice were sacrificed, and the lung tissues were collected. For histological evaluation, the lung tissues were fixed in 10% (v/v) neutral-buffered formalin solution at room temperature for 48 h and embedded in paraffin. The lung tissues were then sliced into 4-μm thick sections with a rotary microtome and stained with hematoxylin (BBC Biochemical Inc.) and eosin (Thermo Fisher Scientific Inc.; H&E) solutions at RT for 30 sec each to estimate the inflammatory response. The lung sections then were visualized using a light microscope (magnification, x100; scale bar, 50 μm) to estimate the recruitment of inflammatory cells. Periodic acid-Schiff (PAS) staining (IMEB, Inc., cat. no. K7308) was performed to estimate the mucus secretion. The degree of inflammatory score and mucus production in each group was assessed by two independent observers in the laboratory using a semi-quantitative scope. The H&E staining was scored as follows: 0, no recruitment of inflammatory cells; 1, small amount of recruitment; 2, moderate recruitment; 3, large amount of recruitment. The PAS staining was scored as follows: 0, no mucus production; 1, mild mucus production; 2, moderate mucus production; 3, distinct mucus production; 4, severe mucus production.

Cell culture. The macrophage cell line RAW264.7 was obtained from the American Type Culture Collection. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin and were incubated at 37˚C in a humidified chamber with 5% CO_2. The cells were activated with lipopolysaccharide (LPS; 0.5 μg/ml) 1 h after PWRE treatment (1.25, 2.5 and 5 μg/ml). The dose of LPS was based on a previous study (23). The level of MCP-1 in the culture supernatant was determined by ELISA.

Statistical analysis. All values are expressed as the mean ± standard deviation of at least three independent experiments. The statistical significance was determined by a two-tailed Student's t-test for comparisons between two groups. One-way analysis of variance followed by Dunnett's multiple groups. Data were analyzed using SPSS 20.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant result.

Results

Effect of PWRE on alleviating the eosinophil numbers in the BALF. The significant increase in eosinophils and macrophages has been well established in OVA-induced pulmonary inflammatory response (24,25). Therefore, the present study focused on the inhibitory effect of PWRE on the cell numbers. To distinguish the inflammatory cells and count the cell numbers, Diff-Quik® staining was performed according to the manufacturer's protocol. As presented in Fig. 1, the numbers of eosinophil and macrophages were significantly increased in the OVA-exposed group compared with the NC group ($P<0.05$). Conversely, this increase in inflammatory cell numbers was significantly decreased in the PWRE-treated group ($P<0.05$; Fig. 1A and B).

Effect of PWRE on attenuating Th2 cytokines in the BALF. The present study next investigated the regulatory effect of PWRE on the production of Th2 cytokines that are deeply associated with the pathophysiology of asthma. ELISAs were performed in order to evaluate the levels of Th2 cytokines. It was revealed that IL-4, IL-5 and IL-13 were significantly increased in the OVA group when compared with the NC group ($P<0.05$; Fig. 2A-C). However, treatment with PWRE decreased the levels of these cytokines induced by OVA. In particular, the inhibitory effects of 15 mg/kg PWRE on the production of cytokines were similar to those of 30 mg/kg MON, which was used as a positive control.

Effect of PWRE on downregulating IgE production. The serum total IgE level is highly elevated in allergic patients such as bronchial asthma and is known to increase with the onset and aggravation of the disease (26,27). A specific IgE test is needed together with total IgE for proper evaluation of allergic diseases (28). Based on the importance of the IgE-mediated immune response in asthma (29), the present study investigated the inhibitory activity of PWRE on OVA-induced IgE production. As presented in Fig. 3, the concentration of total IgE or OVA-specific IgE in the serum were significantly increased in the asthmatic group compared with those in the NC group ($P<0.05$), whereas treatment with PWRE effectively decreased the levels of total IgE and OVA-specific IgE (Fig. 3).

Effect of PWRE on inhibiting inflammatory cell influx and mucus hypersecretion. In order to investigate whether PWRE suppresses the OVA-induced inflammatory cell influx into the lungs, paraffin lung sections were stained with H&E in the present study. A significantly increased level of inflammatory cell influx was observed in the OVA group compared with the NC group ($P<0.05$; Fig. 4A). Notably, this level was downregulated in the PWRE-treated group. The arrows point to the influx of inflammatory cells. The increased secretion of MCP-1 is closely associated with airway inflammation by inducing the influx of inflammatory cells (5,30). Therefore, the present study next assessed the inhibitory effect of PWRE on OVA-induced MCP-1 secretion. As presented in Fig. 4B, the marked increase in MCP-1 was observed in the BALF of the OVA group, whereas treatment with PWRE inhibited this secretion. In order to further investigate the regulatory effect of PWRE on MCP-1 secretion, the inhibitory effect of PWRE on MCP-1 was assessed in LPS-stimulated RAW264.7 macrophages. As

