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Chemical Profile and Anti-inflammatory Effects of *Atractylodes japonica* Rhizome and *Schisandra chinensis* Extract in a PM₁₀-Induced Murine Airway Inflammation Model

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Abstract

Background Airborne pollutants, particularly exposure to particulate matter (PM), is a major risk factor for the development and exacerbation of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and bronchitis. PM can penetrate the lower respiratory tract, disrupt epithelial barrier integrity, and promote the release of pro-inflammatory cytokines and chemokines, leading to persistent airway inflammation and structural remodeling. Recent research has focused on natural products for their potential applications in managing respiratory diseases. *Atractylodes japonica* rhizome has been shown to exert anti-inflammatory, anti-oxidant, mucolytic, and anti-bacterial effects, particularly in bronchitis and respiratory infections. Moreover, a fruit extract of *Schisandra chinensis* containing schisandrin (SZD) can effectively reduce lipopolysaccharide (LPS)-induced acute lung injury and cigarette smoke-induced COPD by reducing inflammatory cytokine production and oxidative stress. These findings provide a strong rationale for respiratory protective effects of CKD-F497, a standardized extract of the rhizome of *A. japonica* and the fruit of *S. chinensis* as a potential therapeutic agent for PM-induced airway inflammation. This study aimed to compare the chemical profiles of CKD-497 and CKD-F497 using qualitative analysis and to evaluate the respiratory protective effects of CKD-F497 in both in vitro and in vivo models of PM₁₀-induced airway inflammation.

Methods CKD-F497 was obtained by extracting a 5:1 mixture of *A. japonica* rhizomes and *S. chinensis* fruits with 50% ethanol at low temperature, followed by concentration and drying. The extract was chemically characterized by high-performance liquid chromatography with diode-array detection (HPLC–DAD), confirming multiple constituents from CKD-F497. For in vivo assessment, a murine airway inflammation model was induced by intratracheal instillation of urban PM₁₀ (200 ug per dose, administrated on days 0, 1, 7, 8, 14, and 15) in BALB/c mice. Mice were orally treated with CKD-F497 or vehicle control once daily for 21 days during the PM₁₀ exposure period. On day 21, assessment was conducted including inflammatory cell infiltration in bronchoalveolar lavage fluid (BALF), measurement of pro-inflammatory cytokines, histopathological evaluation of lung injury and collagen deposition. For in vitro analysis, RAW 264.7 mouse macrophages were pretreated with CKD-F497 to stimulation with LPS, and inflammatory signaling was evaluated. NF-κB/MAPK pathway were analyzed by Western blot to anti-inflammatory effects of CKD-F497.

Results High-performance liquid chromatography–diode array detection analysis of CKD-497 and CKD-F497 extracts revealed highly similar chromatographic patterns, with nine major peaks including the key bioactive compounds

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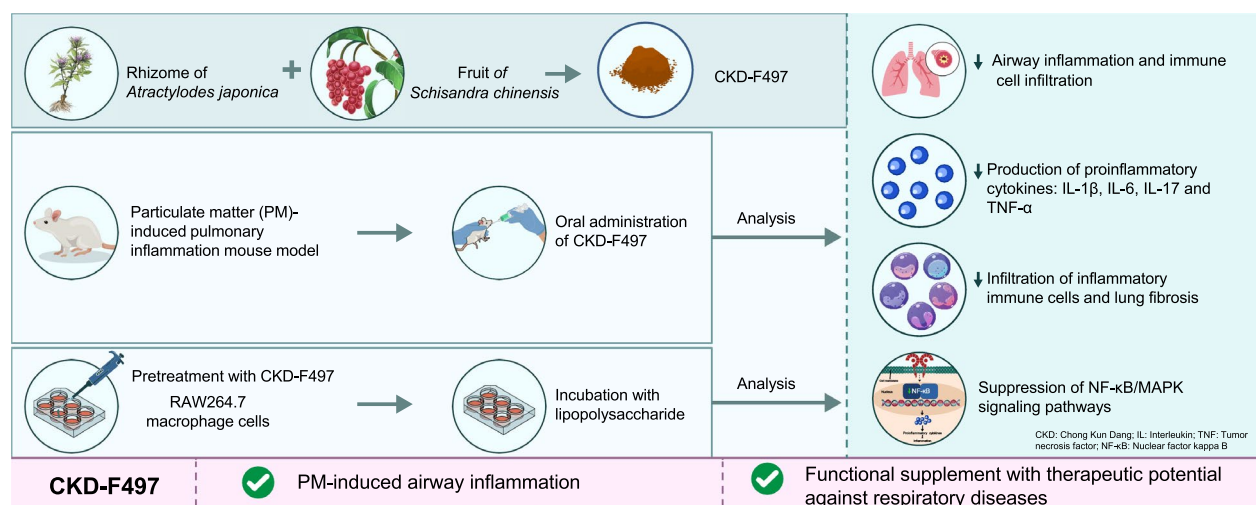
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6(E),12(E)-tetradecadine-8,10-diyne-1,3-diol (TDD) and schisandrin (SZD) consistently detected in both extracts. The strong positive correlation in peak composition confirmed that CKD-F497 retains the chemical integrity and active constituents of the CKD-497, supporting their compositional equivalence. In addition, oral administration of CKD-F497 in a PM₁₀-induced pulmonary inflammation mouse model effectively ameliorated airway inflammation, decreased immune cell infiltration in the BALF, lowered serum levels of pro-inflammatory cytokines, and ameliorated hispathological signs of lung injury and collagen deposition. Furthermore, in LPS-simulated RAW 264.7 mouse macrophage cell, CKD-F497 effectively inhibited LPS-mediated inflammatory response by deactivating NF- κ B through suppression of the MAPK signaling pathway. These findings suggest that CKD-F497 is an effective option for treating airway inflammation and associated respiratory conditions.

Conclusion CKD-F497, extract from *A. japonica* and *S. chinensis*, exhibits potent anti-inflammatory and anti-fibrotic effects in a PM₁₀-induced murine airway inflammation model and mitigates LPS-induced inflammatory activation in macrophages. These results highlight the therapeutic potentials of CKD-F497 for treating airway inflammation caused by air pollutants. CKD-F497 could serve as a functional supplement to protect against respiratory inflammatory diseases.

