



Anti-asthma and antitussive effects of a fermented extract of a mixture of *Ramulus mori*, *Anthriscus sylvestris*, and *Salvia plebeian*

Mirae An¹ · Miae Oh² · Keun-Tae Park² · Ki Hwan Seon³ · Jeong Eun Jo³ · Seong Kweon Lee³ · Jeong-Keun Kim⁴ · Kwang Soon Shin⁵ · Jong-Ho Koh⁶ · Young-Hee Lim^{2,7,8}

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Abstract Respiratory immunity is getting more important recently due to outbreak of respiratory diseases and increasing the concentration of fine dust. The aim of this study was to investigate respiratory protection effect of a fermented extract of medicinal plants (FEMP) containing *Ramulus mori*, *Salvia plebeia*, and *Anthriscus sylvestris*. The expression levels of *IL-8* and *IL-17* in LPS/poly-L-arginine (PLA) and FEMP-cotreated A549 cells were lower than those in LPS/PLA only-treated cells. The levels of IgE, *IL-17*, and *IL-4* in the bronchoalveolar lavage fluid (BALF) and serum of FEMP-treated mice with ovalbumin/

LPS-induced asthma were lower than the control levels. The lung inflammation score and the number of inflammatory cells in the BALF decreased by FEMP treatment. In the citric acid-induced coughing guinea pig, the FEMP treatment decreased the number of coughs. Therefore, FEMP shows anti-asthmatic and antitussive activities without hepatotoxicity and can be used as a compound aiming to improve respiratory health.

Keywords Anti-asthma · Antitussive · Anti-inflammation · Fermented extract of medicinal plant

✉ Young-Hee Lim
yhlm@korea.ac.kr
Mirae An
mran318@naver.com
Miae Oh
aldooh1234@naver.com
Keun-Tae Park
cerex@naver.com
Ki Hwan Seon
skh120701@hanmail.net
Jeong Eun Jo
whwjddms918@naver.com
Seong Kweon Lee
phdlsk1@naver.com
Jeong-Keun Kim
kjkim@kpu.ac.kr
Kwang Soon Shin
ksshin@kyonggi.ac.kr
Jong-Ho Koh
kohjh@kopo.ac.kr

² Department of Integrated Biomedical and Life Sciences, Graduate School, Korea University, Seoul 02841, Republic of Korea
³ R & D Center, Biocean CO. LTD, Seoul 08591, Republic of Korea
⁴ Department of Chemical Engineering and Biotechnology, Korea Polytechnic University, Shihung-si, Gyeonggi-do 15073, Republic of Korea
⁵ Department of Food Science and Biotechnology, Kyonggi University, Suwon 16227, Republic of Korea
⁶ Department of Bio-Food Analysis and Processing, Bio-Campus Korea Polytechnic College, Nonsan 32943, Republic of Korea
⁷ School of Biosystems and Biomedical Sciences, College of Health Science, Korea University, Seoul 02841, Republic of Korea
⁸ Department of Laboratory Medicine, Korea University Guro Hospital, Seoul 08308, Republic of Korea

¹ Department of Public Health Science, Graduate School, Korea University, Seoul 02841, Republic of Korea

Introduction

Respiratory immunity has recently become more important due to increases in the fine dust levels, and consequently, the incidences of respiratory diseases and outbreaks of novel respiratory diseases, such as SARS-CoV, MERS-CoV, and COVID-19, are increasing. Fine dust, also known as particulate matter, is a mixture of particles and droplets with a diameter between 2.5 and 10 μm (Brown et al., 2013) and is considered a global health issue. Fine dust can induce chronic inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease, extrinsic allergic alveolitis, and fibrosing alveolitis. In particular, asthma can be accelerated by the inhalation of fine dust, which can stimulate inflammation in the airway through Toll-like receptor-related signaling. Asthma is also triggered by remodeling airway conditions through aberrant DNA methylation and histone modifications, particularly among people who are exposed to fine dust in their early childhood. The incidences of respiratory diseases caused by novel viruses that induce severe lung damage by dysregulating the immune response and cytokine release syndrome are also increasing. Most respiratory diseases are similarly associated with inflammation caused by various reactions, such as the activation of neutrophils and eosinophils, an imbalance of T helper cells and oxidative stress (Wu et al., 2018). Consequently, the effects of the products or medicines for improving respiratory health are focused on anti-inflammation; for example, fluticasone, budesonide, mometasone, and beclomethasone, which are corticosteroids used to treat symptoms of respiratory diseases, have anti-inflammatory activities (Brightling et al., 2005; Leach et al., 1998). These medicines remarkably improve the symptoms of respiratory diseases but can cause unwanted side effects, such as adrenal suppression, osteoporosis, and diabetes (Phillipps, 1990). Therefore, effective and safe materials need to be developed for the prevention and improvement of respiratory diseases. Medicinal herbs that exert beneficial effects on humans without any side effects might be alternatives for those corticosteroid medicines.

Ramulus mori, the twig of *Morus alba* L. (Moraceae), exerts various beneficial effects on human health, such as antidiarrheic reactions, decreases in blood pressure, and antioxidant and anti-inflammatory activities (Yu et al., 2019). Oxyresveratrol (trans-2,3',4,5'-tetrahydroxystilbene), the representative polyphenol in *R. mori*, is a hydroxy-stilbene with four hydroxyl groups, and has various health-promoting activities, such as anticancer effects and anti-allergic activity (Ashraf et al., 2015). *Salvia plebeia* R. Brown (Lamiaceae) is an annual or biennial plant belonging to the Labiatae family and serves as a folk

remedy for diverse inflammatory diseases, such as hepatitis, influenza, and cough, in Asian countries (Harley et al., 2004). Rosmarinic acid, which is the representative active compound of *S. plebeia* R. Brown, significantly inhibits the expression of cytokines and chemokines related to allergic inflammation, such as IL-4 and IL-5 (Sanbongi et al., 2004). *Anthriscus sylvestris* (Apiaceae), a biennial or short-lived perennial plant of the family Apiaceae, is commonly known as wild chervil or cow parsley and mainly grows in Europe, western Asia, and northwestern Africa. *A. sylvestris* has been traditionally used as a medicinal herb to obtain antitussive effects and for the treatment of asthma and headache (Fejes et al., 2000).