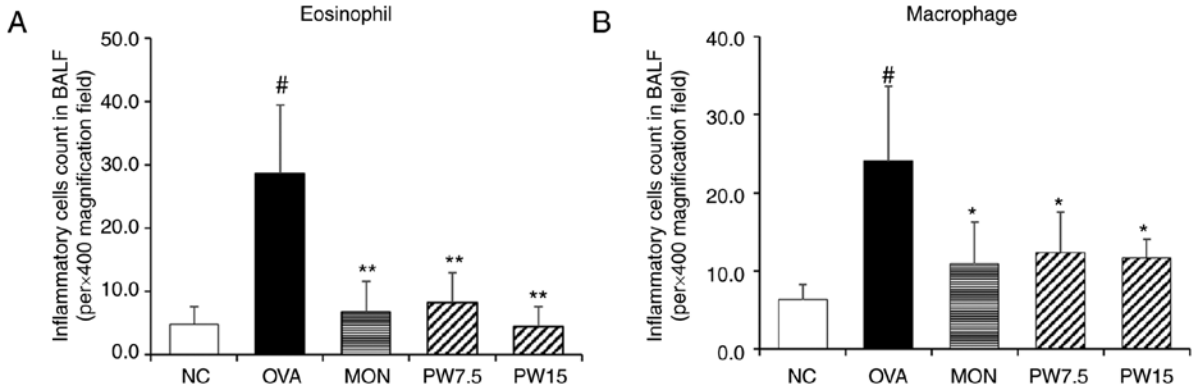

Figure 1. Effect of PWRE on the numbers of inflammatory cells in the BALF of OVA-challenged mice. The count of (A) eosinophils and (B) macrophages in the BALF was determined by Diff-Quik® staining. Data are expressed as the mean ± standard deviation (n=6). #P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. OVA-induced group. NC, normal control mice; OVA group, mice administered ovalbumin; MON group, mice administered MON (30 mg/kg) + OVA; PW 7.5, mice administered *P. weinmannifolia* root extract (7.5 mg/kg) + OVA. OVA, ovalbumin; MON, montelukast; PWRE, *P. weinmannifolia* root extract; PW 7.5, 7.5 mg/kg PW + OVA, PW15, 15 mg/kg PW + OVA; BALF, bronchoalveolar lavage fluid; NC, negative control.

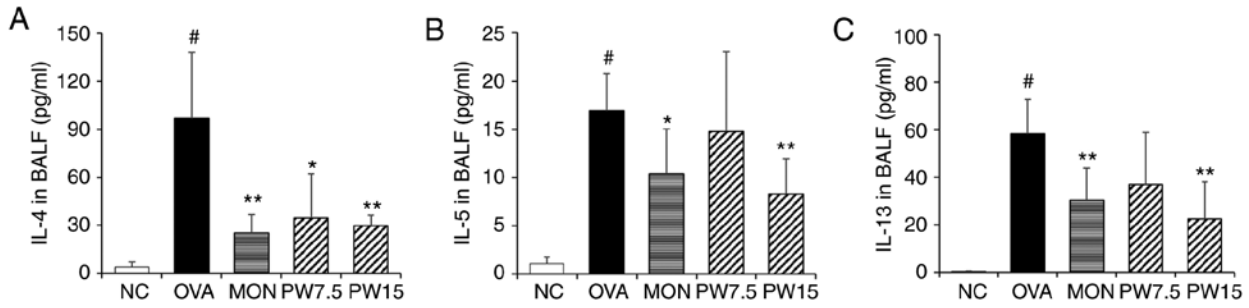

Figure 2. Effect of PWRE on the production of Th2 cytokines in the BALF. The levels of Th2 cytokines, such as (A) IL-4, (B) IL-5 and (C) IL-13, were determined by ELISA kits. The absorbance was measured at 450 nm with a microplate reader. #P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. OVA-induced group. PWRE, *P. weinmannifolia* root extract; IL, interleukin; OVA, ovalbumin; MON, montelukast; BALF, bronchoalveolar lavage fluid; NC, negative control; Th2, T-helper 2.

presented in Fig. 4C, the administration of LPS significantly increased the MCP-1 secretion (P<0.05). However, pretreatment with PWRE significantly downregulated this secretion (P<0.05; Fig. 4C). Mucus hypersecretion is an prominent characteristic in the pathophysiology of allergic asthma (31). Therefore, the present study assessed whether PWRE led to an attenuation of the OVA-induced mucus overproduction. The paraffin lung sections were stained with the PAS staining reagent to measure the mucus production around the airways. As presented in Fig. 5, the levels of mucus production were significantly increased in the OVA group when compared with the NC group (P<0.05). However, a decrease in this level was observed in the PWRE group (Fig. 5). The mucus was stained a purple color by the PAS staining reagent.

