



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Alisol A from *Alisma orientale* modulates SERCA2-dependent calcium signaling as a bioactive triterpenoid with anti-fibrotic potential

Pengli Guo ^{a, b}, Mengnan Zeng ^{a, b, c}, Yuhang Zhang ^{a, b}, Deng-Hui Zhu ^{a, b}, Ziyu Zhang ^{a, b}, Yanxing Wu ^{a, b}, Xiaoke Zheng ^{a, b, c *}, Weisheng Feng ^{a, b, c *}

^a Henan University of Chinese Medicine, 156 Jinshui East Road, Zhengzhou 450046, China

^b The Engineering and Technology Center for Chinese Medicine Development of Henan Province, 156 Jinshui East Road, Zhengzhou 450046, China

^c Co-construction of Collaborative Innovation Center for Chinese Medicine and Respiratory Diseases by Henan & Education Ministry of P.R. China, Zhengzhou 450046, China

ABSTRACT: *Alisma orientale* (AE), a classic “medicine food homology” material rich in protostane-type triterpenoids, has been widely used in dietary therapy (Yakuzen) for metabolic wellness. However, its potential as a functional food ingredient against pulmonary fibrosis (PF) remains unexplored. Here, we evaluated the anti-fibrotic efficacy of AE extract and its major bioactive monomer, Alisol A (AliA), in a bleomycin-induced PF mouse model and transforming growth factor- β 1 (TGF- β 1)-stimulated bronchial epithelial cells. AE and AliA significantly improved survival rates, preserved lung function, and alleviated histopathological injury. At the cellular level, AliA attenuated epithelial-mesenchymal transition (EMT), oxidative stress, and apoptosis. Mechanistically, RNA-seq identified calcium signaling as a key regulated pathway. Crucially, we validated sarco/endoplasmic reticulum Ca^{2+} -ATPase 2 (SERCA2) as a direct target of AliA using molecular docking and cellular thermal shift assays (CETSA), which demonstrated that AliA physically binds to and thermally stabilizes the SERCA2 protein. AliA treatment restored SERCA2 function and corrected intracellular Ca^{2+} homeostasis, whereas co-treatment with the SERCA inhibitor thapsigargin (TG) largely abolished these protective effects. These findings position AliA as a novel natural SERCA2 activator, supporting the development of AE as a nutraceutical for fibrotic disease management.

Keywords: *Alisma orientale*; Alisol A; pulmonary fibrosis; SERCA2; calcium homeostasis; natural functional phytochemicals

1. Introduction

Pulmonary fibrosis (PF) is a chronic, progressive, and life-threatening interstitial lung disease characterized by irreversible alveolar damage, excessive deposition of extracellular matrix (ECM), and declining respiratory function^[1]. Idiopathic pulmonary fibrosis (IPF), the most severe subtype, has a dismal prognosis, with a median survival of only 3–5 years^[2]. Although antifibrotic agents such as PFD and nintedanib are clinically available, their limited efficacy and adverse effects restrict their long-term application^[3], highlighting the urgent need for safer and more effective therapeutic strategies.

*Corresponding author
zhengxk.2006@163.com (X. Zheng), fwsh@hactcm.edu.cn (W. Feng)

Received 4 November 2025
Received in revised form 8 December 2025
Accepted 5 February 2026

Traditional Chinese Medicine (TCM) offers a rich source of bioactive compounds with multitarget therapeutic potential^[4-7]. *Alisma orientale* (Sam.) Juzep. (AE), known as “Zexie” in China, is not only a classic medicinal herb but also a popular “medicine food homology” material widely used in dietary therapy (Yakuzen). In East Asian culture, the rhizome of AE is frequently consumed in the form of medicinal porridges, soups, and herbal teas to promote fluid balance and metabolic wellness. From a phytochemical perspective, this edible agricultural product is known to be rich in protostane-type triterpenoids, particularly Alisol A (AliA). As a major bioactive component derived from this functional food source, AliA has demonstrated various biological activities in other contexts, including anti-inflammatory and organ-protective effects^[8-11].

The pathogenesis of PF involves complex processes, fundamentally driven by fibroblast-to-myofibroblast differentiation, which leads to the overproduction of collagen types I and III and the accumulation of α -smooth muscle actin (α -SMA)^[12-16]. Despite the extensive investigation of AE and its constituents in other disease contexts, their specific role and chemical basis in PF have not been clearly defined. Recent isolation and screening of AE-derived monomers have suggested potential antifibrotic activity, with AliA emerging as a promising candidate.

In this study, we first evaluated the therapeutic effect of the AE extract in a bleomycin-induced mouse model of PF. We then focused on elucidating the antifibrotic mechanisms of its key phytochemical, AliA, through a combination of transcriptomic profiling and mechanistic studies. Notably, recent studies have identified impaired intracellular calcium homeostasis—particularly dysfunction of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), especially the SERCA2a isoform—as a key driver of fibrotic remodeling^[17-19]. Therefore, particular attention was given to determining the role of AliA in modulating SERCA2-mediated calcium regulation in both *in vivo* and *in vitro* models.

2. Materials and methods

2.1 Preparation of AE Extract and Isolation of AliA

AE were collected in December 2023 from Fujian Province, China. The plant material was authenticated as the dried tuber of *Alisma orientale* (Sam.) Juzep. (<http://mpns.kew.org>) belonging to the family Alismataceae by Professor Chengming Dong at Henan University of Chinese Medicine. A voucher specimen is deposited at the Henan Provincial Engineering Technology Research Center for Traditional Chinese Medicine Development. To prepare the extract, 1 kg of AE coarse powder was decocted three times with six volumes of distilled water each time. The aqueous extracts were combined and lyophilized to obtain a dry powder extract (extraction rate of 21.1 %). An appropriate amount of this lyophilized extract was accurately weighed, and stored at 4 °C for subsequent animal experiments and UHPLC-Q-TOF-MS analysis. The components were qualitatively analyzed based on their precise relative molecular masses and secondary mass spectrometry fragmentation provided by mass spectrometry. The base peak diagram is shown in Fig. S1. In positive ion scanning mode, a total of seven components were detected in the AE extract, as shown in Table S1. AliA (CAS: 19885-10-0), a representative compound from AE, was isolated and identified by the

Henan Provincial Engineering Technology Research Center for Traditional Chinese Medicine Development, based on previous research, this compound has been identified as AliA^[20] (Fig. S2A–C).