R. mori, *S. plebeia* R. Brown, and *A. sylvestris* exert beneficial effects independently on various inflammatory or allergic diseases (Jo et al., 2010; Zhang and Shi, 2010). The combination of medicinal herbs can produce synergistic effects compared with those observed with a single ingredient (Guimarães et al., 2011). In this study, a fermented extract of medicinal plants (FEMP) obtained from a mixture of *R. mori*, *A. sylvestris*, and *S. plebeia* R. Brown was prepared through bioconversion to enhance their medicinal effects, focusing on those on respiratory health. We examined the anti-inflammatory activity of the FEMP on cells from the human lung epithelium *in vitro*, and the anti-asthma effect of the FEMP on the mouse model of OVA/LPS-induced asthma and the antitussive activity of FEMP in a guinea pig animal model of CA-induced cough were investigated *in vivo*. Our study demonstrated that the FEMP exerted meaningful effects on respiratory health by inhibiting the expression of inflammatory cytokines and chemokines in experimental animals and human lung tissue cell lines and by showing antitussive activity.

Materials and methods

Preparation of the fermented extract of medicinal plants (FEMP)

Ramulus mori, *Anthriscus sylvestris*, and *Salvia plebeia* R. Brown were purchased from Yong-woo Pharmacy Co (Seoul, Korea). The FEMP was prepared by fermentation of the water extract from a mixture of *Ramulus mori*, *Anthriscus sylvestris*, and *Salvia plebeia* R. Brown (w/w, 1:1:1). The conjugated enzyme cellulose prepared from *Aspergillus niger* (SUMZYME-AC, Shin Nihon Chemical Co., Ltd., Anjo, Aichi, Japan) was added to the mixture with distilled water for bioconversion, and the mixture was then extracted at 45 °C for 60 h and boiled at 90 °C for 30 min to stop enzyme activity. The fermented extract was filtered using filter paper (NO. 50, pore size: 12 μm , HDmicro, Seoul, Korea) and concentrated at 55 °C using a

rotary vacuum evaporator (N-1110, Eyela, Tokyo, Japan) until half of the filtrate remained. The concentrated extract was then lyophilized using a freeze dryer (PVTFD20, Ilshin Biobase, Dongduchen, Korea). The active compounds (oxyresveratrol and rosmarinic acid) in the FEMP were analyzed by high-performance liquid chromatography (HPLC) by using a YL9100 HPLC system (Younglin Instruments, Anyang-si, Korea) using a Luna-Pak® C-18 (5 µm, 4.6 mm i.d. × 250 mm). The mobile phase was acetonitrile (CH₃CN) and 10 mM H₃PO₄ (0 min, 10:90; 0–10 min, 25:75; 10–30 min, 50:50, v/v). Flow rate was 0.7 mL/min, the injection volume was 100 µL and the effluent was monitored at 325 nm with a photodiode array detector. Oxyresveratrol and rosmarinic acid were found at concentrations of 94 mg/kg and 12.144 g/kg in the FEMP, respectively. The HPLC chromatograms of the active compounds are shown in Fig. S1. The FEMP used in this study was deposited under registration number FNCE01 in the R & D Center, Biocean CO. LTD (Seoul, Korea).

Cell culture

The A549 cell line from the human lung was obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (HyClone, Logan, UT, USA) at 37 °C in a humidified atmosphere with 5% CO₂. The culture medium was changed every 2 d during the incubation of the cells. The cells were seeded in a 96-well plate (5 × 10⁴ cells/mL) and in a six-well plate (5 × 10⁴ cells/mL) and treated with various concentrations of the FEMP (2.5, 5, and 10 µg/mL).

Cell viability assay

The cell viability was measured using the 3-[4,5-dimethylthiazol-yl]-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, OH, USA) assay. A 549 cells were seeded at 5 × 10⁴ cells/mL in a 96-well plate, cultured for 24 h, and treated with FEMP (2.5, 5, and 10 µg/mL) for 24 h. At the end of the incubation period, the medium was changed to RPMI containing MTT reagent, and the cells were incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO₂. The formed formazan was extracted with 100 µL of dimethyl sulfoxide (DMSO, Daejung, Siheung, Korea) for 30 min at room temperature, and the absorbance of each well at 540 nm was measured using a microplate reader (SpectraMax 340PC, Molecular Devices, San Jose, CA, USA). The relative cell viability (%) was calculated by comparing the absorbance of the FEMP-treated cells with that of the negative control.

Quantitative real-time polymerase chain reaction (qPCR)

A 549 cells were seeded at 5 × 10⁴ cells/mL in a six-well plate and cultured for 24 h at 37 °C. The medium was then changed to RPMI 1640 without FBS for serum starvation for 24 h. At the end of the incubation period, FEMP (2.5, 5, and 10 µg/mL) and phosphate buffered saline (PBS, negative control) were added. After 1 h, the cells were treated with lipopolysaccharide (LPS) and poly-L-arginine (PLA) (5 µg/mL each) (Sigma-Aldrich, St. Louis, MO, USA) for 8 h to induce the expression of *IL-8* and *IL-17*. The medium was removed by suction, and 1 mL of TRIzol reagent (Thermo Fisher Scientific) was added for the extraction of total RNA according to the manufacturer's manual. The total concentration of RNA was measured using a Nano-Drop (ND-1000 spectrophotometer, Thermo Fisher Scientific). A RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) was used to convert the quantified RNA into cDNA, and the cDNA was used for qPCR using a KAPA SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, NC, USA) and a QuantStudio 6 Flex system (Life Technologies, Carlsbad, CA, USA). The qPCR primers were obtained from Bioneer (Seoul, Korea), and their sequences used were as follows: *GAPDH*, forward 5'-ACC CAC TCC TCC ACC TTT GA-3', reverse 5'-CTG TTG CTG TAG CCA AAT TCG T-3'; *IL-8*, forward 5'-CCA ACA CAG AAA TTA TTG TAA AGC-3', reverse 5'-TGA ATT CTC AGC CCT CTT CAA-3'; and *IL-17*, forward 5'-CAC CTC ACC TTG GAA TCT C-3', reverse 5'-GGA TCT CTT GCT GGA TGG-3'. The qPCR cycle consisted of preheating at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. The relative gene expression was quantified using a comparative Ct method (ΔΔCt method) (Livak and Schmittgen, 2001). *GAPDH* was used as the reference gene.

