Magnolia kobus DC leaf ethanol extract alleviated lipopolysaccharide-induced acute lung inflammation by suppressing NF-κB and Nrf2 signaling

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Abstract

Introduction: Magnolia kobus DC has been used as herbal medicine to treat coughs and is known to exert biological effects such as anti-inflammatory, antioxidant, and antibacterial properties. We aimed to define the pharmacological effects of M. kobus leaf ethanol extract (MLEE) on acute lung inflammation and explore the underlying mechanisms of action.

Methods: For in vitro investigations, RAW 264.7 cells were pretreated with MLEE (1, 10, and 100 μg/mL) and stimulated with lipopolysaccharide (LPS). For in vivo investigations, BALB/c mice were intratracheally administered with LPS for 24 hours after injection of MLEE (0.3, 3, and 30 mg/kg). Hematoxylin and eosin staining was used for histopathology analysis of lung tissue. The phytochemical constituents of MLEE were analyzed using high-performance liquid chromatography.

Results: In RAW 264.7 cells, MLEE reduced the activation of the inflammatory mediators (inducible nitric oxide synthase and cyclooxygenase-2) and the nuclear translocation of nuclear factor (NF)-κB and nuclear factor erythroid-2-related factor 2 (Nrf2). The intraperitoneal injection of MLEE (30 mg/kg) attenuated interstitial edema and immune cell infiltration in LPS-induced acute lung inflammation. MLEE also inhibited the activation of cyclooxygenase-2, NF-κB, and Nrf2 in the lung tissue.

Conclusion: Taken together, MLEE exerted an anti-inflammatory effect by inhibiting inflammatory and oxidative mediators on acute lung inflammation suggesting that it might be used as a natural drug for treating acute lung inflammatory diseases.

Implication for health policy/practice/research/medical education:
The extract of Magnolia kobus leaf has anti-inflammatory and antioxidative effects evidenced by the activated macrophages and acute lung inflammation mouse model. This suggests potential as a natural product candidate for the remedy of acute lung inflammation.


Introduction

Excessive inflammation of the lung causes abscess formation and fibrosis, leading to acute lung inflammatory diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (1). Ultimately, acute lung inflammation exacerbates lung damage through oxidative stress and rapid-onset respiratory failure (2,3) and can provoke chronic obstructive pulmonary disease.
and asthma (4,5). Therefore, it is important to treat acute lung inflammation before chronic stages. In lung inflammation, the infiltrated inflammatory cells and associated cytokine storm develop into lung disease (6). Macrophages, the major innate immune cells in lung tissue, are found in the alveoli, interstitial lung spaces, and airways. Thereby, macrophages process important roles in the lung, including host defence and immune regulation (7). They are a major source of inflammatory mediators such as chemokines and cytokines that contribute to the immune response (8). Hence, the inhibition of macrophage activation might be a primary target for the remedy of lung inflammation.

The treatment for inflammatory lung disease in the acute phase is applying glucocorticoids as anti-inflammatory therapy (9). Despite the strong effects of glucocorticoids, their usage are limited due to known adverse effects, including coagulopathy, gastric ulcers, and osteoporosis (10-12). Therefore, there is a need for effective anti-inflammatory agents to target acute lung inflammation while minimizing negative effects. Natural ingredients and traditional medicines have been studied for decades and may address this need (13).

*Magnolia kobus* is a deciduous broad-leaved tree distributed in Korea and Japan (14). The Dongui Bogam (1613 by Jun Heo) recorded the traditional medicinal use of *M. kobus*. According to this book, the leaves of *M. kobus* are used for cough relief, the flowers for allergic rhinitis, and the stem bark for dermatitis (15,16). Several studies have already shown the effectiveness of *M. kobus* stem bark in relieving contact dermatitis and inflammation (15,16). It is well-known that the leaves of *Magnolia* are particularly rich in lignans, and phytochemical investigations have revealed that various lignans can be isolated from the leaves of *M. kobus* DC, including sesamin, kobusin, and phillygenin (17). Interestingly, previous studies have reported that lignans isolated from plants attenuate acute lung inflammatory diseases such as ALI and pneumonia (18,19). Based on this knowledge, we hypothesized that *M. kobus* leaves may have anti-inflammatory activity in acute lung inflammation. In this study, we focused on the molecular mechanisms of these effects.