Effect of PWRE on decreasing MAPKs and NF-κB activation in the lungs. In order to investigate whether the airway inflammatory response was mediated by MAPK-responsive mechanisms, the present study evaluated the levels of ERK, JNK and p38 phosphorylation. As presented in Fig. 6, the levels of JNK, p38 and ERK were significantly upregulated in the OVA group compared with the NC group (P<0.05). However, 15 mg/kg PWRE significantly downregulated the enhanced activation of JNK, p38 and ERK in the lungs (P<0.05; Fig. 6). In order to further investigate the mechanism of PWRE, the NF-κB signaling pathway was assessed in the present study. As presented in Fig. 7, the activation of NF-κB p65 and IκBα was significantly upregulated in the OVA-exposed group compared with the NC group. However, this increase was effectively blocked by the PWRE treatment.

Effect of PWRE on LPS-stimulated MAPKs and NF-κB activation in RAW264.7 macrophages. In the present study, PWRE exerted a protective effect in pulmonary inflammation in OVA-exposed mice. Its effects were accompanied by MAPK and NF-κB inactivation (Figs. 6 and 7). In particular, NF-κB activation was effectively downregulated upon PWRE administration. The results from the present study also demonstrated that PWRE regulates MCP-1 production in the BALF of OVA-exposure mice and in LPS-stimulated RAW264.7 macrophages (Fig. 4B and C). The regulatory effect of PWRE on LPS-stimulated MAPKs and NF-κB activation was therefore investigated in RAW264.7 macrophages. The administration of LPS significantly upregulated the activation of MAPKs and NF-κB (P<0.05; Figs. 8 and 9). However, the levels of JNK, p38 and ERK activation was not significantly downregulated by PWRE pretreatment (Fig. 8). Similar to those results presented in Fig. 7, the activation of NF-κB p65 and IκBα was significantly downregulated by ≥2.5 μg/ml PWRE pretreatment (P<0.05; Fig. 9).

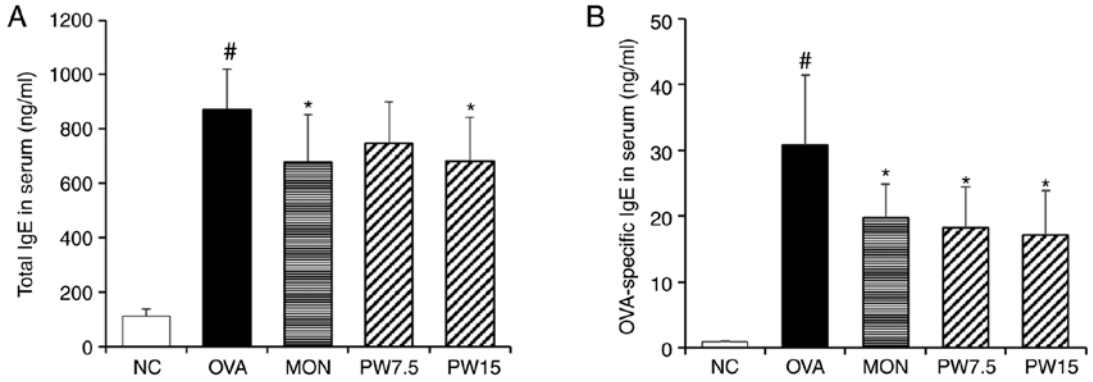

Figure 3. Effect of PWRE on the production of IgE in the serum. The levels of (A) the total or (B) OVA-specific IgE in serum were evaluated by ELISA kits. The absorbance was measured at 450 nm with a microplate reader. #P<0.05 vs. NC group; *P<0.05 vs. OVA group. PWRE, *P. weinmannifolia* root extract; IgE, immunoglobulin E; OVA, ovalbumin; MON, montelukast; NC, negative control.