Ovalbumin (OVA)/LPS-induced asthma model

Five-week-old male BALB/c mice (18–20 g) were obtained from Orient Bio (Seongnam, Korea) and housed under the following conditions: 22 ± 1 °C, illumination intensity of 200 ± 50 lx, 12 h light/12-h dark cycle and 50 ± 5% relative humidity. The experimental protocols were approved by the Korea University Institutional Animal Care and Use Committee (Approval No. KUIACUC-2019-0026). All the mice had free access to their diet (No. 5053, LABDIET, St. Louis, MO, USA) and water. After acclimation for one week, the mice were randomly divided into four groups (8 mice per group): the mice in group 1 (normal control; Nor group) were treated with vehicle (PBS) and were not subjected to OVA/LPS sensitization and challenge, the mice in group 2 (OVA/LPS-induced

asthma control; Model group) were treated with vehicle and subjected to OVA/LPS sensitization and challenge, the mice in group 3 (low FEMP concentration; FEMP50 group) were treated with 50 mg/kg/day FEMP and were subjected to OVA/LPS sensitization and challenge, and the mice in group 4 (high FEMP concentration; FEMP100 group) were treated with 100 mg/kg/day FEMP and subjected to OVA/LPS sensitization and challenge. PBS and the FEMP were orally administered for one week (14–21 days, daily), and the body weight was measured weekly. To induce asthma, the mice were subjected to sensitization and challenge with antigens, namely, OVA (Sigma-Aldrich) and LPS. OVA/LPS sensitization was conducted on days 0 and 7 through the intraperitoneal injection of 0.2 mL of a mixture of 75 µg of OVA, 50 µg of LPS, and 2 mg of aluminum hydroxide (Sigma-Aldrich). OVA/LPS challenge was performed on days 14, 15, and 21 via the inhalation of 1% OVA/LPS for 30 min per day using a nebulizer (NE-C28, Omron, Tokyo, Japan) (Fig. S2). The normal group was exposed to PBS under the same conditions.

Citric acid (CA)-induced cough model

Male Hartley guinea pigs (350–400 g) were obtained from Koatech (Pyeongtaek, Korea) and were housed under the following conditions: 24 ± 1 °C, 12-h light/12-h dark cycle and $50 \pm 5\%$ relative humidity. The experimental protocols were designed in accordance to the guidelines of the Ethics Committee for the Use of Experimental Animals at Kyonggi University (Approval No. 2019–013). All the guinea pigs had free access to their diet (Purina Guinea pig diets, Purina, Seongnam, Korea) and water. After acclimation for one week, the guinea pigs were randomly separated into four groups (8 guinea pigs per group): group 1 (normal control; Nor group) was treated with vehicle (PBS) and was not subjected to stimulation with CA (Sigma-Aldrich), group 2 (CA-induced cough control; Model group) was treated with vehicle and stimulated with CA, group 3 (low FEMP concentration; FEMP50 group) was treated with FEMP at a dose of 50 mg/kg/day and stimulated with CA, and group 4 (high FEMP concentration; FEMP100 group) was treated with 100 mg/kg/day FEMP and stimulated with CA. The vehicle and FEMP were orally administered for one week (0–7 days, daily). One hour after the last administration, the guinea pigs were exposed to a nebulizer filled with 0.2 M citric acid for 10 min to induce coughing, and the number of coughs over the 15-min period immediately following the treatment was counted (Fig. S3).

Serum biochemical analysis

The animals were fasted for 24 h before sacrifice under anesthesia, which was administered via the inhalation of isoflurane (0.25–4%). Blood was collected in a serum separator tube (SST) by cardiac puncture and centrifuged for 10 min at $3000 \times g$ to separate the serum. The serum levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured using FUJI DRY-CHEM slides (FUJIFILM Co., Tokyo, Japan).

Bronchoalveolar lavage fluid (BALF) collection and cell counting

BALF collection was performed immediately after blood collection by inserting a catheter into the trachea. Cold PBS (1 mL) was injected using a syringe into the lungs of mice and sucked three times to obtain the BALF. The BALF was centrifuged at $400 \times g$ and 4 °C for 10 min, and the supernatant was separated and stored at -80 °C for cytokine and immunoglobulin measurements. The cell pellets were resuspended in 200 µL of PBS and centrifuged at $400 \times g$ and 4 °C for 10 min. The supernatant was discarded, and the pellets were resuspended in 200 µL of ACK lysis buffer (NH_4Cl 150 mM, KHCO_3 10 mM, and Na_2EDTA 0.1 mM, pH 7.3) and maintained at 4 °C for 10 min for the lysis of red blood cells. After lysis, 800 µL of PBS was added to stop the reaction, and the mixture was centrifuged at $400 \times g$ and 4 °C for 10 min. The cell pellets were resuspended in 200 µL of PBS, and the total cell count was determined using a hemocytometer under a light microscope (CKX41, Olympus, Tokyo, Japan). A moderate amount of cell suspension was concentrated using a cytocentrifuge (Hanil, Daejeon, Korea) at 12,000 rpm for 10 min. Subsequently, the cytospin smears were stained with Diff-quick stain according to the manufacture's instruction (Sysmex, Kobe, Japan), and the inflammatory cells were observed through a light microscope. Stained immune cells were distinguished by their characteristic staining patterns. Neutrophils are about 12–15 µm and the nucleus, cytoplasm, and granules are blue, pink, and violet, respectively. Eosinophils are about 12–17 µm and the nucleus, cytoplasm, and granules are blue, blue, and red, respectively. Lymphocytes are 9–12 µm and the nucleus is purple/dark blue and the cytoplasm shows typical basophilic characteristics such as thin border containing red granules. Macrophages are 19–22 µm and the nucleus is violet and the cytoplasm is light blue. At least 100 cells were counted randomly.

Histological analysis

The lungs and livers of the mice were removed, immediately fixed with 10% formalin and then embedded in paraffin. The embedded tissues were sectioned at approximately 3–5 μm thickness, dewaxed in xylene, dehydrated in 70, 80, 90 and 100% ethanol, and then stained with hematoxylin and eosin (H&E). The degree of pathological changes in the lung was scored according to the Table S1 (Dubin and Kolls, 2007), (Bayes et al., 2016).

Quantification of cytokines and immunoglobulin in the BALF and serum

The mouse serum and BALF were used to assess the levels of IgE, IL-17, and IL-4 using an IgE mouse uncoated ELISA kit with plates (Invitrogen, Carlsbad, CA, USA), an IL-17 mouse ELISA kit (Invitrogen), and an IL-4 mouse ELISA kit (Invitrogen), respectively, according to the protocols recommended by the manufacturer. After the reaction, the absorbance at 450 nm was measured using a microplate reader (SpectraMax 340PC, Molecular Devices), and the concentrations of IgE, IL-17, and IL-4 were calculated according to the corresponding standard curve.

Statistical analysis

The significance of the differences between the results obtained for the various groups was evaluated through one-way analysis of variance (ANOVA) using the Statistical Package for the Social Science (SPSS) statistical analysis program version 25. The *in vitro* and *in vivo* results are expressed as the mean \pm standard deviation (SD) and the mean \pm standard error of the mean (SEM), respectively. A *p* value was less than 0.05 was considered to indicate a significant difference, and post hoc tests were performed using Tukey's honestly significant difference (HSD) method.