2.2 Animals

Eighty-five SPF-grade male C57BL/6N mice (18–22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SCXK [Jing] 2021-0006). Mice were housed in a clean-grade animal facility at 18–22 °C with free access to food and water. After a 7-day acclimatization period, the mice were used for subsequent experiments. All experimental protocols were approved by the Institutional Animal Ethics Committee (Approval No. DWLL2018080003).

2.3 Establishment of the PF Model and Experimental Grouping

According to a previously published method, a single intratracheal administration of bleomycin (BLM, 5 mg/kg) in 0.02 mL sterile saline was used to induce PF. Bleomycin was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China, product No. B802467). Control group mice received an equal volume of sterile saline. After modeling, the incision was disinfected with povidone-iodine, covered with erythromycin eye ointment, and sprinkled with penicillin powder. During the procedure, mice were kept warm and returned to their cages after full recovery from anesthesia. The detailed experimental design and grouping strategy are illustrated in Fig. 1. Experiment A: BLM-induced mice were randomly divided into the following groups (n = 15 per group): BLM model group, positive control group: pirfenidone (PFD, 270 mg/kg, purchased from Beijing Contini Pharmaceutical Co., Ltd.), Alisma extract low-dose group (ZX-L, 1.67 g/kg), Alisma extract medium-dose group (ZX-M, 3.33 g/kg), Alisma extract high-dose group (ZX-H, 6.67 g/kg). The dosages of the extract were calculated based on the human daily consumption recommended by the Chinese Pharmacopoeia. The human equivalent dose (HED) was converted to the animal dose using the body surface area normalization method^[21]. Drug administration began on day 2 after modeling and continued for 14 consecutive days by gavage. Experiment B: The mice were randomly divided into (n = 15 per group): BLM model group, PFD group (270 mg/kg), AliA low-dose group (AliA-L, 10 mg/kg), AliA high-dose group (AliA-H, 20 mg/kg). The doses of AliA (10 and 20 mg/kg) were selected based on previous pharmacological studies^[22, 23], aiming to evaluate the efficacy at the lower end of the therapeutic window to ensure safety for potential functional food applications. To explore the role of Ca²⁺-ATPase in the mechanism, a subset of model mice received intraperitoneal injections of the Ca²⁺-ATPase inhibitor thapsigargin (TG, 0.5 mg/kg) once per week. TG was purchased from Chengdu Push Biotechnology Co., Ltd. (CAS No. 67526-95-8, purity 98%). The groups were as follows (n = 15 per group): BLM+TG group, PFD+TG group, AliA-L+TG group, AliA-H+TG group, Con+TG group (n = 10). All drug interventions began on the second day after modeling and continued for 14 days. Mouse survival was monitored daily, and survival curves were plotted accordingly. Mice were weighed, and their lung wet weights were recorded. Lung coefficient was calculated as: Lung coefficient (%) = (Lung wet weight / Body weight) × 100%. Mouse survival was monitored daily; survival curves were plotted using the Kaplan-Meier method, and statistical differences between groups were analyzed using the Log-rank (Mantel-Cox) test.

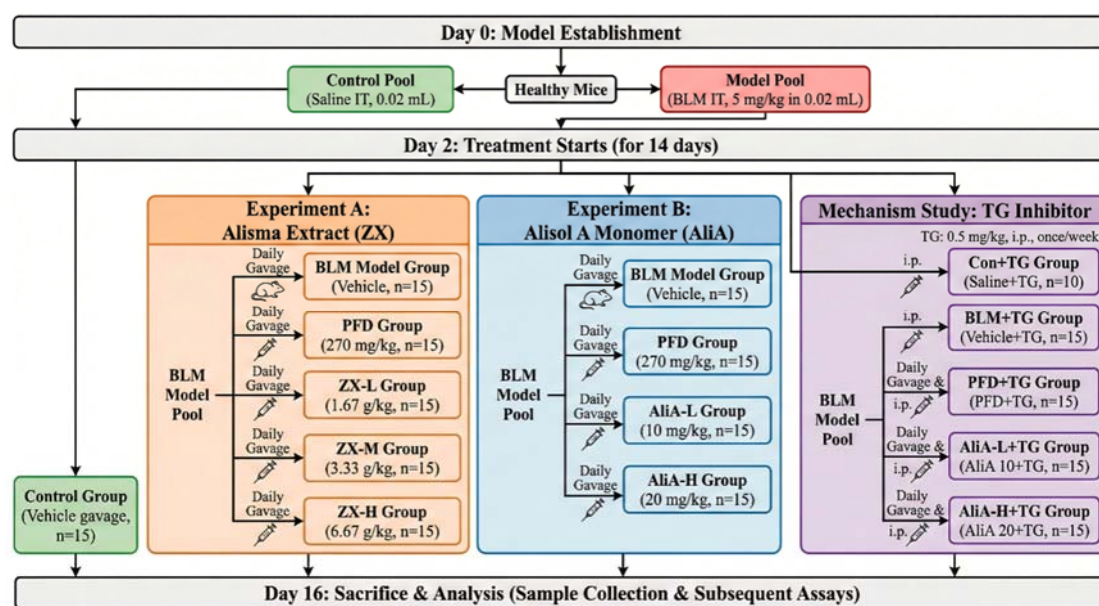


Fig. 1. Schematic diagram of the experimental design. The study timeline includes model establishment (Day 0), drug intervention (Days 2–15), and sacrifice (Day 16). The flowchart details the grouping strategies for Alisma extract (Experiment A), AliA monomer (Experiment B), and the mechanistic study using the SERCA inhibitor Thapsigargin (TG), followed by sample collection and analysis.

2.4 Cell grouping

Human bronchial epithelial cells (BEAS-2B) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a humidified incubator at 37 °C with 5% CO₂ until reaching the logarithmic growth phase. Cells were seeded into 96-well plates at a density of 2×10^4 cells/mL (200 μ L per well). For the cytotoxicity assay, cells were treated for 24 h and divided into a control group (Con) and AliA-treated groups (1–100 μ M). For the cell viability assay, cells were divided into the following groups after 24 h of incubation: control group (Con), model group (TGF- β 1, 1 ng/mL), and AliA-treated groups (1–100 μ M AliA + 1 ng/mL TGF- β 1). Cell viability was assessed using the MTT assay.