Keywords CKD-F497, Fine dust, Respiratory diseases, Anti-inflammatory effect, NF- κ B

Graphical Abstract



1 Introduction

Air pollution, particularly exposure to particulate matter (PM), is a well-established risk factor for the development and exacerbation of respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and bronchitis [1]. PM can penetrate deep into the respiratory track and deposit in the alveoli, triggering the release of pro-inflammatory mediators, which recruit immune cells to the airways and leading to airway inflammation [2, 3]. Prolonged exposure to PM further damages the airway epithelium, resulting in structural remodeling characterized by epithelial thickening, airway narrowing, subepithelial fibrosis, and potentially irreversible impairment of lung

function [4]. In addition to PM exposure also has been shown to prime macrophages for a hyperinflammatory response upon subsequent stimulation, mainly through the upregulation of NF- κ B/MAPK pathway [5]. This macrophage-mediated inflammation amplification contributes to the exacerbation of airway inflammation and tissue remodeling in PM-related respiratory diseases [6].

Recently, there is a growing interest in the study of active compounds derived from botanic resources for their potential applications in managing respiratory diseases [7–9]. The rhizome of *A. japonica* contains compounds known for their anti-obesity, anti-inflammatory, and gastroprotective properties [10–12]. Pharmacological

studies have documented the utilization of the rhizome of *A. japonica* in managing conditions such as bronchitis, and respiratory infectious diseases [13]. Moreover, the *A. japonica* rhizome inhibits the production of pro-inflammatory cytokines that mediate late/chronic allergic inflammatory reactions [13]. In addition, phytochemicals isolated from the rhizome exhibit antibacterial activity against methicillin-resistant *Staphylococcus aureus*, further supporting its potential as an immunomodulatory agent [14]. The dried mature fruit of *S. chinensis* ‘Baillon’, is used in Asia to alleviate symptoms, such as dyspnea, cough, amnesia, and dysentery [15]. Similarly, bioactive constituents of *S. chinensis* and its derivatives exhibit a range of beneficial physiological activities, including anti-cancer, antidiabetic, anti-oxidant, and anti-aging effects [16–18]. Importantly, an extract of *S. chinensis* containing schisandrin (SZD) can effectively reduce lipopolysaccharide (LPS)-induced acute lung injury and cigarette smoke-induced COPD by inhibiting the inflammatory cytokine production and reducing oxidative stress [19–23]. Based on these findings, the rhizome of *A. japonica* and of *S. chinensis* have emerged as promising candidates for the treatment of respiratory diseases. Accumulating data have demonstrated that *A. japonica* extracts protect against PM₁₀-induced pulmonary injury by down-regulating PI3K/Akt and p38 MAPK signaling pathways, reducing oxidative stress and inflammation, as well as suppressing mucus hypersecretion. Notably, *A. japonica* exhibited unique mucolytic expectorant activities absent in dexamethasone-treated mice, thereby supporting its therapeutic potential for respiratory diseases [24].

CKD-F497, a dry, plant-based functional food extract derived from the rhizome of *A. japonica* and the fruit of *S. chinensis*, was developed by the pharmaceutical company Chong Kun Dang (CKD). It has been optimized for uniform composition by drying the CKD-497 soft extract, known for its effectiveness in ameliorating airway inflammation and preventing lung injury by inhibiting the NF- κ B/MAPK pathway [25]. The aim of this study was to compare the chemical profiles of CKD-497 and CKD-F497 through qualitative analysis and to verify the respiratory protective effects of CKD-F497 in both in vitro and in vivo experimental models. Our findings demonstrated that CKD-F497 ameliorates PM₁₀-induced airway inflammation and lung fibrosis, suggesting that CKD-F497 is an effective approach protecting the respiratory-related diseases.

2 Materials and Methods

2.1 Materials

2.1.1 CKD-F497 Dry Extract

CKD-497 was extracted as previously described [25]. The CKD-497 soft extract and dextrin drying agent were

mixed at a 1:1 ratio, powdered, and then dried to obtain a dry extract. Finally, CKD-F497, the optimal raw material containing 6(E),12(E)-tetradecadine-8,10-diyne-1,3-diol (TDD) and schisandrin (SZD), the active compounds in the rhizome of *A. japonica* and fruit of *S. chinensis*, was obtained.

2.2 Methods

2.2.1 Qualitative Analysis of High-performance Liquid

Chromatography–Diode Array Detection (HPLC–DAD)

A diode array detector (DAD; Agilent, USA) was used with an Agilent 1260 series HPLC instrument (Agilent) for chromatographic qualitative analysis of nine marker compounds. The system control and data analyses were processed with Agilent OpenLAB CDS ChemStation software (Agilent). Chromatographic separation was performed using a Phenomenex Gemini column (4.6 mm $\times$ 250 mm, 5 μ m particle size) at 30 °C. The mobile phases used were water (A) and acetonitrile (B). Ten microliters of the samples was injected into the column and eluted at a flow rate of 1 ml/min following gradients: initial 1 min/8% B; 16 min/8%–18% B; 17 min/42% B; 18–21 min/55% B; 50 min/58% B; 55 min/80% B; 56–60 min/8% B; UV detection was set at 254 nm.

2.2.2 Cell Culture

Raw 264.7, a mouse macrophage cell line (TIB-71™, American Type Culture Collection Manassas, VA, USA), was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (#11,965,092; Gibco, Thermo Fisher Scientific Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, #A3160501; Gibco), 300 mg/l L-glutamine, 25 mM HEPES, and 25 mM NaHCO₃, 100 U/ml penicillin, and 100 μ g/ml streptomycin (#15140122; Gibco). The cells were routinely passaged at 70% confluency and maintained at 37 °C in a humidified incubator containing 5% CO₂.