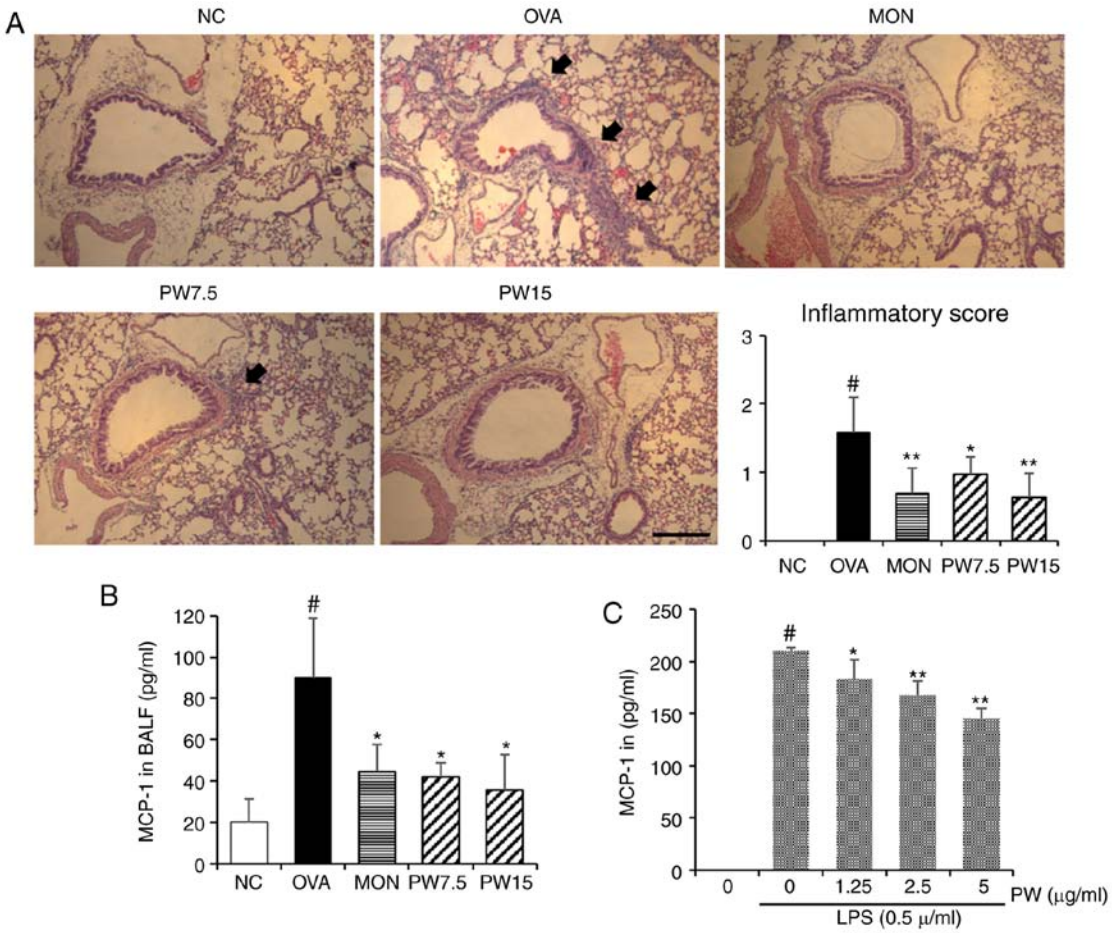

Figure 4. Effect of PWRE on the influx of inflammatory cells into the lungs and on the downregulation of MCP-1 secretion in LPS-stimulated RAW264.7 macrophages. (A) Hematoxylin and eosin staining was used to determine the level of inflammatory cell influx (peribronchial lesion; magnification, x100; scale bar, 50 μm) and the degree of the inflammation score was assessed by two independent observers. An ELISA was used to determine the MCP-1 secretion level in the (B) BALF samples of allergic asthma and in the (C) LPS-stimulated RAW264.7 macrophages. #P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. OVA group. PWRE, *P. weinmannifolia* root extract; MCP-1, monocyte chemoattractant protein-1; OVA, ovalbumin; MON, montelukast; BALF, bronchoalveolar lavage fluid; NC, negative control; LPS, lipopolysaccharide.

Discussion

Previously, studies have demonstrated that PWRE exerts anti-inflammatory effects via downregulation of inflammatory molecules including IL-6 and IL-8, which are important parameters in chronic obstructive pulmonary disease (16,18,20). The present study extended the results of these previous publications, which demonstrate the protective effects of PWRE in OVA-induced pulmonary inflammation.

The airway inflammatory response is well known as a major cause of allergic asthma and is caused by a variety of