Results and discussion

Effect of FEMP on cell viability

A549 cells were used to evaluate the anti-inflammatory effect of the FEMP *in vitro*. Compared with that of the negative control group, cell viabilities show that the concentrations of FEMP used in this study did not exhibit any cytotoxicity (Fig. S4).

Reducing effect of FEMP on the cytokines gene expression level in cells treated with LPS/PLA

LPS is commonly found in the environment and can be a crucial asthma-aggravating factor in the case of its absorption (Zhang et al., 2016). PLA can imitate the function of major basic proteins that cause airway hyper-responsiveness by stimulating airway myocytes (Duan et al., 2004) and induce an inflammatory pathology by injuring the cell membrane (Zhang et al., 2016). Therefore, A549 cells were cotreated with LPS and PLA to induce inflammation in this study, and the effect of FEMP on the expression of genes related to inflammatory cytokines in A549 cells was assessed by qPCR. IL-4 is a main factor that has a significant impact on asthma. In the case of LPS-induced cell experiments, we analyzed IL-8 that affects to asthma related to inflammation instead of IL-4 because LPS-induced cell model has limitations in inducing IL-4 that is secreted by basophilic granulocytes and eosinophils (Sokol et al., 2008) and then induces the differentiation of naïve helper T cells to Th2 cells that produce IL-4, and IL-4 subsequently acts as an autocrine. IL-8 is a chemokine produced by airway epithelial cells and smooth muscle cells, and its main function is chemotaxis to activate neutrophils and eosinophils (Hedges et al., 2000). IL-17 is a proinflammatory cytokine produced by T helper 17 cells, which are generated by cytokines such as IL-6, IL-1 β , IL-23, and TGF- β (Kolls and Lindén, 2004). TNF- α and IL-1 β are typical proinflammatory cytokines that stimulate inflammatory responses and they show increased circulation in the patients with asthma (Berry et al., 2007; Lambrecht et al., 2019). The *IL-8* expression level in the cells treated with LPS/PLA alone was 4.7-fold higher than that of the negative control (Fig. 1A). The *IL-8* expression levels in the cells cotreated with LPS/PLA and FEMP (2.5, 5, and 10 $\mu\text{g}/\text{mL}$) significantly decreased compared with the cells treated with LPS/PLA alone in a dose-dependent manner. The *IL-17* expression level in the cells treated with LPS/PLA alone was 1.9-fold higher than that of the negative control (Fig. 1B). The *IL-17* expression levels in the cells cotreated with LPS/PLA and FEMP (2.5, 5, and 10 $\mu\text{g}/\text{mL}$) significantly decreased in a dose dependent manner compared with the cells treated with LPS/PLA alone. Expression level of *TNF- α* in the cells treated with LPS/PLA alone was 1.9-fold higher than that of the negative control (Fig. 1C). Although it was not significant, expression level of *TNF- α* decreased in the cells cotreated with LPS/PLA and FEMP compared with the cells treated with LPS/PLA alone. Expression level of *IL-1 β* in the cells treated with LPS/PLA alone was 1.5-fold higher than that of the negative control, but it was not significant (Fig. 1D). Unexpectedly, although it was not significant, expression level of *IL-1 β* in the cells cotreated with LPS/PLA and

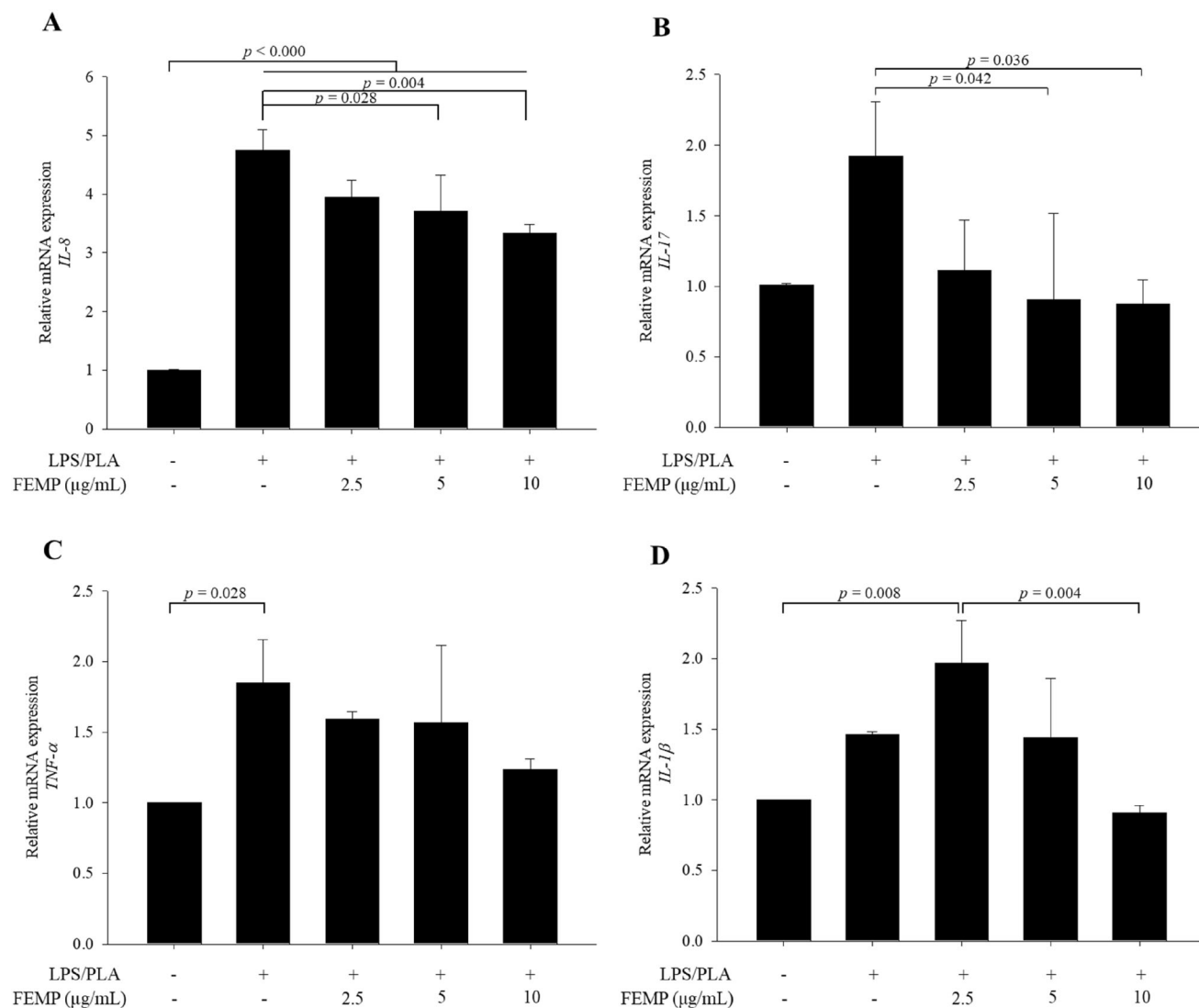


Fig. 1 The mRNA expression levels related to inflammatory cytokines decreased by FEMP treatment. A549 cells (5×10^4 cells/mL) exhibited inflammation induced by LPS/PLA and were treated with the FEMP and then the mRNA expression levels of *IL-8* (A), *IL-*