**Materials and Methods**

**Reagents, chemicals, and Magnolia kobus extract**

Lipopolysaccharides (from Escherichia coli 055:B5) and water-soluble dexamethasone (Dexa) were dissolved in phosphate-buffered saline (PBS). The leaves of *M. kobus* were collected from the Korea National Arboretum plantation (Pocheon, South Korea) in 18 August 2020. The medicinal plant was taxonomically identified and a voucher specimen was deposited with the number JMC15122 at the Korea National Arboretum (Pocheon, South Korea). Leaves of *M. kobus* were collected on 18 August 2021, and the plant was identified with a number JMC15122 at the Korea National Arboretum (Pocheon, South Korea) (14). Prof. Gil-Saeng Jeong conducted an experiment to extract the fresh specimens immediately upon receipt, using a method described previously (14).

In brief, *M. kobus* leaves were washed and thoroughly dried. Then, 75.2 g of dried leaves were immersed in 70% ethanol for 24 hours. Next, the sample was extracted by reflux at a constant temperature of 25 °C ± 1 °C through solvent evaporation and condensation for 4 hours. The extraction was performed using a high-performance liquid chromatography-grade ethanol at room temperature for 2 days. Finally, a total of 3.99 g (yield 5.61%, w/w) was obtained from *M. kobus* leaf. It was maintained at −70 °C until use and dissolved in PBS prior to use.

**Animals and ethics statement**

Male BALB/c mice (5 weeks; 21–23 g) were purchased from the Dae-Han Experimental Animal Center (Daejeon, South Korea). Mice were maintained at 55% ± 5% relative humidity and 22 °C ± 2 °C with a light:dark cycle in a laminar airflow chamber. Animal care was carried out in accordance with the guidelines established by the Public Health Service Policy on Human Care and Use of Laboratory Animals.

**Induction of acute lung inflammation in mice**

Experimental mice (*n = 35*) were divided into seven treatment groups (*n = 5/group*): control (sham), MLEE (30 mg/kg), LPS (8 mg/kg), LPS + MLEE (0.3, 3, or 30 mg/kg), and Dexa (3 mg/kg) (20). PBS was used as the vehicle.

MLEE and Dexa were administered by intraperitoneal (IP) injection 1 hour before intratracheal injection of LPS. Then, a ketamine:xylazine:PBS (2:1:7, v/v) mixture solution was IP injected to anesthetize the mice, and LPS was intratracheally injected to induce acute lung inflammation. After 24 hours, mice were euthanized by IP injection of ketamine:xylazine:PBS (7:2:1, v/v) mixture solution. Subsequently, the collected blood was centrifuged to obtain serum. The spleen was collected and weighed. The lungs of the mice were collected for wet/ dry weights, histological observation, qPCR, and Western blot.

**Cell culture**

The RAW 264.7 cells (monocyte/macrophage-like cells, American Type Culture Collection TIB-71™) were maintained in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) at 37 °C in a 5% carbon dioxide atmosphere. The subculture media contained 10% fetal bovine serum and 1 × antibiotic–antimycotic.

**Reactive oxygen species (ROS) detection assay**

MLEE (1, 10, or 100 μg/mL)-pretreated RAW 264.7 (5 × 10⁶ cells/24-well plate) cells were stimulated with LPS (1 μg/mL). After 24 hours, for the cellular ROS detection
Nitric oxide (NO) assay
RAW 264.7 cells were pretreated with 1, 10, or 100 μg/mL of MLEE or 50 μg/mL of Dexta and stimulated with LPS. After 24 hours, the supernatant and an equal volume of Griess reagent were transferred onto a 96-well plate, based on a previous protocol (22). Nitrate levels were measured and quantified using a nitrate standard curve using a spectrophotometer (VersaMax™ Microplate Reader).

Histopathological observation
After euthanasia, the right middle lung lobe was fixed using 10% formalin and sectioned at 6 μm thickness. The slides stained with hematoxylin and eosin were observed for changes in inflammatory cells infiltrating the alveolar area of lung tissue using a bright-field microscope (Carl Zeiss microscope) at 400× magnification. Lung inflammation scores were assessed blindly by five independent investigator using recently published criteria, divided into five groups: None = 1, Mild = 2, Moderate = 3, Severe = 4, and Diffuse = 5.