For Ca²⁺-ATPase inhibition experiments, BEAS-2B cells in the logarithmic growth phase were seeded at densities of 2×10^4 cells/mL (96-well plate, 200 μ L/well) and 4×10^4 cells/mL (6-well plate, 3 mL/well). After adherence, cells were assigned to the following groups: control group (Con), model group (TGF- β 1, 1 ng/mL), drug treatment group (AliA, 10 μ M), and corresponding groups with Ca²⁺-ATPase inhibitor thapsigargin (TG, 1 μ M). TG was added 2 h prior to drug administration.

For gene overexpression experiments, BEAS-2B cells in the logarithmic growth phase were seeded at densities of 5×10^4 cells/mL (24-well plate, 1 mL/well) and 3×10^4 cells/mL (96-well plate, 0.2 mL/well). After adherence, cells were divided into the following groups: control group (Con), model group (TGF- β 1, 1 ng/mL), drug treatment group (AliA, 10 μ M), and SERCA2 (NM_001681.4) overexpression plasmid group (OE, 500 ng/well for 24-well plates or 100 ng/well for 96-well plates). Transfection was carried out using Lipofectamine™ 3000 (Cat. No. L3000008, Invitrogen, USA) at a 2:1 ratio of reagent to plasmid. After 6 h, the transfection medium was removed, cells were washed twice with 1 mL PBS, and fresh culture medium (500 μ L/well) was added. AliA was added 24 h post-transfection.

2.5 Blood Oxygen Saturation

After the final administration, mice were anesthetized with isoflurane and fixed in the prone position on a thermostatic pad. The left hind paw was clipped with a pulse oximeter probe (PhysioSuite system), and red-light signal intensity was adjusted. SpO₂ levels were continuously recorded for 5 minutes.

2.6 Hydroxyproline (HYP) Determination

Approximately 20 mg of lung tissue was used to detect HYP content using a hydroxyproline assay kit (Product No. A030-2-1, Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer's protocol.

2.7 Whole-Body Plethysmography (WBP) for Lung Function

WBP (FinePointe™) was calibrated and operated according to the manufacturer's instructions. After 5 minutes of adaptation in the chamber, measurements were recorded for another 5 minutes, including: Peak inspiratory flow (PIF), Peak expiratory flow (PEF), Tidal volume (TVb), Inspiratory time (Ti), Expiratory time (Te), Expiratory flow at 50% tidal volume (EF50).

2.8 Histopathological Analysis

Lung tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining and Masson's trichrome staining were performed. Fibrosis severity was semi-quantitatively scored using the Szapiel and Ashcroft systems. Collagen deposition was quantified using ImageJ software.

2.9 Flow Cytometric Analysis of Immune Cell Populations in Mice

Peripheral blood (100 µL) was collected from the orbital sinus and labeled with the following antibodies (all purchased from Invitrogen, USA unless otherwise specified): Dendritic cells (DCs): CD11c-PE (Cat. No. 12-0114-83), CD86-FITC (Cat. No. 11-0862-82), Myeloid-derived suppressor cells (MDSCs): CD11b-APC (Cat. No. 17-0112-81), LY-6G-PE (Cat. No. 12-5931-81), Regulatory T cells (Tregs): CD4-FITC (Cat. No. 11-0041-82), CD25-PE (Cat. No. 12-0251-81), FOXP3-APC (Cat. No. 17-5773-82), Macrophages and neutrophils: CD45-FITC (Cat. No. 11-0451-85), LY-6G-PE (Cat. No. 12-5931-81), F4-80-APC (Cat. No. 565853, BD Biosciences, USA). After lysis of red blood cells with 1× lysis buffer, the cells were washed twice with PBS and resuspended for analysis using a BD FACS Aria™ III flow cytometer. For FOXP3 detection, cells were permeabilized and stained according to the instructions of the FOXP3 staining kit.

Lung tissue was minced into ~1 mm³ pieces, digested with 0.25% trypsin for 40 min at 37 °C, and filtered through a 70 µm cell strainer to obtain single-cell suspensions. The following antibodies were used for flow cytometry (all from Invitrogen, USA unless otherwise noted): DCs: CD45-PE-CY7 (Cat. No. 25-0451-82), CD11c-PE (Cat. No. 12-0114-83), CD86-FITC (Cat. No. 11-0862-82), MDSCs: CD45-PE-CY7 (Cat. No. 25-0451-82), CD11b-APC (Cat. No. 17-0112-81), LY-6G-PE (Cat. No. 12-5931-81), Tregs: CD45-PE-CY7 (Cat. No. 25-0451-82), CD4-FITC (Cat. No. 11-0041-82), CD25-PE

(Cat. No. 12-0251-81), FOXP3-APC (Cat. No. 17-5773-82), Macrophages and neutrophils: CD45-PE-CY7 (Cat. No. 25-0451-82), LY-6G-PE (Cat. No. 12-5931-81), F4-80-APC (Cat. No. 565853, BD Biosciences, USA). After staining, cells were washed twice with PBS and analyzed. For FOXP3 staining, cells were permeabilized according to the manufacturer's protocol before antibody labeling.

Lung primary cells were isolated as described above. Reactive oxygen species (ROS) were detected using DCFH-DA fluorescent probe (CA1410, Solarbio). Apoptosis was analyzed using Annexin V-PE/7-AAD Apoptosis Detection Kit (Cat. No. 559763, BD). Intracellular Ca^{2+} levels were measured with Fluo-4, AM probe (Cat. No. F8500, Solarbio). Samples were analyzed using BD FACS Aria™ III.

Following 24 h of drug treatment, cells from 6-well and 24-well plates were collected and stained according to the instructions of the PE Annexin V apoptosis detection kit and the Fluo-4, AM calcium detection kit. After staining, cells were transferred to flow cytometry tubes and analyzed using a flow cytometer. All experiments were independently repeated three times.

2.10 Measurement of Oxidative Stress Markers in Lung Tissue and Serum

The supernatant of lung tissue homogenate and serum samples were collected and subjected to biochemical analysis using commercial assay kits, following the manufacturers' instructions. The levels of malondialdehyde (MDA; Cat. No. A003-1-2), total superoxide dismutase (T-SOD; Cat. No. A001-1-2), and glutathione peroxidase (GSH-Px; Cat. No. A005-1-2) in lung tissue were measured using kits from Nanjing Jiancheng Bioengineering Institute (China).