17 (B), *TNF-α* (C), and *IL-1β* (D) were measured by qPCR. The data are expressed as the mean \pm SD and were analyzed by one-way ANOVA/Tukey's HSD method

FEMP (2.5 μ g/mL) increased compared with the cells treated with LPS/PLA alone, however, expression levels of *IL-1β* in the cells cotreated with LPS/PLA and FEMP (10 μ g/mL) significantly decreased compared with the cells cotreated with LPS/PLA and FEMP (2.5 μ g/mL). These results show that the LPS/PLA-induced expression of inflammatory cytokines genes was significantly reduced by treatment with the FEMP in a dose-dependent manner, which suggests that the FEMP exerts anti-inflammatory effects in lung tissue.

Effect of FEMP on the body weight change and hepatotoxicity in OVA/LPS-induced asthma mice

To evaluate the anti-inflammatory effect of FEMP *in vivo*, we used an OVA/LPS-induced asthma mouse model. To induce asthma, OVA is commonly used for whole-body sensitization, and the co-administration of OVA with LPS is also used to induce asthma and the corresponding immune response in studies of bronchial asthma pathogenesis. During the first two weeks after sensitization, the body weights of the mice with OVA/LPS-induced asthma, regardless of whether the mice were orally administered the FEMP, were lower than those of the mice belonging to the normal group; however, no significant difference in

body weight was found among the groups, including the normal group, at the end of the experiment (Fig. 2A). In addition, the serum GOT and GPT levels did not show a significant difference among the groups (Table 1). To investigate the histological changes in liver tissue, H&E staining was performed, and no differences in tissue morphology were detected (Fig. 2B). The results suggest that the oral administration of the FEMP within the concentration range used in this study did not result in hepatotoxicity.

Suppression of secreting immunoglobulin E and inflammatory cytokines by FEMP in OVA/LPS-induced asthma mice model

IgE plays a vital role in type I hypersensitivity, such as allergic asthma, allergic rhinitis and atopic dermatitis (Gould et al., 2003). IL-4, which, similarly to IL-17, is a proinflammatory cytokine, stimulates the activation of B cells, T cells and naïve T helper cells, and the overproduction of IL-4 is also related to allergic diseases, which are caused by disproportionate immune responses involving T helper 2 cells and allergens (Steinke and Borish, 2001). Therefore, IgE, IL-4, and IL-17 are the major factors responsible for the induction of allergic diseases, including asthma, by activating T helper cells and leading to the secretion of other inflammatory cytokines (Wong et al., 2001). To investigate the anti-asthmatic effect of the FEMP on OVA/LPS-induced asthmatic mice, the expression levels of IgE and cytokines (IL-4 and IL-17) in the

BALF and serum were measured by ELISA. The secretion of IgE in both the BALF and serum was significantly higher in the model group than in the normal group, whereas the secretion of IgE in the BALF and serum was significantly decreased in the FEMP50 and FEMP100 groups, respectively, compared with the model group (Fig. 3A). No significant difference in the secretion of IL-17 in serum was found among the groups, regardless of FEMP treatment, whereas the secretion of IL-17 in the BALF was significantly lower in the FEMP100 group than in the model group (Fig. 3B). The secretion of IL-4 in the BALF was significantly increased in the model group compared with the normal group, whereas that in the FEMP100 group was significantly lower than that in the model group (Fig. 3C). No IL-4 secretion was detected in serum. Both IL-4 and IL-17 are proinflammatory cytokines that are produced in response to physical triggers of the immune response, such as infections, toxins, medications, and allergens. Once antigen-presenting cells are stimulated, naïve T cells undergo differentiation into various T helper subsets according to the trigger of the immune response. In the case of allergen triggers, naïve T cells are mostly stimulated to differentiate into T helper 2 cells that secrete IL-4 and IL-2, and helper T2 cell-induced inflammation increases IgE synthesis, airway hyperresponsiveness, and mucus hypersecretion in the airway (Heuer et al., 2013). IL-17 is secreted by T helper 17 cells that are differentiated in response to infections or autoimmunity. Recent studies revealed that IL-17 contributes to the development of severe asthma (Ramakrishnan et al., 2019). The secretion

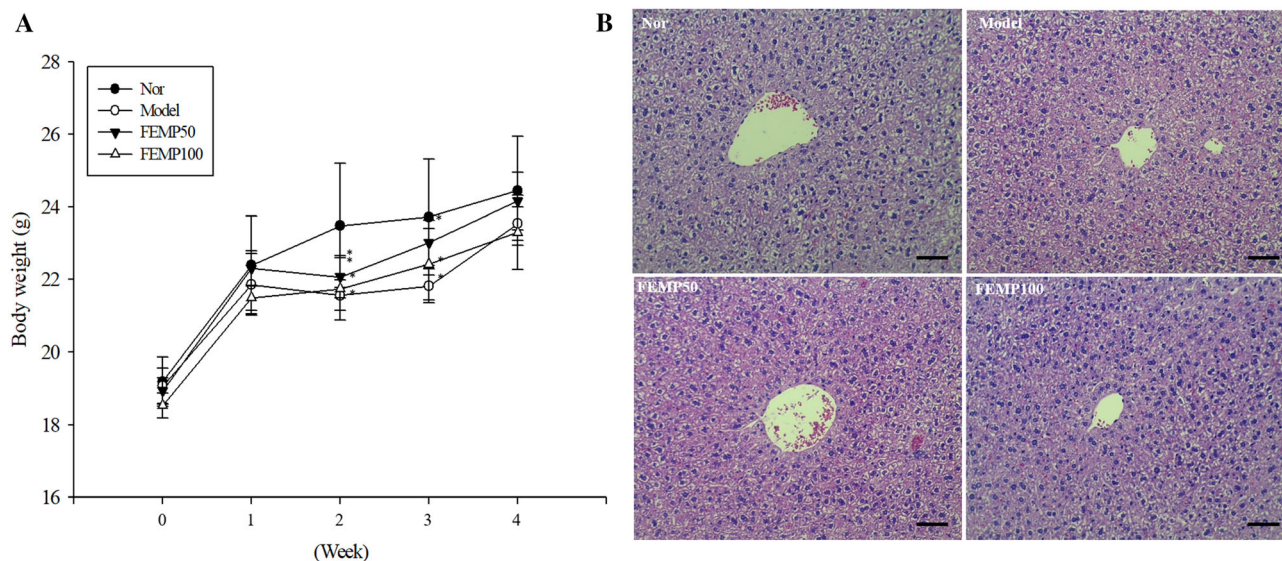


Fig. 2 Effect of the FEMP on the body weight changes (A) and liver tissue morphology (B) in OVA/LPS-induced asthma mice (n = 8/group). Nor, normal control; Model, OVA/LPS-induced asthma control; FEMP50, OVA/LPS-induced asthmatic mice treated with 50 mg/kg/day FEMP; FEMP100, OVA/LPS-induced asthmatic