Lung wet/dry (W/D) weight measurement
Lung weights were measured using an electronic scale (PAG214, OHAUS Co., Parsippany, NJ, USA). The dissected left lung was weighed immediately after the mice were sacrificed. The lungs were then dried in 60 °C slide warmer (C&A Scientific, Manassas, USA) for 72 hours, then the dried lungs were weighed (23). It was calculated using this equation: wet weight/dry weight.

Statistical analysis
GraphPad 7 software (San Diego, CA, USA) was used for statistical analysis. A one-way analysis of variance followed by Dunnett’s multiple comparison test was performed. Statistical significance was determined by a P value of <0.05. Results were expressed as mean ± standard error of the mean (SEM).

Results

Anti-inflammatory effect of Magnolia kobus leaf ethanol extract (MLEE) on RAW 246.7 cells
The photograph in Figure 1A shows the dried leaves of M. kobus before ethanol extraction. The extraction method of MLEE is presented in Figure 1B. First, to rule out the cytotoxic effect of MLEE, the MTT assay was used as an indicator of cell viability and cytotoxicity. The MTT assay indicated that MLEE at concentrations up to 100 μg/mL did not exhibit cytotoxicity in RAW 264.7 cells (Figure S1C, Supplementary file 1). Thus, MLEE was used at concentrations up to 100 μg/mL in vitro experiments.

To assess the anti-inflammatory effect of MLEE, RAW 264.7 cells were activated with lipopolysaccharide (LPS), which amplifies the inflammatory response (24). LPS stimulation increased the gene expression levels of TNF-α, IL-1β, and IL-6 (Figure 1A), whereas MLEE pretreatment suppressed their expression. MLEE also suppressed the secreted proteins of TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW 264.7 cells (Figure 1B). Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are highly upregulated during the inflammatory stage (25). In our experiments, MLEE (100 μg/mL) significantly inhibited the activation of iNOS and COX-2 (Figure 1C). The activation of nuclear factor (NF)-κB is responsible for the expression and production of these pro-inflammatory mediators (26). Therefore, we identified the effect of MLEE in LPS-stimulated RAW 246.7 cells on the nuclear translocation of NF-κB and degradation of IκBα. Results showed that MLEE inhibited IκBα degradation and NF-κB translocation (Figure 1D).

Antioxidative effect of MLEE in RAW 246.7 cells
When LPS-induced inflammation occurs, immune cells are recruited to cause oxidative stress, which includes ROS and nitric oxide (NO) generation. Therefore, inflammation and oxidative stress are closely linked and play important roles in LPS-induced pathogenesis (27). RAW 264.7 cells were photographed to evaluate the effect of MLEE on ROS production. ROS production was found to be notably increased in LPS-stimulated RAW 264.7 cells; however, MLEE reduced ROS production (Figure 2A), in addition to the NO production (Figure 2B) in a concentration-dependent manner. H₂O₂ is another major oxidant produced during the inflammatory response. Macrophages can produce large quantities of H₂O₂ and acute exposure to high concentrations of H₂O₂ causes cell death (28). According to our results, MLEE significantly recovered cell viability at 10 and 100 μg/mL (Figure 2C). Nrf2, a transcription factor that plays a protective role against oxidative stress, is activated by ROS and also has an anti-inflammatory role (29). Stimulated macrophages express high levels of Nrf2, which induces an antioxidant response. In our study, LPS activated the nuclear translocation of Nrf2 in RAW 264.7 cells, which was significantly alleviated by MLEE (Figure 2D). Comprehensively, this indicates that MLEE suppresses Nrf2 nuclear translocation by reducing the production of oxidative stress inducers during the inflammatory response.

Effect of MLEE on symptoms of acute lung inflammation
Acute inflammatory lungs result in histopathological changes such as increased lung mass due to fluid accumulation and immune cell infiltration (30).
Effects of *Magnolia kobus* on lung inflammation

**Figure 1.** Effects of *Magnolia kobus* leaf ethanol extract (MLEE) on lipopolysaccharide (LPS)-induced inflammation. (A) qPCR was performed to assess the expression levels of TNF-α, IL-1β, and IL-6. (B) ELISA was conducted to assess the impact of MLEE on the levels of TNF-α, IL-1β, and IL-6 cytokines. *P < 0.05 vs. LPS-stimulated RAW 264.7 cells group. Data set is displayed as the mean ± SEM (n = 3). (C) Western blot analysis expressed bands for iNOS, COX-2, and β-actin. (D) Translocation of NF-κB was detected using Western blotting in cell fraction protein. Dexa: dexamethasone.