2.2.3 Nuclear and Cytoplasmic Fractionation

To evaluate NF- κ B nuclear translocation, nuclear and cytoplasmic protein fractions were isolated using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Fisher Scientific) following the manufacturer's protocol. RAW 264.7 cells were seeded in 6-well plates (1 $\times$ 10⁶ cells per well) and incubated overnight at 37 °C in a humidified 5% CO₂ incubator. The cells were stimulated with LPS for 4 h. After treatment, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested by scraping. For cytoplasmic extraction, cell pellets were resuspended in Cytoplasmic Extraction Reagent I (CER I), vortexed vigorously for 15 s, and incubated on ice for 10 min. Next, Cytoplasmic Extraction Reagent II (CER II) was added,

followed by vortexing and centrifugation at $16,000\times g$ for 5 min at 4 °C. The supernatant containing the cytoplasmic fraction was collected in a new tube and stored at −80 °C. For nuclear extraction, the remaining pellet was resuspended in Nuclear Extraction Reagent (NER), vortexed for 15 s, and incubated on ice for 40 min, with vortexing every 10 min. The suspension was then centrifuged at $16,000\times g$ for 10 min at 4 °C, and the supernatant containing nuclear proteins was collected and stored at −80 °C. To confirm the purity of the fractionated proteins, western blot analysis was performed using β -actin as a cytoplasmic marker and lamin B1 as a nuclear marker.

2.2.4 Western Blot

Raw 264.7 cells (2×10^6 cells/ml) cultured in six-well plates were incubated at 37 °C in 10% DMEM overnight. On the next day, the cells were subjected to 2 h medium deprivation, followed by 1 h pretreatment with CKD-F497 (100, 200 and 400 μ g/ml). Subsequently, the cells were exposed to LPS (#L2880; Sigma–Aldrich) for an additional 2 h. Thereafter, whole-cells lysates were harvested with ice-cold cell extraction buffer (#FNN0011; Sigma–Aldrich) containing protease inhibitor cocktail (A32965; Thermo Fisher Scientific). The cell lysates were quantified using QuickStart™ Bradford Protein Assay Kit 2 (#5000202; Bio-Rad, Hercules, CA, USA). The cell lysates (15 μ g) were resolved on 4–12% Bis–Tris gel (NP0322BOX; Thermo Fisher Scientific) and subsequently transferred onto nitrocellulose membranes. The membranes were treated with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and probed with specific primary antibodies overnight. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (#31,460 and #31,430, respectively; Thermo Fisher Scientific) for 1 h at room temperature. β -Actin was used as a loading control. The following primary antibodies were obtained from Cell Signaling Technology (CST; Danvers, MA, USA) and were diluted at a 1:1000 ratio: phospho-phospho–NF- κ B inhibitor (I κ B)- α (#9246), I κ B- α (#9242), phospho-NF- κ B p65 (#3031), NF- κ B p65 (#8242), phosphor-p38 (#9211), p38 (#9212), lamin B1 (#12568), and β -Tubulin (#2146). The other primary antibody used was β -actin (1:2000; #MA5-15739; Thermo Fisher Scientific). Images of the membranes were visualized using a chemiluminescence imaging system (ChemiDoc MP; Bio-Rad), and band intensities were quantitated using ImageJ software.

2.3 Animals and Experimental Design

2.3.1 Ethics Approval and Experimental Mice

Six-week-old female BALB/c mice were procured from a specific pathogen-free facility at Koatech (Gyeonggi-do, Republic of Korea). They were housed under standard conditions (12 h light/12 h dark cycles, temperature 23 ± 3 °C, and $55\pm 15\%$ humidity). The in vivo efficacy study was conducted at HLB Biostep Co., Ltd. (formerly Knotus; Incheon, Republic of Korea), a contract research organization. Approval to conduct the study was granted by the Institutional Animal Care and Use Committee of HLB Biostep (IACUC 22-KE-0467). Humane endpoints were established in accordance with IACUC guidelines to minimize animal suffering. The predefined human endpoints included: (1) severe impairment of movement or difficulty walking on all four limbs, (2) a body temperature drop of more than 4 °C below normal, (3) a reduction in body weight exceeding 10% of the normal weight, (4) severe skin ulceration or lesions occurring in 3–4 areas of the dorsal region, and (5) the occurrence of other complications that significantly restrict movement and behavior. At the end of the experimental period, all mice exhibited normal behavior, feeding activity, and body temperature, without any signs of distress or abnormal clinical symptoms such as diarrhea. No animals reached any of the predefined humane endpoints during the study. At the conclusion of the study, all mice were humanely euthanized under avertin anesthesia (240 mg/kg, intraperitoneal injection) followed by cervical dislocation. Death was confirmed by the complete cessation of respiration and heartbeat, as well as the loss of reflexes.

2.3.2 PM₁₀-Induced Lung Inflammatory Mouse Model

The mice were assigned to five groups: control (only treated with phosphate buffered saline, PBS), PM₁₀ (PM₁₀-treated model), PM₁₀+CKD-F497 67.7 mg/kg (PM₁₀ and CKD-F497 67.7 mg/kg-treated model), PM₁₀+CKD-F497 135.4 mg/kg (PM₁₀ and CKD-F497 135.4 mg/kg-treated model), and PM₁₀+CKD-F497 180.4 mg/kg (PM₁₀ and CKD-F497 180.4 mg/kg-treated model). Standardized reference materials (SRM 2787 (fine PM<10 μ m)) were procured from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). To establish a murine model of PM₁₀-induced lung inflammation (PM₁₀ model), mice were anesthetized using Avertin (2,2,2-tribromoethanol (#T48402; 240 mg/kg of body weight; Sigma, Germany), administered via intraperitoneal injection. After anesthesia, the mice received an intratracheal instilled with 200 μ g of PM₁₀ dissolved in 20 μ l PBS on multiple days (days 0, 1, 7, 8, 14, and 15). Control mice were

intranasally treated with 20 μ l PBS on the same days. The PM₁₀+CKD-F497 (67.7, 135.4, and 180.4 mg/kg) group mice were orally administered CKD-F497 once per day for 21 days using a Zonde needle. The three doses (67.7, 135.4 and 180.4 mg/kg) used in the in vivo efficacy study were selected based on their equivalent clinical doses.