2.11 ELISA Detection of Cytokines and Fibrosis Markers

Mice were fixed in a supine position, and tracheal intubation was performed. The lungs were lavaged four times with 0.5 mL of sterile saline. The bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 3000 rpm for 15 min, and the supernatant was stored at -80°C . Clean lung tissues were weighed and homogenized with 9 volumes of saline, followed by centrifugation at 3000 rpm for 15 min. The supernatant was collected and stored at -80°C . Serum, BALF, and lung homogenates were analyzed for cytokines and fibrosis markers using ELISA kits from Jiangsu Meimian Industrial Co., Ltd (China), according to the manufacturer's protocols: Inflammatory cytokines: IL-1 β (Cat. No. MM-0040M1), IL-6 (Cat. No. MM-0163M1), TNF- α (Cat. No. MM-0132M1), TGF- β 1 (Cat. No. MM-0135M1). Fibrosis markers: Collagen I (Cat. No. MM-0993M1), Collagen III (Cat. No. MM-1102M1), ECM (Cat. No. MM-46514M1), α -SMA (Cat. No. MM-44558M1).

2.12 Immunofluorescence Staining of Lung Tissue

Lung tissue sections were processed as previously described (deparaffinized, rehydrated, and subjected to antigen retrieval). Sections were blocked with 3% fetal bovine serum for 30 min at room temperature, then incubated overnight at 4°C with the following primary antibodies: Collagen I (Cat. No. GB11022, Servicebio, Wuhan, China), Collagen III (Cat. No. GB111629, Servicebio, Wuhan, China), α -SMA (Cat. No. A17910, Abclonal, China), SERCA2 (Cat. No. bsm-61408R, Bioss, Beijing, China). After washing,

sections were incubated with fluorescent secondary antibodies for 50 min in the dark. Nuclei were counterstained with DAPI (10 min), followed by washing and mounting. Images were captured using a fluorescence microscope (Nikon Eclipse C1, Japan), and average fluorescence intensity was quantified using ImageJ software.

2.13 Transcriptome Sequencing and Analysis of Lung Tissues

The processing of lung tissue samples, mRNA sequencing, and subsequent bioinformatics analyses were conducted by Hangzhou Lianchuan Biotechnology Co., Ltd. (Hangzhou, China).

2.14 Quantitative Real-Time PCR (qRT-PCR) Analysis

Clean lung tissue was collected from mice, and cells were collected 24 hours after drug treatment, total mRNA was extracted from the cells using a Total RNA Extraction Kit. Reverse transcription was performed using HiScript® III RT SuperMix to synthesize complementary DNA (cDNA). qRT-PCR was then carried out in a 20 µL reaction system containing cDNA template, forward and reverse primers, and 2× ChamQ Universal SYBR qPCR Master Mix. The relative expression levels of target mRNAs were calculated using the $2^{-\Delta\Delta C_t}$ method. Primer sequences used for qRT-PCR are listed in Table 1, and were synthesized by Servicebio (Wuhan, China).

Table 1 Primer sequences

Gene	Primer sequence (5'-3')
Mouse <i>Colla1</i>	S: GAGAGGTGAACAAGGTCCCG A: AAACCTCTCTCGCCTCTTGC
Mouse <i>Col3a1</i>	S: GTGGCAATGTAAAGAAGTCTCTGAAG A: GGGTGCATATCTATGATGGGTAG
Mouse α -SMA	S: GTACCACCATGTACCCAGGC A: GAAGGTAGACAGCGAAGCCA
Mouse <i>spp1</i>	S: CTTTCACTCCAATCGTCCCTAC A: CCTTAGACTCACCGCTCTTCAT
Mouse <i>Atp2a2</i>	S: GGCAAAGTGTATCGACAGGACA A: TCAGTATGCTTGATGACGGAGA
Mouse <i>GAPDH</i>	S: CTGGAGAAACCTGCCAAGTATG A: GGTGGAAGAATGGGAGTTGCT
Human <i>SPP1</i>	S: AGTTTCGCAGACCTGACATCC A: TTCCTGACTATCAATCACATCGG
Human <i>ATP2A2</i>	S: CCTGATCCTCTATGTCGAACCCT A: GCATTATGAGCAGCACAAACG
Human α -SMA	S: CAATGTCCTATCAGGGGGCAC A: CGGCTTCATCGTATTCTGTT
Human <i>Colla1</i>	S: TGGCAAAGATGGACTCAACG A: TCACGGTCACGAACCACATT
Human <i>GAPDH</i>	S: GGAAGCTTGTTCATCAATGGAAATC A: TGATGACCCTTTTGGCTCCC

2.15 Cellular immunofluorescence

Cells cultured in 96-well plates were fixed with 4% paraformaldehyde (150 µL/well) at room temperature for 20 min. After removing the fixative, cells were permeabilized with 0.25% Triton X-100 (150 µL/well) for 10 min at room temperature. Following PBS washes, cells were blocked with 1% BSA (50 µL/well) for 1 h at room temperature. Primary antibodies against α -SMA, Collagen I, osteopontin

(SPP1), SERCA2, and β -actin were then added, and the cells were incubated overnight at 4 °C. After three washes with PBST (5 min each), the appropriate secondary antibodies (Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 647-conjugated goat anti-rabbit, 1:1000, 50 μ L/well) were added and incubated for 1 h in the dark. Cells were then washed three times with PBS (5 min each) in the dark and stained with ready-to-use DAPI solution for 5 min at room temperature. After two final PBS washes (5 min each), imaging and data analysis were performed using the PerkinElmer high-content imaging system.

2.16 Molecular Docking

The crystal structure of the SERCA2 protein was retrieved from the Protein Data Bank (PDB ID: 6LN5). The 3D structure of AliA was obtained from the PubChem database. Molecular docking was performed using AutoDock Vina. The protein and ligand were prepared by removing water molecules, adding hydrogen atoms, and computing Gasteiger charges. The binding affinity (kcal/mol) and binding interactions were analyzed using PyMOL.

2.17 Cellular Thermal Shift Assay (CETSA)

CETSA was performed to evaluate the drug-target interaction in living cells. Briefly, cells were treated with AliA (100 μ M) or DMSO for 2 h. After treatment, cells were harvested, washed with PBS, and resuspended in lysis buffer supplemented with protease inhibitors. The cell suspensions were divided into aliquots and subjected to a temperature gradient (37–67°C) for 3 min using a thermal cycler, followed by cooling at room temperature for 3 min. The samples were then lysed by three freeze-thaw cycles using liquid nitrogen. The soluble fraction (supernatant) was separated by centrifugation at 20,000 g for 20 min at 4°C and analyzed by Western blotting using anti-SERCA2 antibody.

