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

JAE-WON LEE\(^1\)*, JAE-HONG MIN\(^1\)*, MIN-GU KIM\(^1\), SEONG-MAN KIM\(^1\), OK-KYOUNG KWON\(^1\), TAE KYU OH\(^2\), JAE KYOUNG LEE\(^2\), TAE YOUNG KIM\(^2\), SANG WOO LEE\(^3\), SANGHO CHOI\(^3\), WAN-YI LI\(^4\), HYUNG WON RYU\(^1\), KYUNG-SEOP AHN\(^1\) and SEI-RYANG OH\(^1\)

\(^1\)Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju, Chungcheongbuk-do 28116; \(^2\)BTC Corporation, Technology Development Center, Ansan, Gyeonggi-do 15588; \(^3\)International Biological Material Research Center, KRIIB, Daejeon 34141, Republic of Korea; \(^4\)Institute of Medicinal Plants, Yunnan Academy of Agricultural Sciences, Kunming, Yunnan 650200, P.R. China

Received January 15, 2019; Accepted September 24, 2019

DOI: 10.3892/ijmm.2019.4367

**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.

**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.
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 after the final OVA inhalation and incubated in 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.

**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 (p-IκB; cat. no. 9285; 1:1,000; Cell Signaling Technology, Inc.), anti-p-inhibitor of NF-κB (p-IkBα; 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-IkBα (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).

**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.
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, a decrease in this level was observed in the PWRE group (Fig. 6). The mucus was stained a purple color by the PAS staining reagent.

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 regulatory effect of PWRE on LPS-stimulated MAPKs and NF-κB activation was therefore investigated in RAW264.7 macrophages. The regulatory effect of PWRE on LPS-stimulated MAPKs and NF-κB activation was therefore investigated in RAW264.7 macrophages.
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.
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 on MAPKs activation was assessed in the present study. It was subsequently confirmed that OVA-induced NF-κB 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 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-IκBα was performed by densitometric analysis. *P<0.05 vs. NC group; **P<0.01 vs. OVA group; PWRE, *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.
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<0.01 vs. NC group; †P<0.05 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 eosin 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.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Ministry of Trade, Industry and Energy and the Korea Institute for the Advancement of Technology (grant no. N0002410, 2017).

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.

References

11. Subhashini, Chauhan PS, Dash D, Paul BN and Singh R: Intestinal curcumin ameliorates airway inflammation and obstruction by regulating MAPKinas activation (p38, Erk and JNK) and prostaglandin D2 release in murine model of asthma. Int Immunopharmacol 31: 200-206, 2016.


This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.