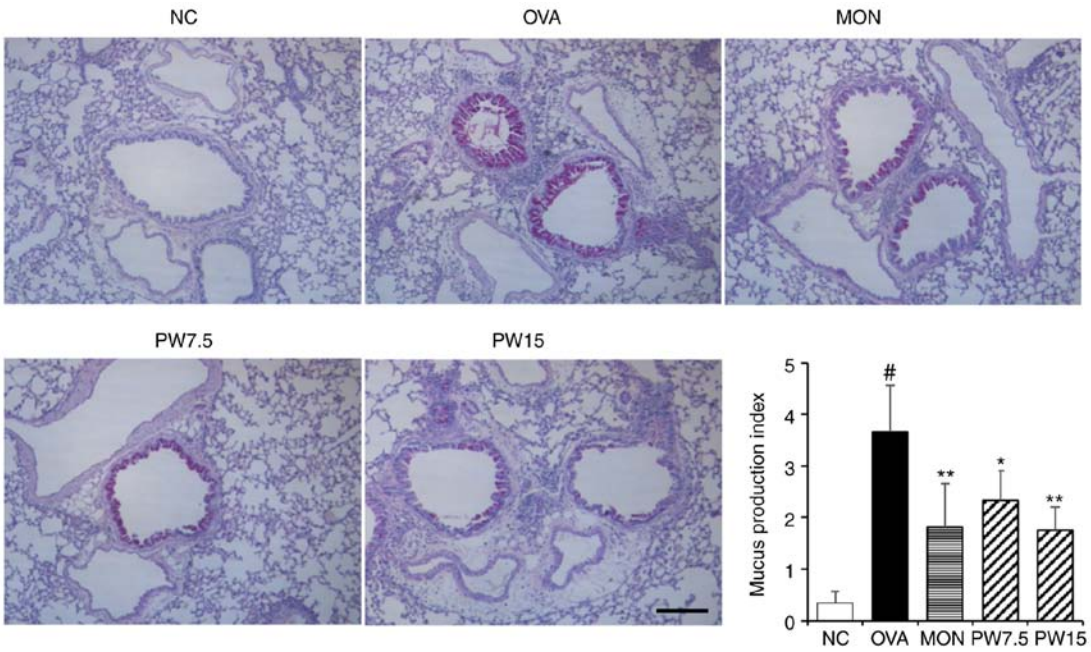

Figure 5. Effect of PWRE on OVA-induced hyperproduction of mucus in the lungs. PAS staining was used to assess mucus production (peribronchial lesion, magnification, x100; scale bar, 50 μm) and the degree of mucus production was assessed by two independent observers. $^{\#}$P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. OVA group. PWRE, *P. weinmannifolia* root extract; NC, negative control; OVA, ovalbumin; MON, montelukast.

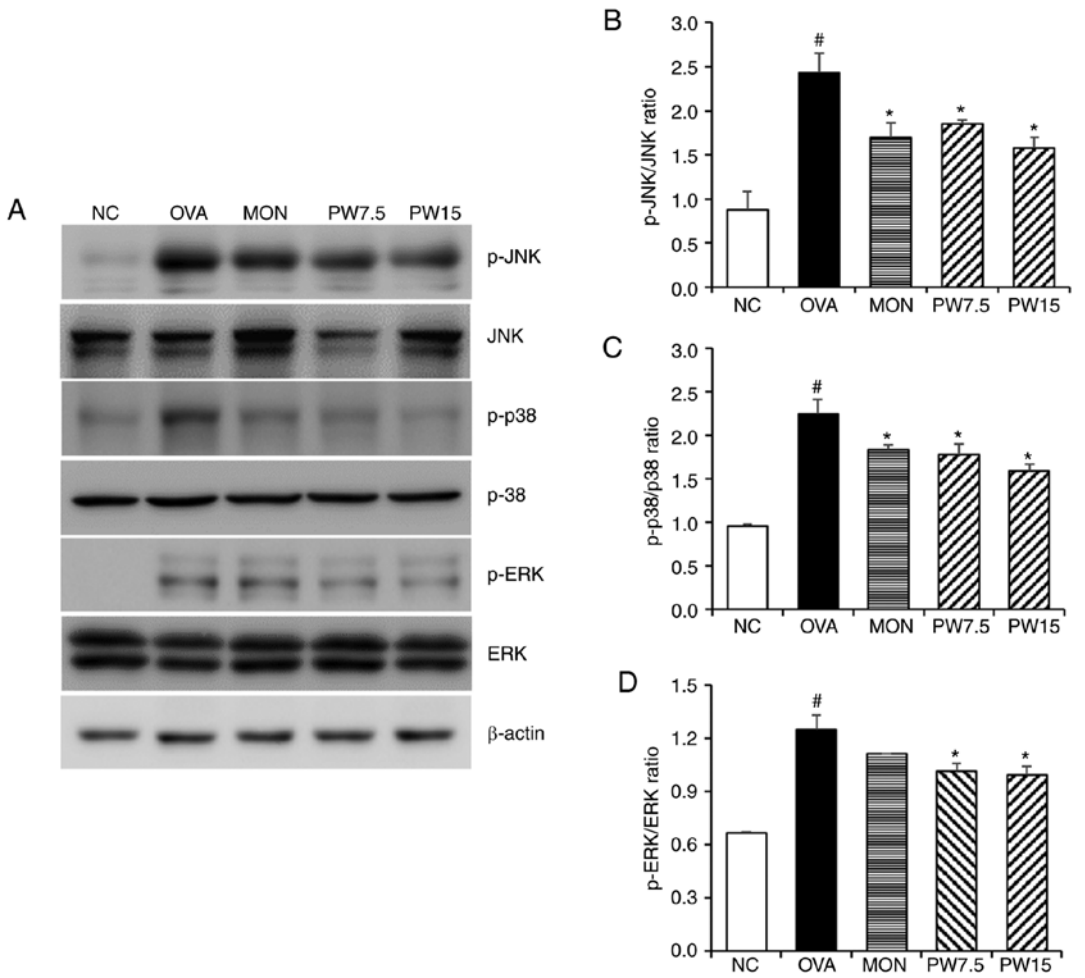

Figure 6. Effect of PWRE on OVA-induced activation of MAPK molecules in the lungs. (A) The levels of JNK, p38 and ERK activation in the lung tissues were determined via western blot analysis. (B-D) Quantitative analysis of p-JNK, p-p38 and p-ERK was performed by densitometric analysis. $^{\#}$P<0.05 vs. NC group; *P<0.05 vs. OVA group. PWRE, *P. weinmannifolia* root extract; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; p-ERK, phosphorylated-extracellular signal-regulated kinase; OVA, ovalbumin; MON, montelukast.