2.3.3 Collection and Analysis of Serum and Bronchoalveolar Lavage Fluid (BALF)

Serum and BALF samples were collected on day 21. Briefly, each mouse was anesthetized using avertin (240 mg/kg) via intraperitoneal injection and a 1-ml syringe was inserted into the abdominal vena cava to collect whole blood. A total of 1 ml blood samples were collected from each mouse in a single blood collection, and the serum was obtained by centrifuging the sample at 201 $\times$ g for 10 min. BALF was collected according to a previously reported method [19]. Briefly, the tracheal bronchus was carefully functured, and the right bronchus was ligated using 3–0 silk. A catheter was then inserted into the left trachea to wash the lungs with 1 ml of ice-cold 1 $\times$ PBS three times and centrifuged at 400 $\times$ g for 5 min. The inflammatory cytokine levels in serum samples were measured. Mouse cytokine enzyme-linked immunosorbent assay (ELISA) kits were used to measure the levels of IL-6, TNF- α , IL-1 β , and IL-17A (#MLB00C, #MTA00B, #M6000B, and #M1700, respectively; R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.3.4 Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells from the lung and BALF were stained with the indicated antibodies in staining solution (PBS containing 0.5% BSA; pH 7.2) for 10 min on ice. The stained cells were analyzed using two-color flow cytometry on FACS-Calibur with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). All antibodies, CD45, Ly6G, CD11b, CD11c, SiglecF, and F4/80 (#63–0451-82, #11–9668-82, #47–0112-82, #47–0112-82, #45–0114-82, #50–1702-82, and #25–4801-82), were purchased from Invitrogen (Carlsbad, CA, USA).

2.3.5 Histopathological Analysis of the Lung and Tracheal Tissues

Mouse lungs were fixed in 4% paraformaldehyde in PBS (Sigma, Germany) at 4 °C for 48 h, embedded in paraffin, sectioned at 5- μ m thickness, and stained with hematoxylin and eosin (H&E). Lung fibrosis was assessed by staining with a Picro-Sirius Red kit (#ab150681; Abcam, Cambridge, MA, USA). To determine the degree of inflammatory cell infiltration, the peribronchial cell count was quantified in the airway epithelium in a blind

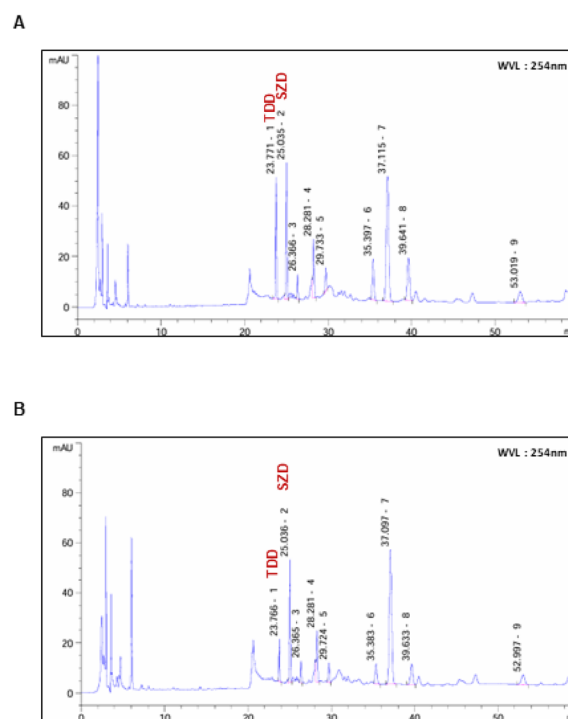


Fig. 1 Chromatographic comparison of CKD-497 and CKD-F497. Mobile phase: water/acetonitrile (92:8, v/v), flow rate: 1.0 mL min⁻¹ at 30 °C, and UV detection at 254 nm. High-performance liquid chromatography with diode-array detection (HPLC–DAD) was employed for simultaneous determination of nine natural substances. **A** Extracted compounds from CKD-497 and **B** CKD-F497. Peaks marked with 1 and 2 are 6(E),12(E)-tetradecadiene 8,10-diyne-1,3-diol (TDD) and schisandrin (SZD) respectively. Peaks 3 and 4 represent atratylenolide III and atratylenolide I, respectively

manner using a 4-point grading system. Images were obtained using an optical microscope (BX53; Olympus, Japan).

2.3.6 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM). The significance of differences was determined using one-way analysis of variance, followed by Dunnett's multiple comparison test. Results with P values less than 0.05 were considered statistically significant and denoted as “*”, “**”, and “***”, respectively.

3 Results

3.1 Chemical Profiling Analysis of CKD-497 and CKD-F497

HPLC–DAD analysis was performed to identify the similarity between the soft extract CKD-497 and dry extract CKD-F497. The analysis revealed a representative HPLC

chromatogram of CKD-497 and CKD-F497 (Fig. 1A, B). Among the peaks occupying an area of >1% of the total peak area, nine peaks with confirmed repeatability and precision were considered as major peaks constituting the component profile. Additionally, the sum of the area of these nine peaks was >60% of the total peak area. The Pearson correlation coefficient for the similarity between CKD-497 and CKD-F497 was 0.970, indicating a strong positive correlation. The marker compounds TDD and SZD in CKD-F497 were chromatographed at retention times of 23.771 and 25.035 min (Fig. 1A), whereas those in CKD-F497 were analyzed at 23.766 and 25.036 min, indicating that the retention time was similar between the two (Fig. 1B).

We further performed quantitative analysis of TDD and SZD present in CKD-497 and CKD-F497. A presentative HPLC chromatogram of CKD-497 and CKD-F497 was obtained (Supplementary Fig. 1A, B), with peaks mainly identified based on reference substances (Supplementary Fig. 1C). The standard solution of TDD and SZD showed retention times of 14.557 and 18.584 min, respectively. Two major peaks were identified from CKD-497 and CKD-F497, matching their retention times of the reference standards. Quantitative analysis revealed the presence of 4.368 mg/g (TDD) and 2.033 mg/g (SZD) in CKD-497 and 2.610 mg/g (TDD) and 1.169 mg/g (SZD) in CKD-F497 (Table 1). These results indicate that CKD-497 and CKD-F497 are similar, with no changes in the TDD and SZD content by the dextrin drying agent.