mice treated with 100 mg/kg/day FEMP. The data are expressed as the mean \pm SEM and were analyzed by one-way ANOVA/Tukey's HSD method. Significant differences are marked with asterisks: * p < 0.05 and ** p < 0.01. The scale bar indicates 50 μ m, and the tissue was observed at 200 \times magnification

Table 1 Effect of the FEMP on the serum GOT and GPT levels in mice with OVA/LPS-induced asthma (n = 8/group)

	Nor	Model	FEMP50	FEMP100
GOT (U/L)	76.2 ± 14.2	68.4 ± 19.8	70.4 ± 22.4	70.4 ± 21.0
GPT (U/L)	25.2 ± 1.3	19.8 ± 0.9	22.4 ± 1.7	21.6 ± 0.7

Nor, normal control; Model, OVA/LPS-induced asthma control; FEMP50, OVA/LPS-induced asthmatic mice treated with 50 mg/kg/day FEMP; FEMP100, OVA/LPS-induced asthmatic mice treated with 100 mg/kg/day FEMP. The data are expressed as the mean ± SEM

levels of IgE and IL-4 were elevated after the inhalation of OVA/LPS; however, the secretion of IL-17 after stimulation was not higher than that obtained with the normal group, and overall, the secretion levels of IgE, IL-4, and IL-17 in the FEMP-treated groups were lower than those in the model group. The observed decreases in the IgE and IL-4 levels suggest that the FEMP might exert an anti-asthma effect by reducing these factors. Consequently, the reduction of IgE and IL-4 production obtained with the FEMP could alleviate various symptoms of asthma. No significant differences in IL-17 secretion in the BALF and serum was found between the normal and model groups. Although the IL-17 level in the BALF of the FEMP100 group was significantly lower than that obtained with the model group, the secretion of IL-17 in the BALF and serum generally did not show a significant difference among the groups. In this study, severe asthma was not induced by OVA and LPS, which might explain why the secretion of IL-17 was not significantly stimulated in the model group compared with the normal group; consequently, no significant decrease in the IL-17 levels in the BALF was found in the FEMP-treated groups with the exception of the FEMP100 group. Therefore, further studies are needed to evaluate the anti-severe asthma effect of the FEMP.

Reducing inflammatory cells and alleviation of lung inflammation by FEMP in OVA/LPS-induced asthma mice model

After asthma is induced, inflammatory cells, such as eosinophils, neutrophils, and macrophages, usually infiltrate in the lung, and the morphology of the airway smooth muscle layer in the lung becomes thickened compared with that of the normal lung (Ichinose, 2009). As lung inflammation progresses, peribronchial infiltration and alveolar involvement become severe (Dubin and Kolls, 2007). Lung inflammation was observed around bronchial tubes (Fig. 4A), and the lung inflammation score of the model group was also significantly higher than that of the normal group (Fig. 4B). In contrast, the lung inflammation scores of the FEMP-administered groups significantly decreased compared with the model group in a dose dependent manner. The BALF was used to investigate the infiltration of inflammatory cells. The number of total cells in the

BALF of the model group was significantly higher than that in the BALF of the normal group (Fig. 4C). Compared with those found for the model group, the cell numbers were significantly decreased by FEMP treatment in a dose-dependent manner. In addition, compared with those found for the model group, the numbers of macrophages and eosinophils were significantly decreased by FEMP treatment in a dose-dependent manner (Fig. 4D). Asthmatic lung tissue has characteristic features, which generally include increased thickness of the lamina propria. The lamina propria of lung tissue is a thin layer that usually involves the mucosa, connective tissue, and blood vessels, whereas the asthmatic lamina propria is filled with diverse inflammatory cells, such as eosinophils, neutrophils, macrophages, and mast cells; consequently, asthma is associated with a characteristic structure in lung tissue (Pesci et al., 1993). The administration of FEMP significantly alleviated lung tissue inflammation and decreased inflammatory cell infiltration in the BALF; in particular, the number of eosinophils was significantly decreased by the FEMP in a dose-dependent manner, and the histological analysis indicated that this treatment alleviated lung tissue inflammation by reducing inflammatory cells. The results suggest that administration of the FEMP significantly inhibits the secretion of inflammatory cytokines, which decreases inflammatory cell infiltration in the BALF and thereby induces the remodeling of lung tissue damaged by inflammation.

Suppression of cough by FEMP in citric acid (CA)-induced coughing guinea pig model

The cough reflex is essential under physiological conditions and is considered an ordinary symptom of respiratory diseases, such as chronic obstructive pulmonary disease. Chronic cough not only negatively impacts health-related quality of life but might induce an increase in inflammatory mediators by imposing physical stress on airway cells (Wex and Bouyssou, 2015). Cough is also a symptom of asthma. Aggravated inflammatory conditions in the airway stimulate sensory nerves, and this stimulation cause coughing. Chronic cough is caused by cough reflex hypersensitivity, which is generated by mast cell activation and neurogenic inflammation (Qiu et al., 2011). In this