2.18 Assessment of acute toxicity in a zebrafish model

Wild-type (AB strain) adult zebrafish were maintained under standard husbandry conditions (28.5 °C, 14/10-h light/dark photoperiod). Embryos were obtained via natural spawning, rinsed with E3 embryo medium, and cultured in a temperature-controlled incubator at 28.5 °C. Healthy larvae were selected at 48 hours post-fertilization (hpf) and randomly allocated to 24-well plates at a density of 10 larvae per well in 2 mL of E3 medium. Each experimental condition was performed in triplicate. The study included a blank control (E3 medium), a vehicle control (0.1% v/v DMSO in E3 medium), and treatment groups exposed to AliA at concentrations of 0.1, 1, 5, 10, 50, and 100 μ M. The final DMSO concentration was maintained at 0.1% (v/v) across all groups to prevent solvent-induced toxicity. Larvae were incubated for a 72 h exposure period at 28.5 °C. Following incubation (at 120 hpf), all larvae were examined under a stereomicroscope to assess toxicological endpoints, including mortality rates and the incidence of gross morphological abnormalities.

2.19 Statistical Analysis

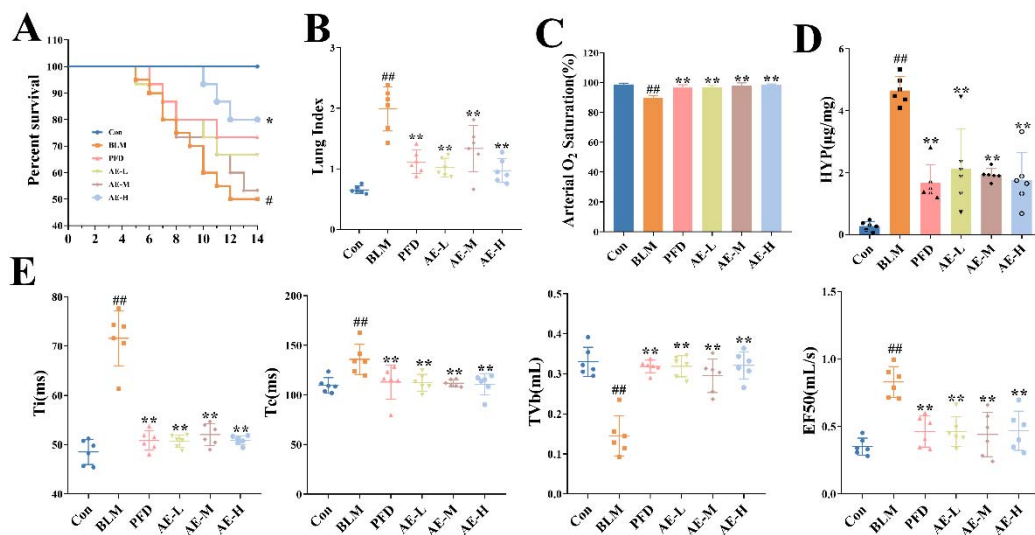
All data are presented as mean $\pm$ standard deviation ($\bar{x} \pm$ SD). Statistical analyses were performed using SPSS 26.0 software. One-way analysis of variance (ANOVA) followed by LSD-t post hoc test was used to

evaluate differences among groups. Survival data were analyzed using the Kaplan-Meier method followed by the Log-rank (Mantel-Cox) test. A value of $P < 0.05$ was considered statistically significant

3. Results

3.1 AE improves PF in mice

The therapeutic effects of AE on bleomycin-induced PF were systematically evaluated. AE treatment significantly improved survival in fibrotic mice, whose mortality reached 50% in the model group (Fig. 2A). The lung coefficient (lung wet weight/body weight ratio), a marker of pulmonary edema and congestion, was markedly increased in the model group but significantly reduced following AE administration (Fig. 2B). Additionally, AE significantly restored blood oxygen saturation levels, which were severely decreased in fibrotic mice, indicating improved gas exchange function (Fig. 2C). Hydroxyproline content, reflecting collagen deposition, was substantially elevated in the lungs of the model group but markedly reduced by AE treatment (Fig. 2D). Consistently, Masson's trichrome staining showed extensive collagen accumulation in the model group, with increased fibrotic area (Fig. 2G, 2J) and Ashcroft score (Fig. 2I), all of which were significantly attenuated by AE. Pulmonary function, assessed via whole-body plethysmography, revealed significant impairment in the model group; however, key functional parameters were notably improved after AE treatment (Fig. 2E). Histological examination by H&E staining demonstrated severe alveolar destruction, inflammatory infiltration, and interstitial thickening in fibrotic lungs, which were alleviated by AE, as reflected by the reduced Szapiel score (Fig. 2F, 2H). Collectively, these results indicate that AE effectively improves survival, attenuates lung injury and fibrosis, and restores pulmonary function in mice with bleomycin-induced PF.