3.2 CKD-F497 Downregulated NF-κB/MAPK Signaling in LPS-Activated Murine RAW 264.7 Cells

Based on our results that CKD-497 and CKD-F497 are similar, we determined whether CKD-F497 could alleviate the inflammatory response similar to CKD-497 [20]. We assessed the expression of inflammatory proteins in

LPS-stimulated RAW 264.7 cells. After LPS stimulation, we measured p38 and NF-κB p65 protein translocation using western blot analysis. As demonstrated in Fig. 2A, LPS stimulation significantly increased the phosphorylation of p38, IκBα, and p65 in RAW 264.7 cells compared with that in control cells. We confirmed that CKD-F497 treatment suppressed LPS-induced nuclear translocation of NF-κB p65 (Supplementary Fig. 1). In addition, the level of p38 phosphorylation considerably decreased in the CKD-F497-treated group (Fig. 2B). These results show that CKD-F497 reduced NF-κB nuclear translocation in a dose-dependent manner, suggesting its inhibitory effect on NF-κB/MAPK activation.

3.3 CKD-F497 Alleviated Pulmonary Pathological Changes Induced by PM₁₀

Based on the observed anti-inflammatory effects of CKD-F497 in RAW 264.7 cells, we evaluated the long-term (once daily for 21 days) effects of 3-week supplementation of CKD-F497 (Fig. 3A). Histopathological changes and inflammatory responses in PM₁₀-treated mice indicated significant pulmonary damage and accumulation of multiple inflammatory cells in peribronchiolar and perivascular spaces after 3 weeks. Peribronchiolar and perivascular infiltration of immune cells in the PM₁₀ group was significantly higher than that in the control group. However, oral administration of CKD-F497 (67.7, 135.4 and 180.4 mg/kg) significantly reduced pulmonary injury, accompanied by a reduction in alveolar structure remodeling and a decrease in immune cell infiltration into the alveoli (Fig. 3B). In particular, long-term administration of CKD-F497 at 180.4 mg/kg had anti-inflammatory effects in both perivascular and peribronchiolar regions (Fig. 3C). These results suggest that long-term

Table 1 Quantification of TDD and SZD contents in CKD-497 and CKD-F497 extracts

	TDD content (mg/g)	Average (mg/g)	SZD content (mg/g)	Average (mg/g)
CKD-497	4.390	4.368 (RSD % 0.446)	2.043	2.033 (RSD% 0.450)
	4.363		2.031	
	4.352		2.026	
CKD-F497	2.625	2.610 (RSD % 0.538)	1.158	1.169 (RSD% 0.854)
	2.597		1.171	
	2.607		1.178	

The contents of TDD and SZD in CKD-497 and CKD-F497 were quantified using HPLC analysis. A calibration curve was generated using reference compound (TDD: 5–60 mg/kg, SZD: 1–15 mg/kg), and the concentrations were calculated accordingly. Relative standard deviation (RSD) values were ≤0.538% for TDD and ≤0.854% for SZD. Data represent the mean values from three independent experiments

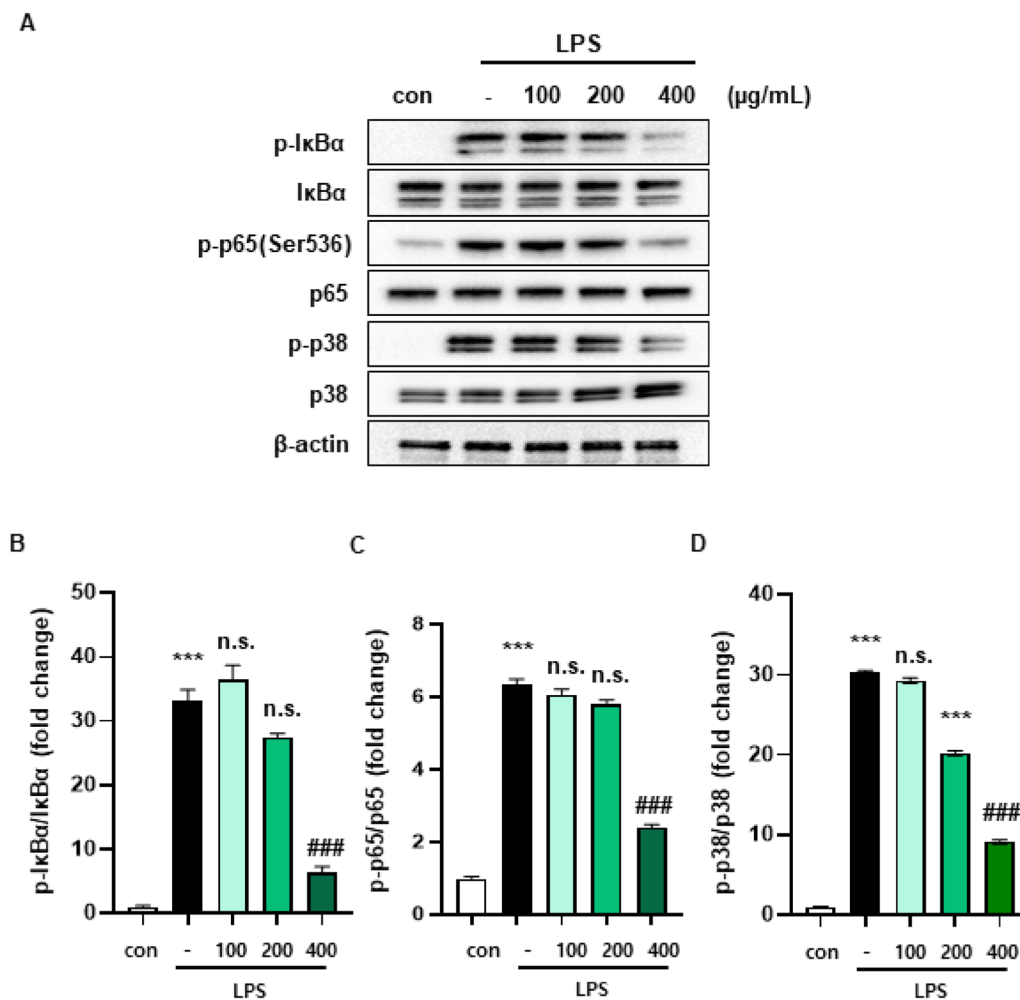


Fig. 2 CKD-F497 reduced LPS-induced NF-κB/MAPK signaling and alleviated the inflammatory response in RAW 264.7 cells. A RAW 264.7 cells were pretreated with CKD-F497 for 2 h followed by 2 h of LPS treatment. Protein expression levels of phospho-p65 NF-κB (p-p65), p65, phospho-IκBα, IκBα, phospho-p38, and p38 were analyzed using western blotting; β-actin was used as the loading control. B-D Quantitative analysis of western blot results of p-p65 per p65, p-IκBα per IκBα, and p-p38 per p38 using Image J software. All data were analyzed using a one-way analysis of variance followed by Dunnett's multiple comparison test. *** $P < 0.001$ vs. control. ### $P < 0.001$ vs. PM₁₀. Results are presented as mean ± SEM