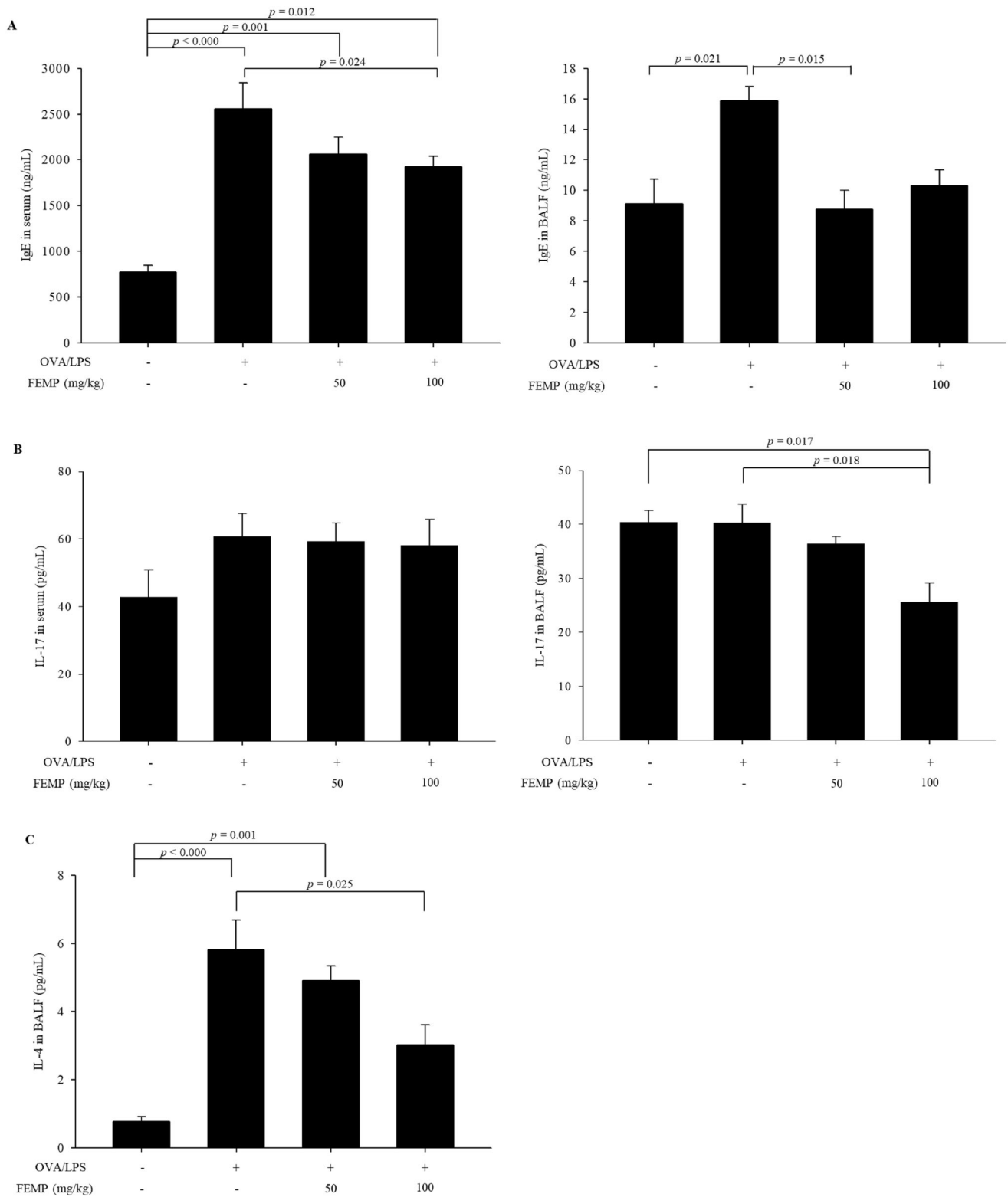


Fig. 3 The FEMP decreased the secretion levels of IgE and IL-4 in an OVA/LPS-induced asthma mouse model ($n = 8/\text{group}$). BALB/c mice exhibited asthma induced by OVA/LPS, and the levels of IgE and cytokines were measured by ELISA. Secretion level of IgE in the

BALF and serum (A); secretion level of IL-17 in the BALF and serum (B); secretion level of IL-4 in the BALF (C). The data are expressed as the mean \pm SEM and were analyzed by one-way ANOVA/Tukey's HSD method