**Figure 2.** Effects of *Magnolia kobus* leaf ethanol extract (MLEE) on oxidative stress and inflammation. (A) Reactive oxygen species (ROS) were stained with 2',7'-dichlorofluorescin diacetate and observed under fluorescence microscopy. Green color = ROS; magnification: 200×; scale bar: 50 μm. (B) Nitric oxide production was assessed by a Griess reaction assay. Data sets are displayed as the mean ± SEM (n = 5). (C) Cell viability with H₂O₂ treatment was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. *P < 0.05 vs. H₂O₂-treated group only. (D) Western blot analysis expressed bands for Nrf2 and β-actin from cytosolic proteins and nucleus-Nrf2 and TATA-binding protein from nuclear proteins. LPS: lipopolysaccharide, H₂O₂: hydrogen peroxide, Dexa: dexamethasone.
An experimental scheme was designed to assess the pharmacological effects of MLEE on acute lung inflammation (Figure 3A). MLEE or LPS did not cause significant changes in spleen weight (Figure 3B). There were no histopathological changes in the lung tissues between the control group and the 30 mg/kg MLEE alone group. LPS-induced mouse lungs showed interstitial edema and infiltrating inflammatory cells in the alveoli; however, MLEE decreased these histopathological changes (Figure 3C). Similarly, lung inflammation scores significantly reduced in 3 mg/kg and 30 mg/kg MLEE groups (Figure 3D). MLEE also decreased the lung Wet/Dry weight ratio increased by LPS (Figure 3E).

**Effect of MLEE on inflammatory mediators in acute lung inflammation**

To assess the role of MLEE in immune response in acute lung inflammation, the serum levels of pro-inflammatory cytokines (TNF-α and IL-6) and immunoglobulin (IgG2a) were measured by ELISA. Compared to the LPS-induced group, the serum levels of TNF-α, IL-6, and IgG2a were reduced by MLEE injection in a dose-dependent manner (Figure 4A). In addition, the LPS only group showed a reduction in serum SOD activity, while it was significantly restored in the MLEE-treated group.

To confirm the effect of MLEE on the gene expression of acute lung inflammation-associated cytokines and chemokines, we performed a qPCR analysis from lung lesions. C-C motif chemokine ligand (CCL3) (as macrophage inflammatory protein 1-α), F4/80 (as mouse macrophage surface marker), IFN-γ (as macrophage activating cytokine), and IL-1β (as pro-inflammatory cytokines) were decreased by MLEE (Figure 4B).

COX-2 and NF-κB activities are known as associated lung inflammatory signaling pathways (31). LPS-induced acute lung inflammation tissue showed significant increases in Nrf2, COX-2, and NF-κB levels; however, MLEE decreased the levels of these proteins (Figure 5).

**Identification of the phytochemicals in MLEE**

To verify the phytochemical underlying the anti-inflammatory and antioxidant effects of the MLEE, high performance liquid chromatography-diode array detector (HPLC-DAD) was performed (Figure 6A), and the isolated constituents were characterized using mass spectrometry (Figure 6B). Chromatographic separation
Effects of Magnolia kobus on lung inflammation

of MLEE revealed the isolation of three bioactive lignans, which were characterized as kobusin, syringaresinol-di-O-glucoside, and phillygenin. The retention times of these chemicals were respectively 23.1, 27.3, and 31.2 minutes, which showed peaks corresponding to reference standards.

Discussion

Acute lung inflammation causes high morbidity and mortality, as it leads to ALI and ARDS. Although glucocorticoids have potent anti-inflammatory effects, they are not a perfect cure, and their side effects are constantly reported (32). Consequently, the development

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**Figure 4.** Effects of Magnolia kobus leaf ethanol extract (MLEE) on immune response in lipopolysaccharide (LPS)-induced acute lung inflammation model mice. (A) ELISA kits were utilized to quantify the serum levels of TNF-α, IL-6, and IgG2a. Serum superoxide dismutase (SOD) activity level was measured using SOD assay kits. (B) The mRNA expression of CCL3, F4/80, IFN-γ, IL-1β, COX-2, and iNOS in LPS-induced acute lung inflammation lesions were measured by qPCR analysis. *P < 0.05 vs. LPS-treated group only. Data sets is displayed as the mean ± SEM (n = 5). Dexa: dexamethasone.