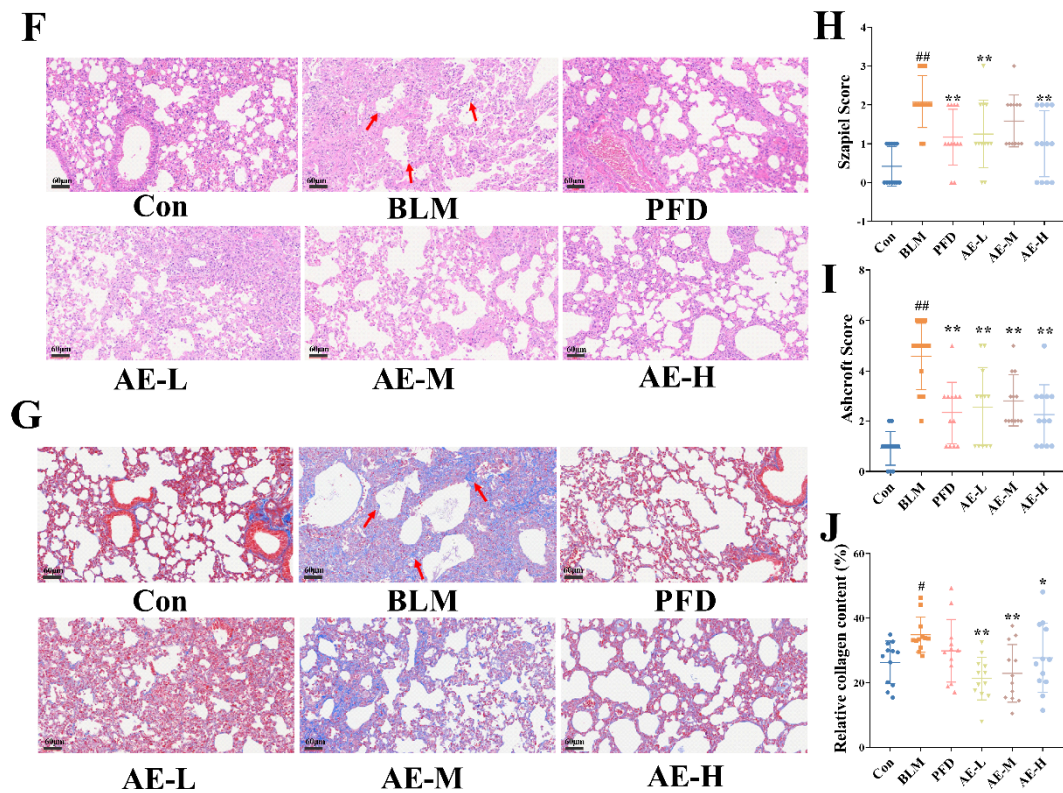


Fig. 2. AE improves PF in mice. (A) Kaplan-Meier survival curves of mice in each group (n=15). (B) Lung coefficient (n=6). (C) Blood oxygen (n=3). (D) Hydroxyproline content in lung tissue (n=6). (E) WBP detection of lung function in mice (n=6). (F) HE histopathological staining of lung tissue (n=3). (G) Masson's trichrome staining of lung tissue (n=3). (H) Szapiel Score. (I) Ashcroft Score. (J) Quantification of collagen area. Data are presented as the mean ± SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. * $P < 0.05$, ** $P < 0.01$ vs. Con. * $P < 0.05$, ** $P < 0.01$ vs. BLM. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AE-L: Alisma orientate low dose group, AE-M: Alisma orientate medium dose group, AE-H: Alisma orientate high dose group.

3.2 Therapeutic inhibition of fibrogenic markers by AE in PF

During the progression of PF, α -SMA serves as a marker of activated myofibroblasts, which excessively synthesize and deposit Collagen I and Collagen III, leading to scarring, stiffening, and loss of lung function. Immunofluorescence analysis revealed that BLM exposure significantly increased the expression of α -SMA, Collagen I, and Collagen III in lung tissues, whereas AE treatment markedly downregulated their levels (Fig. 3A and 3B). Consistent with these findings, ELISA results showed that AE significantly reduced the levels of fibrotic markers in both serum and lung tissue (Fig. 3C and 3D). Moreover, qPCR analysis further confirmed the downregulation of α -SMA and collagen III mRNA levels following AE treatment (Fig. 3E), aligning with the immunofluorescence data. These results suggest that AE effectively inhibits myofibroblast activation and ECM accumulation, thereby attenuating fibrotic remodeling in the lungs.