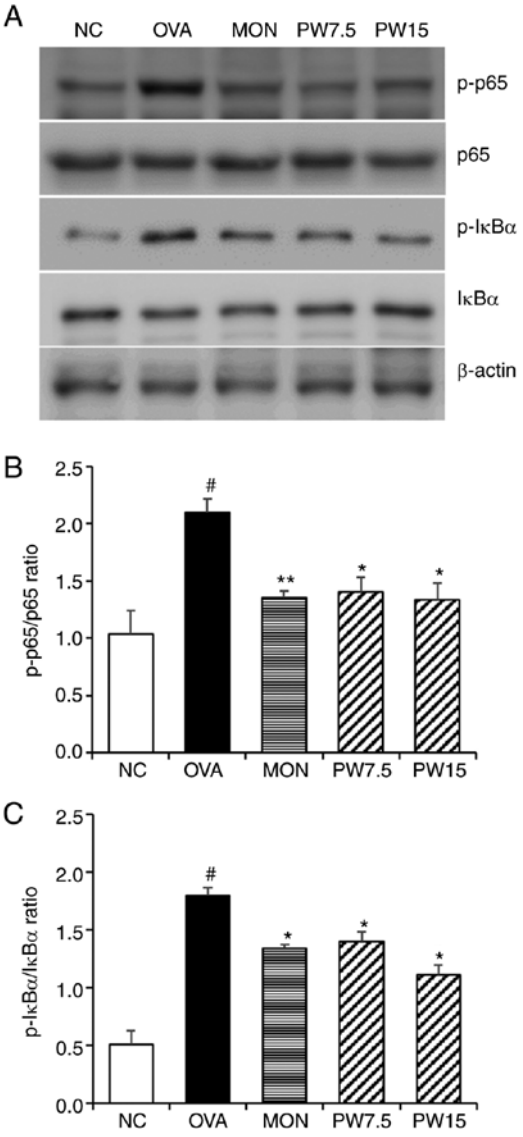

Figure 7. Effect of PWRE on OVA-induced activation of NF-κB p65 and IκBα molecules in the lungs. (A) The levels of NF-κB and IκB activation in the lung tissues were assessed via western blot analysis. (B) Quantitative analysis of p-NF-κB p65 and (C) p-IκB was performed by densitometric analysis. #P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. OVA group. PWRE, *P. weinmannifolia* root extract; NF-κB, nuclear factor κB. IκB, inhibitor of NF-κB; OVA, ovalbumin; MON, montelukast.

inflammatory cells and molecules. IL-4 has been reported to differentiate native T cells into Th2 cells and induce class switching in B cells to IgE production (32,33). IL-5 has an important role in the maturation and recruitment of eosinophils, and IL-13 is recognized as a dominant factor for IgE class switching, eosinophil inflammation and mucus production (9). Eosinophil infiltration is well known as an indispensable indicator in airway inflammation and the increase of eosinophil cationic proteins leads to airway hyper-responsiveness (34). The high level of macrophages is also well known as a characteristic of the allergic asthma murine model and macrophage-derived MCP-1 is known as a potent eosinophil chemoattractant (5,30). Therefore, the regulation of eosinophil influx, Th2 cytokine secretion and IgE production are important therapeutic approaches in the treatment of asthma. OVA has been used as an allergen in asthma animal models and the utility of OVA-induced asthma model has been well established and this model has been widely used to evaluate anti-asthmatic effects and immunological mechanisms involved in the pathogenesis of asthma (35). In this study an allergic asthma mouse model, in which the levels of Th2 cytokines, IgE and mucus production were successfully upregulated by OVA compared with the NC control was established. In the present study, it was confirmed that PWRE administration attenuated OVA-induced eosinophils and macrophage recruitment. OVA-induced IL-4, IL-5, IL-13 and IgE were suppressed by the treatment of PWRE. In addition, the increased levels of MCP-1 were downregulated following PWRE treatment in both *in vivo* and *in vitro* studies. Therefore, the results from the present study suggest that PWRE has a protective role against OVA-induced pulmonary inflammation.