**Figure 5.** Effects of Magnolia kobus leaf ethanol extract (MLEE) on Nrf2, COX-2, and NF-κB activity in lipopolysaccharide (LPS)-induced lung lesions. Western blot analysis expressed bands for COX-2, NF-κB, Nrf2, and β-actin. The band represents three separate experiments. *P < 0.05 vs. LPS-treated group only. The band intensity displays as the mean ± SEM (n = 3). Dexa: dexamethasone.
of a new drug for acute lung inflammation with minimal side effects is urgently needed. From this point of view, interest in researching the effectiveness of natural products is expanding (33). *M. kobus* DC, belonging to the *Magnolia* family, is well known for its various biological functions, such as antioxidative, anti-inflammatory, and antibacterial effects (16,34,35). In various studies, the bioactive components of *Magnolia* have been reported to help treat diverse diseases, including allergy, heart disease, and neuronal inflammation (36-38). In this research, we defined the impact of MLEE in modulating acute inflammatory responses mediated by macrophages in the lung.

Macrophages are essential for innate immunity and the synthesis of inflammatory mediators in the lungs (39). LPS induces NF-κB nuclear translocation by inducing the production of inflammatory mediators by activating the Toll-like receptor 4 (TLR4) pathway in macrophages (40). Thus, LPS-stimulated macrophages increase pro-inflammatory cytokines and chemokines, and ultimately trigger an inflammatory cascade (41). NF-κB is involved in regulating innate and adaptive immune function and mediates the inflammatory responses pivotally (42). In particular, LPS as a TLR4 ligand differentiate macrophage toward M1 phenotype (43). We found that the expression of inflammatory cytokines (TNF-α, IL-1β, IL-6) was decreased by MLEE treatment in activated macrophages. iNOS and COX-2 are pro-inflammatory enzymes that are induced by LPS or inflammatory cytokines (44). MLEE treatment blocked COX-2/iNOS activation, making it possible to inhibit the inflammatory response. Furthermore, MLEE suppressed the nuclear translocation of NF-κB. These results demonstrate that MLEE might be used in the treatment of acute lung inflammation by its inhibitory effect on the inflammatory process in activated macrophages.

Macrophages generate ROS and H₂O₂ by LPS, a substance involved in cellular stress response (45). The production of ROS and H₂O₂ during oxidative stress can cause DNA damage, leading to cell death and tissue damage (46). Nrf2 is a transcription factor that acts as a sensor, regulating genes associated with oxidative stress (47). In this study, MLEE inhibited both Nrf2 activation in LPS-stimulated macrophages as well as ROS and NO production due to its antioxidant effects. These findings suggest that MLEE possesses antioxidant properties in macrophages. Overall, our results showed that MLEE treatment suppressed the inflammatory and oxidative responses of macrophages.

The induction of acute lung inflammation increases alveolar epithelium permeability and inflammatory cell accumulation, eventually leading to pulmonary edema (48). Based on this knowledge, we developed a mouse model of LPS-induced acute lung inflammation that mimicked lung inflammatory lesions. Our results showed that MLEE administration alleviated histopathological changes such as immune cell infiltration and lung edema, and reduced the inflammation score and lung weight ratio.

The spleen is an important part of the immune system, and lymphatic system problems cause an enlarged spleen (49). However, MLEE treatment did not significantly affect the size of the spleen. This result indicates that MLEE treatment relieves the symptoms of acute lung inflammation without significantly affecting the immune system.