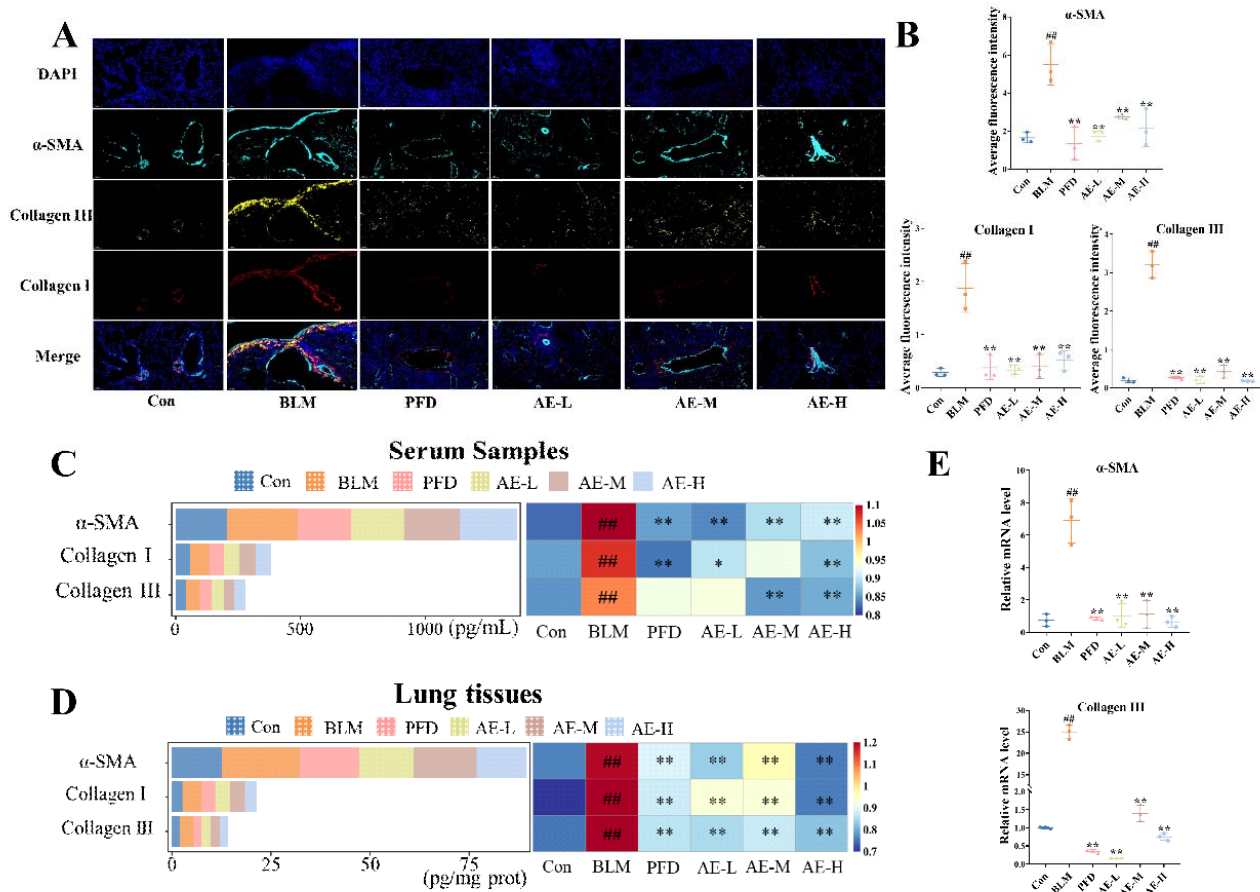


Fig. 3. Therapeutic inhibition of fibrogenic markers (α -SMA/Collagen I/III) by AE in PF. (A) Immunofluorescence detection of α -SMA, Collagen I, and Collagen III levels in lung tissue ($n=3$). (B) Quantification of immunofluorescence results ($n=3$). (C) Levels of α -SMA, Collagen I, and Collagen III in serum ($n=6$). (D) Levels of α -SMA, Collagen I, and Collagen III in lung tissue ($n=6$). (E) Detection of mRNA levels of α -SMA and collagen III genes by PCR ($n=3$). Data are presented as the mean $\pm$ SD. For assays with limited sample size ($n=3$), representative data from independent experiments with consistent trends are shown. # $P < 0.05$, ## $P < 0.01$ vs. Con. * $P < 0.05$, ** $P < 0.01$ vs. BLM. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AE-L: Alisma orientate low dose group, AE-M: Alisma orientate medium dose group, AE-H: Alisma orientate high dose group.

3.3 AE improves the levels of oxidative stress and inflammatory factors in mice with PF.

To assess oxidative stress in fibrotic mice, we measured ROS levels in primary lung cells, along with MDA (a lipid peroxidation marker) and antioxidant enzymes including SOD and GSH-Px in serum and lung tissue. Compared to the BLM group, AE treatment significantly reduced ROS production in lung cells and MDA levels in serum, while markedly increasing the expression of SOD and GSH-Px (Fig. 4B–D). In addition, AE significantly attenuated apoptosis in lung cells, as shown in Fig. 4A. The positive control drug PFD also effectively suppressed inflammation, oxidative stress, and lung cell apoptosis in fibrotic mice. Furthermore, AE treatment reduced systemic inflammation, as evidenced by decreased inflammatory markers (Fig. 4E and 4F). These findings indicate that AE alleviates PF by attenuating oxidative stress, inhibiting apoptosis, and suppressing inflammation.

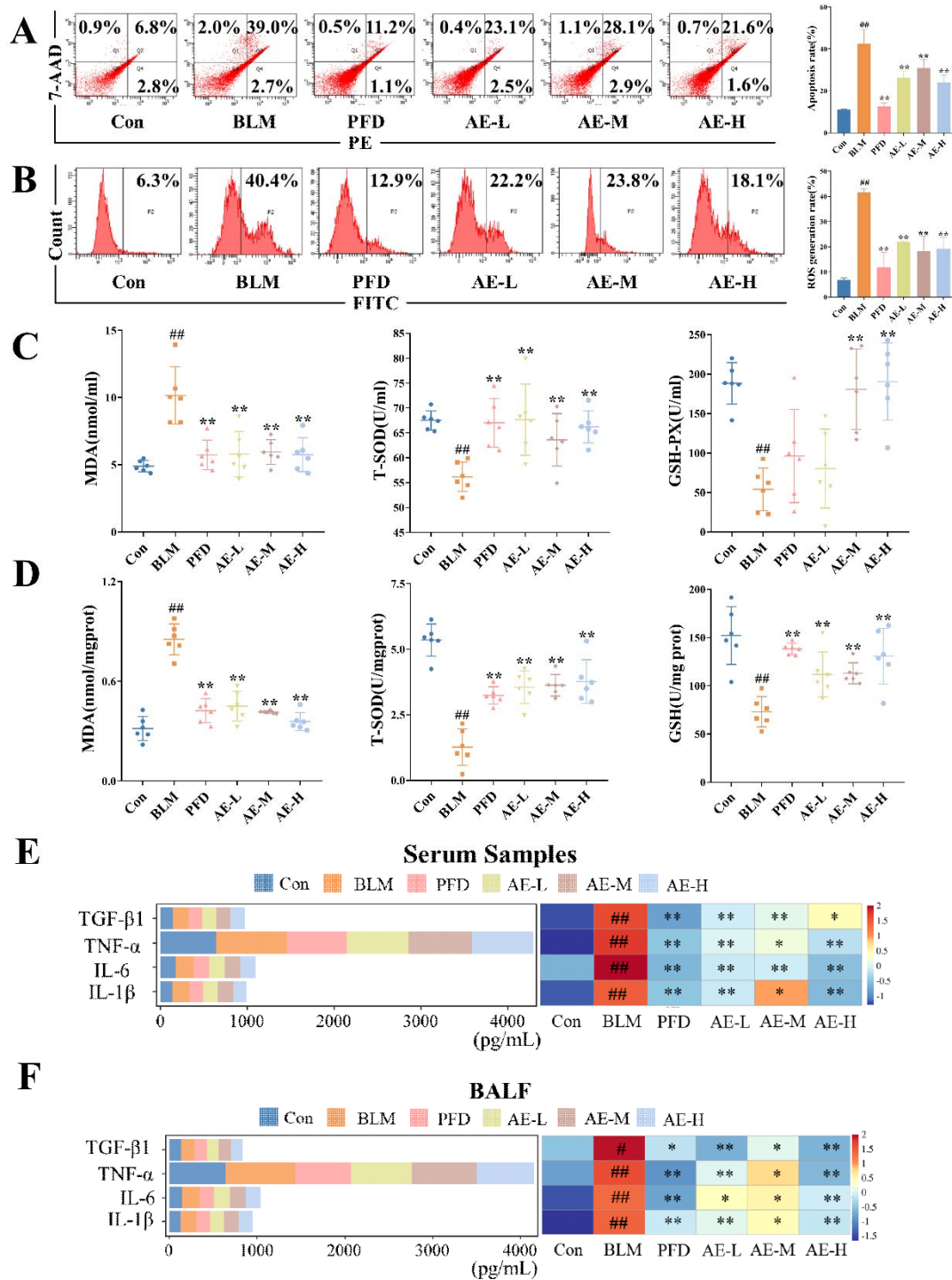


Fig. 4. AE improves the levels of oxidative stress and inflammatory factors in mice with PF. (A) Flow cytometry analysis of apoptosis levels in mice with PF (n=3). (B) Flow cytometry analysis of ROS levels in mice with PF (n=3). (C) Levels of MDA, T-SOD, and GSH-PX in serum (n=6). (D) Levels of MDA, T-SOD, and GSH-PX in lung tissue (n=6). (E) Levels of TGF- β 1, TNF- α , IL-6, and IL-1 β in serum (n=6). (F) Levels of TGF- β 1, TNF- α , IL-6, and IL-1 β in BALF (n=6).