In normal circumstances, goblet cell-derived mucus exerts protective roles against harmful agents. However, the excessive production of mucus could easily obstruct breathing (36,37). Therefore, the regulation of mucus hypersecretion may be a valuable therapeutic strategy in alleviating airway obstruction. MUC5AC is a major oligomeric mucin in airway mucus and its level is upregulated in patients with asthma (38). The inhibitory activities of PWRE on MUC5AC secretion in PMA-stimulated airway epithelial cells have already been confirmed (20). Therefore, the regulatory effect of PWRE on mucus overproduction was expected in the present study and it was observed that PWRE ameliorated the OVA-induced mucus hypersecretion.

The MAPK and NF-κB signaling pathways are known as key mediators in allergic asthma, and are closely associated with the activation of various immune cells (39,40). Accumulating evidence emphasizes the importance of the inhibition of the MAPK pathway in airway inflammatory diseases such as asthma (9). Accordingly, the inhibitory effect of PWRE on MAPKs activation was assessed in the present study. It was subsequently confirmed that OVA-induced MAPKs activation was significantly decreased by PWRE treatment. In LPS-stimulated RAW264.7 macrophages, PWRE did not exert any inhibitory effects on MAPKs activation. It is well established that the activation of IκB leads to airway inflammation by inducing NF-κB activation and production of inflammatory molecules (41-43); therefore, the present study next investigated the ability of PWRE to inactivate NF-κB and IκB. Notably, PWRE exerted an inhibitory effect on OVA-induced NF-κB p65 and IκBα activation. Similar to the results presented, the inhibitory effect of PWRE was observed in IκBα and NF-κB activation in LPS-stimulated RAW264.7 macrophages. Therefore, the results from the present study suggest that the molecular mechanism underlying the protective effects of PWRE on pulmonary inflammation primarily regard the downregulation of NF-κB activation.

In the present study, PWRE inhibited the pulmonary inflammatory response by diminishing the recruitment of inflammatory cells and the concentration of IL-4, IL-5, IL-13 and IgE. PWRE also downregulated the levels of MCP-1 and mucus production. Notably, the effects of PWRE were accompanied by MAPKs and NF-κB inactivation.

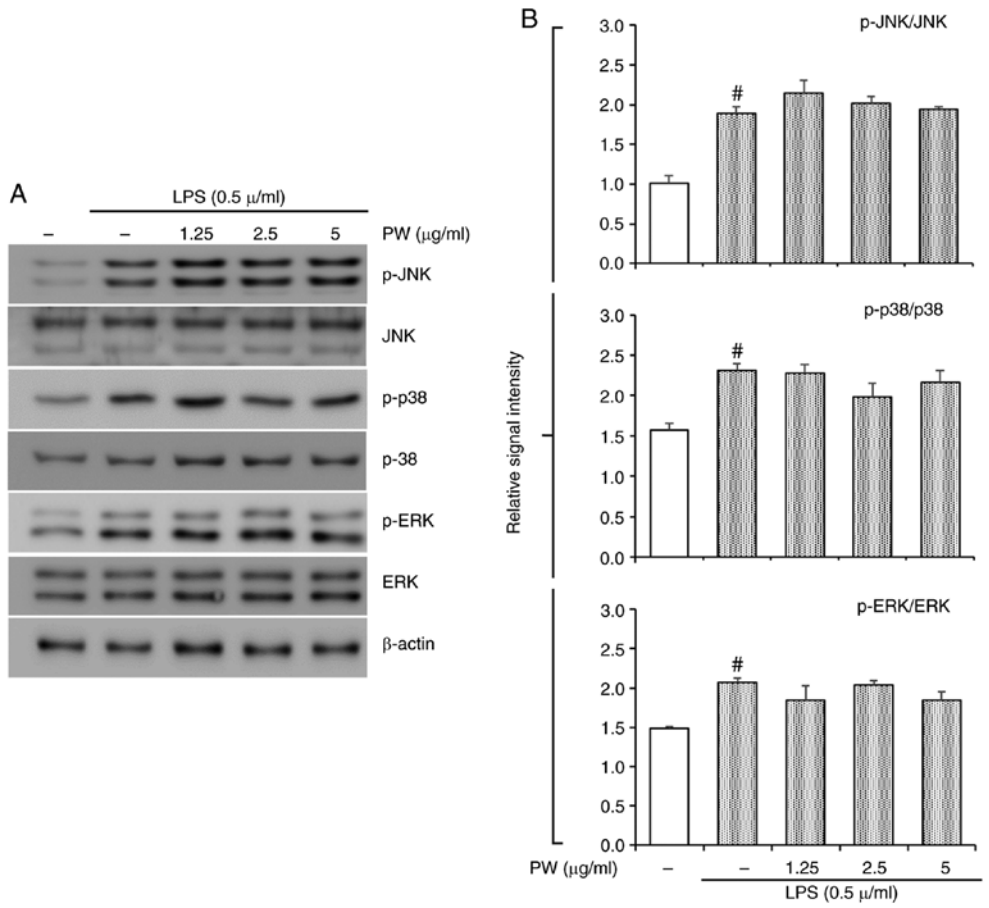

Figure 8. Effect of PW on LPS-stimulated activation of MAPK in RAW264.7 cells. (A) The levels of JNK, p38 and ERK activation were determined via western blot analysis. (B) Quantitative analysis of p-JNK, p-p38 and p-ERK was performed by densitometric analysis. #P<0.05 vs. NC group. PW, *P. weinmannifolia* root extract; LPS, lipopolysaccharide; p-ERK, phosphorylated-extracellular signal regulated kinase; JNK, c-jun n-terminal kinase; NC, negative control; MAPK, mitogen-associated protein kinase.