In the onset of acute lung inflammation, systemic inflammation is amplified by the release of inflammatory mediators such as TNF-α, IL-1β, and IL-6 (50). With the continuation of acute lung inflammation, the migration

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**Figure 6.** High performance liquid chromatography (HPLC) characterization of *Magnolia kobus* leaf ethanol extract (MLEE). HPLC-diode array detector (DAD) analysis was performed using a Capcell Pak C18 column (5 μm × 4.6 mm × 250 mm) at 240 nm. The peaks were confirmed by the addition of standards for kobusin, syringaresinol-di-O-glucoside, and phillygenin in MLEE.
of Th1 cells to the lung induces the production of IgG2a via IFN-γ, leading to a chronic condition (51). Therefore, to determine whether MLEE regulates the release of inflammatory mediators, we investigated the serum levels of pro-inflammatory cytokines (IL-6 and TNF-α) and IgG2a in LPS-induced acute lung inflammation model mice. As a result, the intraperitoneal administration of MLEE resulted in a reduction of TNF-α, IL-6, and IgG2a levels in the serum of acute lung inflammation model mice. In addition, MLEE induces SOD activity, which catalyzes the removal of free radicals in the antioxidant system (52). Because tissue damage caused by acute lung inflammation is directly correlated with macrophages, inhibition of macrophage activity has been shown to decreased lung damage in several experimental models (53,54). We showed that MLEE inhibited the expression of macrophage-associated CCL3 and F4/80, as well as inflammation-related IL-1β, IFN-γ, COX-2, and iNOS expression in lung-inflamed tissues. These inflammatory mediators alleviated lung inflammation by inhibiting COX-2, NF-κB, and Nrf2 activation in lung lesions. These results indicate that MLEE might inhibit the conversion from acute to chronic inflammation because it reduced inflammatory mediators, both systemically and locally.

Phytochemical researchers have verified that lignans and neolignans can be isolated from M. kobus, M. denudata Desr. and M. kobus var. boreali (17,55,56). Lignans have been widely studied because they have the same chemical structure as steroids, which are used to treat inflammatory diseases (57). Analogously, MLEE also contains three lignan compounds: kobusin, syringaresinol-di-O-glucoside, and phillygenin. Previous studies have shown that kobotin, syringaresinol-di-O-glucoside, and phillygenin exhibit anti-inflammatory effects. In particular, kobusin has been found to possess anti-inflammatory properties in macrophages (58); syringaresinol-di-O-glucoside exerts antitumor effects in human synovial sarcoma cells associated with soft tissues like muscles and lungs (59); phillygenin alleviates inflammation and apoptosis in lung epithelial cells (60). Based on this knowledge, we suggest that the anti-inflammatory and antioxidant effects of MLEE might be related to these three phytochemicals.

In this study, we have demonstrated that MLEE exhibits anti-inflammatory and antioxidant effects by inhibiting NF-κB and Nrf2. We propose that MLEE holds promise as a prospect candidate for addressing inflammatory diseases, particularly acute lung inflammation. The notion that the active constituents of plants differ among various organs is well supported by numerous studies (61,62). Thus, our investigation, which focuses on the pharmacological effects of specific plant organs, could offer valuable insights for the development of pharmaceuticals and functional foods. However, further in-depth research on the characterization of phytochemicals, compound profiling, and the specific pharmacological mechanisms of MLEE is crucial.

Conclusion
This study demonstrates that MLEE inhibits NF-κB and Nrf2, resulting in anti-inflammatory and antioxidant properties in LPS-induced in vitro and in vivo models. MLEE suppressed the activation of inflammatory mediators in macrophages and locally relieved acute inflammatory symptoms in a mouse model of acute lung inflammation. We propose that MLEE could be an excellent alternative therapeutic agent for acute lung inflammation.

Acknowledgments
The authors would like to express their gratitude to all members of the Garden Plant Resources Department of the Korea National Arboretum for their collaboration in identifying and providing the fresh plants.

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Conflict of interests
Authors declare no conflict of interests.

Ethical considerations
All mouse experiments were approved by the animal care center at Kyungpook National University (Institutional Animal Care and Use Committee, IRB# 2022-0389; Daegu, South Korea).

Funding
This research was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2021R1A5A2021614, 2022M3A9G8018189, and 2020M3A9D303894), as well as the Korea National Arboretum for their collaboration in identifying and providing the fresh plants.
Arboretum in South Korea (grant number KNA 1-2-34, 17-9).

**Supplementary files**

Supplementary file 1 contains the following segments: phytochemical identification, cell viability, qPCR, ELISA, and western blotting. Additionally, the supplementary file includes Table S1 and Figure 1.

**References**


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