administration of CKD-F497 exerts anti-inflammatory and protective effects in lung tissues.

3.4 CKD-F497 Decreased PM₁₀-Induced Inflammatory Responses in BALF and Serum

We examined the effect of CKD-F497 on immune cell infiltration into the airways by conducting flow cytometry analysis of BALF obtained from PM₁₀-induced mice. We assessed CD45 expression on the whole leukocyte population, including lymphocytes, monocytes, and granulocytes, in BALF. The proportions of total lymphocytes in the BALF of mice from the PM₁₀-treated group was significantly higher than those of mice from the control group, indicating ongoing pulmonary inflammation in the airway compartment. Conversely, mice treated

with CKD-F497 (at doses of 67.7, 135.4 and 180.4 mg/kg) showed significantly decreased numbers of CD45⁺ leukocytes (Fig. 4A). We also quantified macrophages, lymphocytes, and neutrophils among the leukocytes in BALF. Significant recruitment of CD45⁺F4/80⁺ macrophages, CD45⁺LY6G⁻CD11b⁻ lymphocyte and CD11c⁻CD11b⁺Ly6G⁺ SiglecF⁻ neutrophils was accompanied in PM₁₀-treated mice. In contrast, the populations of macrophages, lymphocytes, and neutrophils in the CKD-F497-treated group (at doses of 67.7, 135.4 and 180.4 mg/kg) were lower than those in PM-treated group. In particular, the numbers of alveolar macrophages and neutrophils were significantly low in the CKD-F497 (180.4 mg/kg) group (Fig. 3A). PM₁₀ exposure also

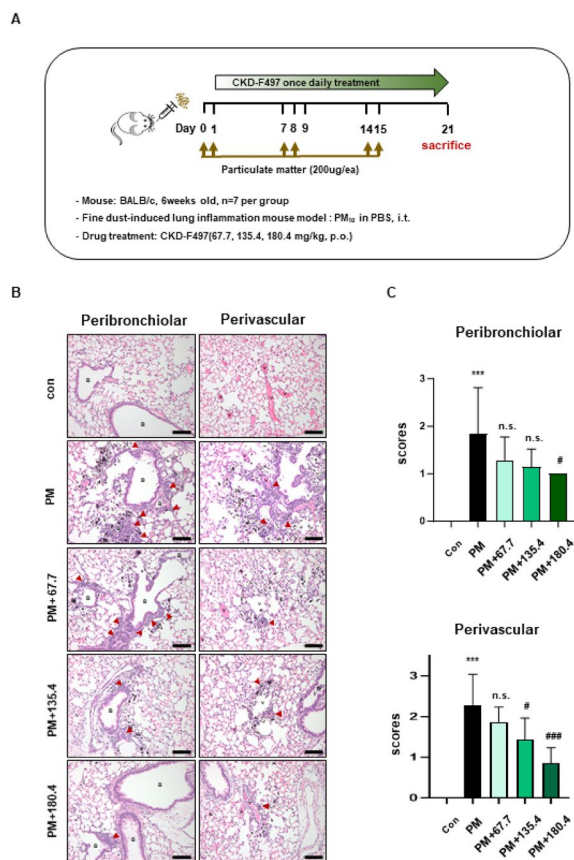


Fig. 3 Effects of CKD-F497 on PM₁₀-induced histological changes in lung tissues. **A** Outline of the experimental schedule. **B** Representative images of peribronchiolar lesions (left panel) and perivascular lesions (right panel). Lung tissues were stained with H&E to visualize inflammatory cell infiltration. Infiltrated immune cells are highlighted with red arrows. Magnification: 200×, con: control group; PM: PM₁₀-treated pulmonary inflammation group; PM + 67.7: PM₁₀ treated with CKD-F497 67.7 mg/kg/day group; PM + 135.4: PM₁₀ treated with CKD-F497 135.4 mg/kg/day group; PM + 180.4: PM₁₀ treated with CKD-F497 180.4 mg/kg/day group. **C** A four-point inflammatory score was used to evaluate the accumulation of inflammatory cells in H&E-stained lung sections. n = 7 per group. All data were analyzed using one-way analysis of variance, followed by Dunnett's multiple comparison test. ****P* < 0.001 vs. control. #*P* < 0.05 vs. PM₁₀. Results are presented as mean ± SD

upregulated inflammation-related cytokine production in the serum. we quantified IL-1 β , IL-6, IL-17, and TNF- α in the serum using ELISA. PM exposure elevated serum IL-1 β , IL-6, IL-17, and TNF- α levels by approximately 8.6-, 18.5-, 14.2- and 33.9-fold, respectively, compared with those in the con group (Fig. 4B). However, these inflammatory cytokines could not be assessed in mice treated with CKD-F497 (180.4 mg/kg). Therefore, these results suggest that PM₁₀ instillation strongly induced

airway inflammation, and that CKD-F497 reduced the recruitment of immune cells into the BALF and cytokine secretion.

3.5 CKD-F497 Attenuated Pulmonary Fibrosis Induced by PM₁₀

Interstitial pulmonary fibrosis is characterized by an altered cellular composition of the alveolar region with excessive deposition of collagen and remodeling of the lung architecture [26]. Sirius Red staining revealed that exposure to PM₁₀ increased the severity of pulmonary fibrosis. After 3 weeks of CKD-F497 treatment, Sirius Red staining intensity in CKD-F497 (180.4 mg/kg)-treated mice was lower than that in the PM₁₀ only group (Fig. 5). These results suggest that CKD-F497 decreases the accumulation of collagen by inhibiting pulmonary fibrosis.