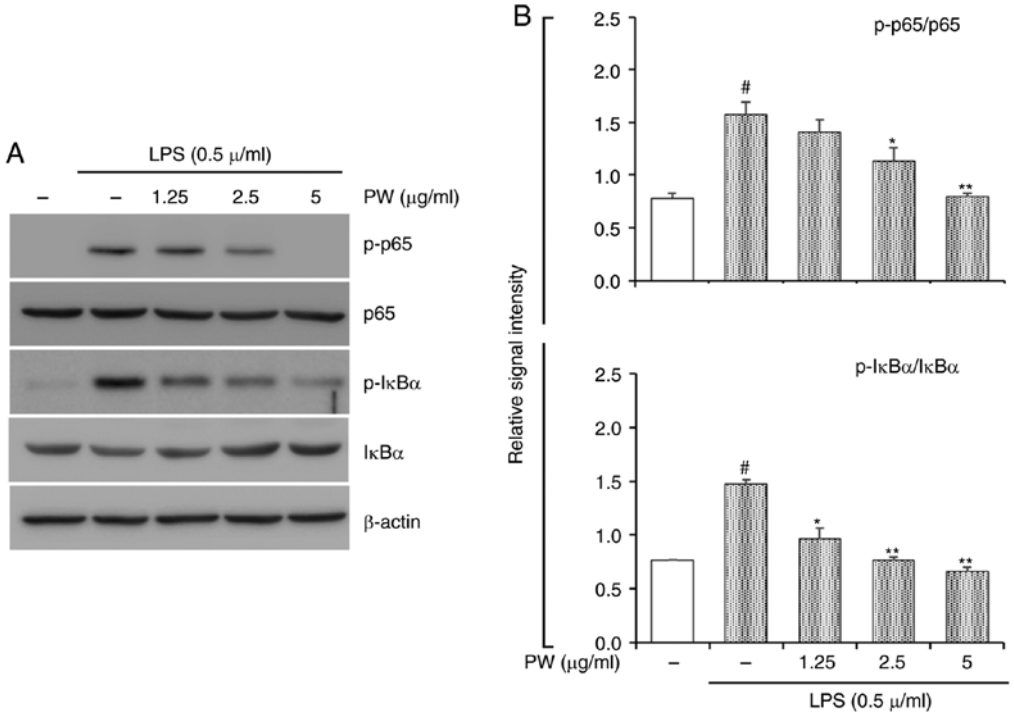

Figure 9. Effect of PW on LPS-stimulated activation of NF-κB p65 and IκBα in RAW264.7 cells. (A) The levels of NF-κB and IκB activation were evaluated via western blot analysis. (B) Quantitative analysis of p-NF-κB p65 and p-IκB was performed by densitometric analysis. #P<0.05 vs. NC group; *P<0.05 and **P<0.01 vs. LPS only group. PW, *P. weinmannifolia* root extract; LPS, lipopolysaccharide; NF, nuclear factor.

Abnormal weight changes and toxicological changes (such as intraperitoneal changes) were not observed after administration of PWRE. Therefore, the results from the present study suggest that PWRE may ameliorate airway inflammation and mucus hypersecretion in allergic asthma as a potential anti-inflammatory adjuvant. However, there was no evaluation of accurate count of inflammatory cells using flow cytometry. The levels of T-cell activation and eotaxin production in the pathogenesis of OVA-induced pulmonary inflammation have also not been investigated. It is necessary to confirm the inhibitory effect of PWER on MCP-1 in alveolar macrophages. These limitations should be addressed in the near future. In addition, the present study has limitations on the efficacy of PWRE in OVA-induced pulmonary inflammation. Therefore, clinical trials should be performed to elucidate this efficacy.

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Availability of data and materials

All data generated and/or analyzed during the present study are included in this published article.

Authors' contributions

JWL performed the *in vivo* experiments and wrote the manuscript. JHM, MGK and SMK performed the *in vivo* experiments and contributed to the interpretation of the results. OKK performed the *in vitro* experiments. TKO, JKL and TYK contributed to the acquisition of data. SWL, SC, WYL, HWR and KSA made substantial contributions to the conception and design of the present study, acquisition of data, and the analysis and interpretation of data. SRO designed the present study and was involved in revising it critically for important intellectual content. All authors discussed the results and read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (permit no. KRIBB-AEC-18054).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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