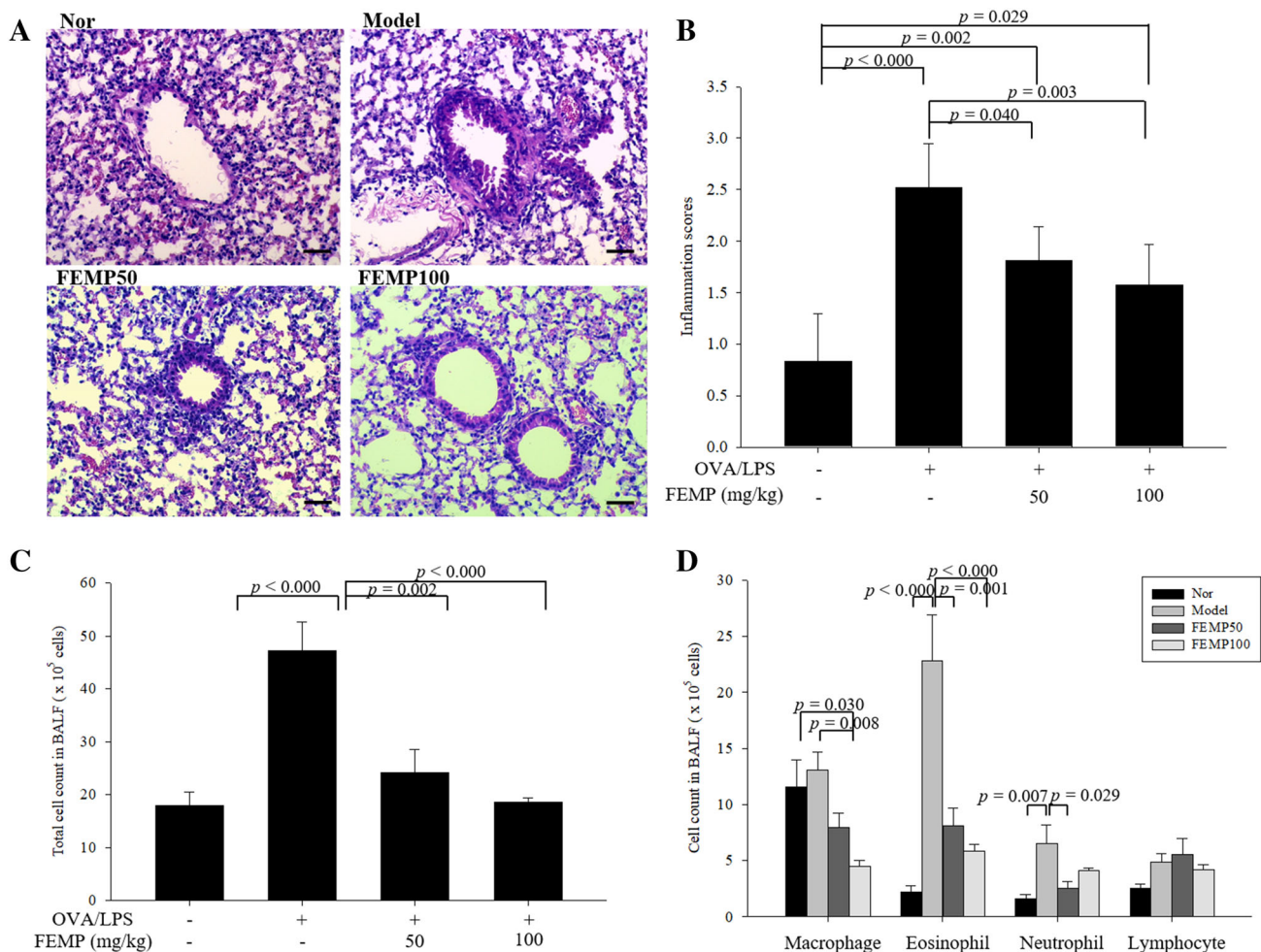


Fig. 4 The FEMP relieved lung tissue inflammation and inflammatory cell infiltration in mice with OVA/LPS-induced asthma ($n = 8/\text{group}$). H&E staining of lung tissues of mice with OVA/LPS-induced asthma (A). The scale bar indicates 50 μm , and the tissue was observed at 200 \times magnification. Lung inflammation scores (B); total number of inflammatory cells (C); numbers of

macrophages, eosinophils, neutrophils and lymphocytes (D). Nor, normal control; Model, OVA/LPS-induced asthma control; FEMP50, OVA/LPS-induced asthmatic mice treated with 50 mg/kg/day FEMP; FEMP100, OVA/LPS-induced asthmatic mice treated with 100 mg/kg/day FEMP. The data are expressed as the mean \pm SEM and were analyzed by one-way ANOVA/Tukey's HSD method

study, a CA-induced coughing model was used to investigate the antitussive effect of the FEMP. CA has been commonly used as a tussive material in animal studies and stimulates A δ -fiber, C-fiber and A-fiber terminals to induce coughing (Takahama et al., 1993). The active components oxyresveratrol and rosmarinic acid in FEMP have influential anti-inflammatory and antioxidant effects. Antioxidants inhibit cough hypersensitivity induced by hyperoxia (Brozmanova and Hanacek, 2018). Compared with that in the CA-induced control group, the number of CA-induced coughs was significantly decreased by FEMP treatment in a dose-dependent manner (Fig. 5). These results show that the FEMP effectively inhibits CA-induced cough. To examine the effects of FEMP on CA-induced coughing guinea pigs in terms of their body weight or hepatotoxicity, we measured the body weights of the animals and major

hepatotoxicity indicators, such as GOT and GPT. Administration of the FEMP did not result in any difference in body weight, and the GOT and GPT measurements indicated no hepatotoxicity (Table S2). These results suggest that the FEMP might protect respiratory health without inducing hepatotoxicity. Although CA-induced coughing does not occur through the asthmatic pathway, the results suggest that the FEMP may inhibit asthma-induced coughing by reducing the secretion of inflammatory cytokines.

Fermentation uses enzymes in microbes to degrade high molecular weight compounds into low molecular weight ones and induces bioconversion, which increases extraction yield and produces new compounds. It converts the compounds that are difficult to absorb into absorbable forms. It also undergoes biochemical reactions to make more

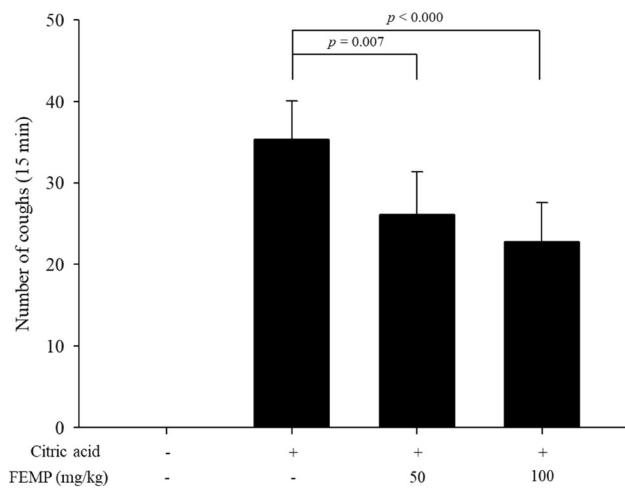


Fig. 5 The FEMP reduced the cough reflex sensitivity in CA-induced coughing guinea pigs ($n = 8/\text{group}$). The FEMP was administered for one week. Coughing was induced by exposure to 0.2 M citric acid for 10 min, and the number of coughs over the subsequent 15 min period was counted. The data are expressed as the mean \pm SEM and were analyzed by one-way ANOVA/Tukey's HSD method

effective compounds from less effective compounds. Mulberroside A, a stilbene glycoside in the *Morus alba* L. is converted to oxyresveratrol by removing glycoside through bio-conversion, which shows more beneficial activities, for instance, more antioxidative and anti-inflammatory activities (Chung et al., 2003). Rosmarinic acid, an effective component of *S. plebeia* R. Brown, can be produced by bioconversion from L-phenylalanine and L-tyrosine using *Saccharomyces cerevisiae* (Babaei et al., 2020). In our further study, we will analyze the chemical composition of FEMP. Oxyresveratrol is a polyphenolic molecule and exerts anti-inflammatory effects by inhibiting the phosphorylation of PI3K/AKT and suppressing NF- κ B signaling pathway (Hankittichai et al., 2020). Rosmarinic acid is also a potential anti-inflammatory agent and is known as a strong antioxidant (Colica et al., 2018). Asthma is a complicated disease occurred by diverse causes such as environmental factors, genetic factors, and hygiene hypothesis. Ultimately, these causes induce long-term inflammation in the airways. Especially, oxidative stress induced inflammatory responses are the main cause of exacerbation of asthma pathogenesis (Mishra et al., 2018). Therefore, the anti-inflammatory mediators and antioxidants can have a therapeutic effect to asthma.

In conclusion, fine dust, which causes respiratory diseases, including asthma, has become an important issue worldwide. The fermented water extract of a mixture of medicinal plants, including *Ramulus mori*, *Salvia plebeia* R. Brown, and *Anthriscus sylvestris*, improved asthma and tussive symptoms through its anti-inflammatory activity. In this study, the FEMP exerted anti-inflammatory effects on

a lung epithelium cell line and lung tissue from an asthma mouse model through the inhibition of proinflammatory cytokines and exhibited antitussive activity in guinea pigs with CA-induced cough. Moreover, the FEMP did not exhibit cytotoxicity or hepatotoxicity *in vitro* and *in vivo*, respectively. Therefore, the FEMP could be used as a potential material for inclusion in functional foods aiming to improve respiratory health.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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