4 Discussion

The efficacy of a herbal extract is largely determined by its chemical profile, and solvent selection is critical in the extraction processes as it directly affects the profile of the final extracts [19]. Based on the results of content analysis of the marker compounds TDD and SZD in CKD-497, 50% ethanol was selected as the solvent for extracting CKD-497.

CKD-497 is known to relieve cough and facilitate sputum expectoration [27]. However, owing to its non-uniform properties, we obtained the optimized CKD-F497 by drying the CKD-497 soft extract. To confirm the equivalence between the soft extract CKD-497 and dry extract CKD-F497, we utilized the HPLC–DAD technique, commonly employed for quantifying components in food and pharmaceutical products [28]. The HPLC–DAD chromatograms of CKD-497 and CKD-F497 showed that the peaks for both extracts appeared at the same relative retention times and that they exhibited identical whole wavelength scanning patterns for each compound peak (Fig. 1). Specifically, two main marker compounds, TDD and SZD, were detected at retention times of = 23.771 min (TDD, CKD-497)/23.766 min (TDD, CKD-F497) and 25.035 min (SZD, CKD-497)/25.036 min (SZD, CKD-F497) in the HPLC chromatogram. The correlation coefficient indicated a positive relationship between CKD-497 and CKD-F497, suggesting qualitative equivalence. TDD isolated from the rhizome of *A. japonica* showed antibacterial activity against methicillin-resistant *S. aureus*. Individual components of *S. chinensis*, schisandrin A, shisandrin B, and gomisins N, have been reported to exhibit anti-inflammatory and anti-oxidative effects [29]. Consistent with previous studies, we observed that CKD-F497 containing TDD and SZD possesses anti-inflammatory

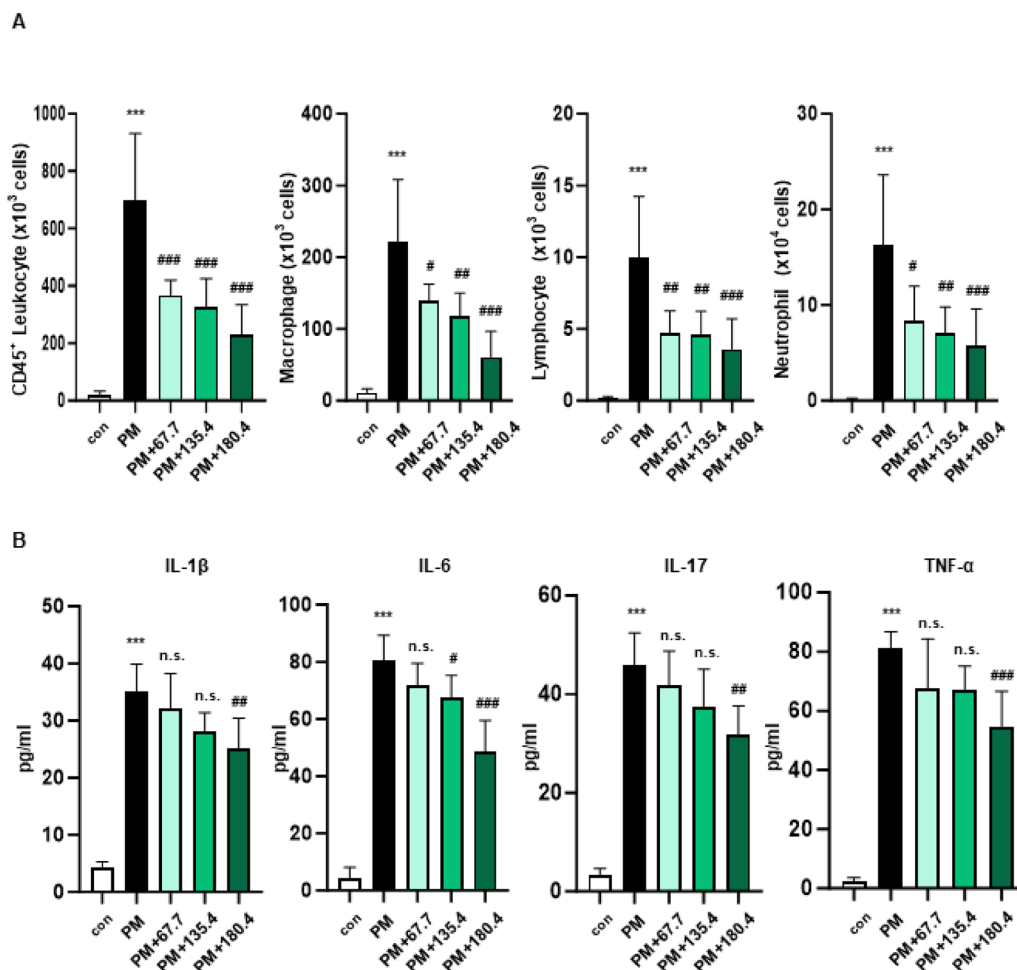


Fig. 4 Effects of CKD-F497 on PM₁₀-induced inflammatory responses in BALF and serum. **A** Total cell count, including CD45⁺ leukocytes, macrophages, lymphocytes, and neutrophils, in BALF. **B** Pro-inflammatory cytokine levels in the serum. Concentrations of IL-1 β , IL-6, IL-17, and TNF- α in the serum. $n=7$ per group. All data were analyzed using one-way analysis of variance, followed by Dunnett's multiple comparison test. *** $P<0.001$ vs. control. # $P<0.05$ vs. PM₁₀. Results are presented as mean $\pm$ SD

properties. An *in vitro* study on LPS-induced inflammation model of RAW 264.7 macrophages showed that CKD-F497 effectively inhibited the inflammatory response through the NF- κ B/MAPK signaling pathway. Inflammation is a key contributing factor to respiratory diseases [30]. In particular, consistent PM exposure induces excessive immune system response, leading to various health issues, including infections, hypersensitivity, and malignancies [31–33]. Moreover, PM triggers the innate immune response, prompting airway epithelial cells and alveolar macrophages to release pro-inflammatory cytokines and chemokines such as IL-1 β , IL-6, and TNF- α . In our study, intratracheal administration of PM₁₀ to mice induced a systemic inflammatory response, increasing these cytokine levels. Chronic exposure to PM is a well-known risk factor for chronic lung disease, such as chronic obstructive pulmonary disease (COPD)

and pneumoconiosis. PM₁₀-induced airway inflammation mouse model may partially mimic key inflammatory mechanisms of these human respiratory diseases. Although species-specific differences exist, this model provides valuable insights into the impact of prolonged PM exposure on lung inflammation, contributing to a better understanding of disease mechanisms relevant to human conditions [34, 35]. Long-term administration of CKD-F497 markedly reduced the systemic inflammation induced by PM₁₀ exposure, decreasing the production of pro-inflammatory cytokines, including IL-1 β , IL-6, IL-17, and TNF- α . Additionally, CKD-F497 suppressed PM₁₀-induced excessive collagen deposition in the interstitial extracellular matrix of mouse lung tissue, as evidenced by decreased Sirius Red staining intensity. Pulmonary fibrosis is a chronic progressive lung disease that gradually disrupts lung architecture and ultimately

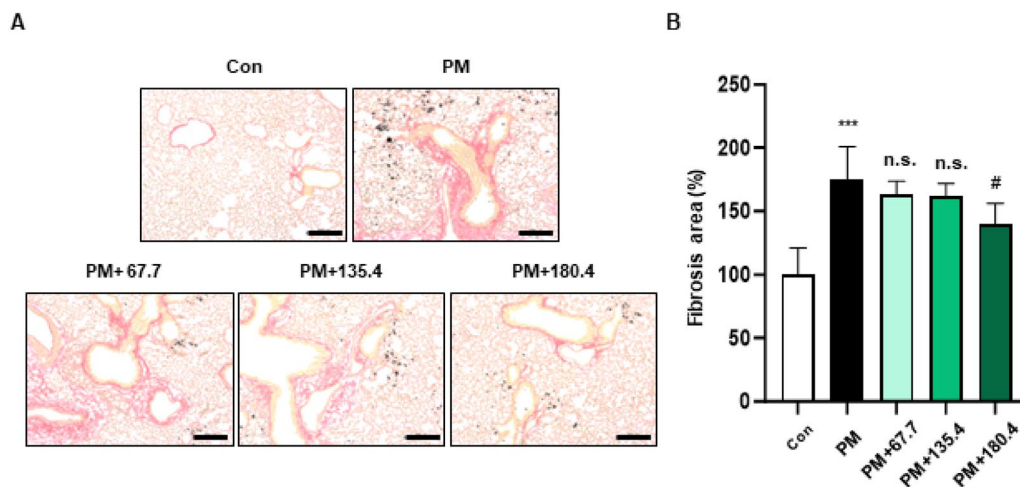


Fig. 5 Effects of CKD-F497 on PM₁₀-induced collagen deposition in mouse pulmonary tissue. **A** Representative images of Picro-Sirius Red-stained lung sections. Red color in Picro-Sirius Red staining indicates collagen (n = 7). **B** Quantification of Sirius Red-stained positive area (%), indicative of pulmonary fibrosis area. Magnification: 100 ×. All data were analyzed using a one-way analysis of variance, followed by Dunnett's multiple comparison test. ****P* < 0.001 vs. control. #*P* < 0.05 vs. PM₁₀. Results are presented as mean ± SD

leads to respiratory failure [36]. Notably, highest dose (180.4 mg/kg) resulted in a marked reduction in alveolar macrophages and neutrophils in BALF, along with significant inhibition of collagen accumulation in lung tissue. Collectively, these findings indicate that long-term administration of CKD-F497 not only mitigates systemic inflammation but also prevents fibrotic progression in a PM-induced lung inflammation model. CKD-F497 may thus serve as a promising therapeutic candidate for the management of chronic lung diseases and related respiratory conditions.

Abbreviations

BALF	Bronchoalveolar lavage fluid
CKD	Chong Kun Dang
CST	Cell Signaling Technology
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
H&E	Hematoxylin and eosin
HPLC-DAD	High-performance liquid chromatography-diode array detection
LPS	Lipopolysaccharide
NIST	National Institute of Standards and Technology
PBS	Phosphate buffered saline
PM	Particulate matter
PM ₁₀ + CKD-F497 135.4 mg/kg	PM ₁₀ and CKD-F497 135.4 mg/kg-treated model
PM ₁₀ + CKD-F497 180.4 mg/kg	PM ₁₀ and CKD-F497 180.4 mg/kg-treated model
PM ₁₀ + CKD-F497 67.7 mg/kg	PM ₁₀ and CKD-F497 67.7 mg/kg-treated model
PM ₁₀	PM ₁₀ -treated model
SZD	Schisandrin
TBST	Tris-buffered saline with Tween 20
TDD	6(E),12(E)-tetradecadine-8,10-diyne-1,3-diol

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s44424-025-00011-5>.

Below is the link to the electronic supplementary material. Supplementary file1 (DOCX 227 KB)

Author Contributions

Hyejeong Kim conceptualized and supervised the study, managed the project, and drafted the manuscript. Jihye Choi contributed to investigation, data analysis, and manuscript review. Jieun Lee participated in experimental investigation and manuscript editing. Jaeok Seo conducted experiments and contributed to manuscript preparation. Dong Hyuk Nam contributed to conceptualization, methodology development, and manuscript writing. Taewon Jeong conducted the in vivo experiments and contributed to data acquisition and manuscript revision. All authors reviewed and approved the final manuscript.

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Availability of Data and Materials

No datasets were generated or analysed during the current study.

Declarations

Conflict of Interest

The authors declare no competing interests.

Ethical Approval and Consent to Participate

All animal experiments were approved by the Institutional Animal Care and Use Committee at HLB Biostep Co., Ltd (formerly Knotus) in the Republic of Korea (approval number: KNOTUS IACUC 22-KE-0467), and were performed in accordance with the IACUC guidelines.

Consent for Publication

Not applicable.

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