Data are presented as the mean $\pm$ SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. $\#P < 0.05$, $\#\#P < 0.01$ vs. Con. $*P < 0.05$, $**P < 0.01$ vs. BLM. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AE-L: Alisma orientate low dose group, AE-M: Alisma orientate medium dose group, AE-H: Alisma orientate high dose group.

3.4 AE regulates immune cell levels in mice with PF

Infiltration of various immune cell populations, including neutrophils, macrophages, DCs, Tregs, and MDSCs, is a key pathological feature of PF and plays critical roles in lung injury and repair. To further

explore the immunomodulatory effects of AE, we analyzed the levels of these immune cells in lung tissue and peripheral blood using flow cytometry. As shown in Fig. S3 and 5, AE treatment significantly increased the proportion of DCs while decreasing the levels of Tregs, MDSCs, macrophages (F4/80⁺), and neutrophils (Ly-6G⁺) compared to the model group. Similarly, the positive control drug PFD also modulated the distribution of immune cells in fibrotic mice. These results suggest that AE may attenuate PF, at least in part, through regulating immune cell infiltration and maintaining immune homeostasis.

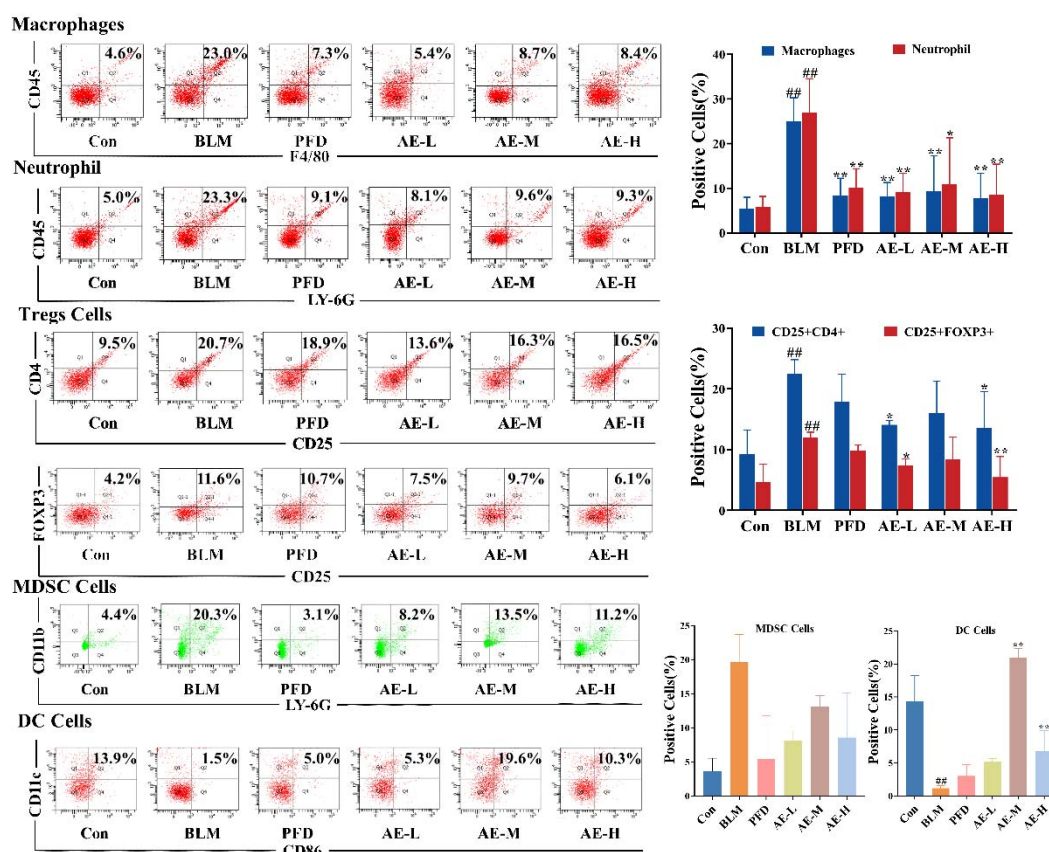


Fig. 5. AE regulates the levels of immune cells in the lung tissue of mice with PF. Data are presented as the mean $\pm$ SD. For assays with limited sample size ($n=3$), representative data from independent experiments with consistent trends are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Con. $^*P < 0.05$, $^{**}P < 0.01$ vs. BLM. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AE-L: Alisma orientate low dose group, AE-M: Alisma orientate medium dose group, AE-H: Alisma orientate high dose group.

Tregs Cells: regulatory T cells, MDSC Cells: myeloid-derived suppressor cells, DC Cells: dendritic cells.

3.5 Transcriptomics research on the intervention mechanism of AE in PF

To explore the potential molecular targets underlying the therapeutic effects of AE in PF, transcriptomic analysis was conducted. As shown in Fig. 6A, compared with the control group, the BLM group exhibited 1,083 upregulated and 1,747 downregulated genes. In contrast, AE treatment resulted in 1,306 upregulated and 515 downregulated genes relative to the BLM group. Intersection analysis identified a total of 1,302 differentially expressed genes (DEGs) common to both comparisons. KEGG enrichment analysis of these DEGs revealed that pathways such as ECM-receptor interaction and calcium signaling played pivotal roles in the anti-fibrotic effects of AE (Fig. 6B). GSEA (Fig. S5A and S5B) and heatmap analyses further supported these findings, indicating significant enrichment of ECM-receptor interaction-related genes, including *Spp1* and *collagen 1* (Fig. S5C), and calcium signaling-related genes

such as *Atp2a2* (the gene encoding SERCA2) (Fig. 6C). Volcano plots of DEGs are shown in Fig. 6D and 6E. These candidate genes were subsequently validated by qPCR. As shown in Fig. 6F, BLM administration significantly upregulated *Spp1* and *collagen I* while downregulating *Atp2a2*. AE treatment markedly reversed these changes, suggesting that AE mitigates ECM deposition and fibrosis progression by modulating calcium signaling and ECM-related gene expression. Taken together, these transcriptomic findings suggest that AE may exert its anti-fibrotic effects, at least in part, through regulating the Ca^{2+} signaling pathway and ECM remodeling in BLM-induced PF.

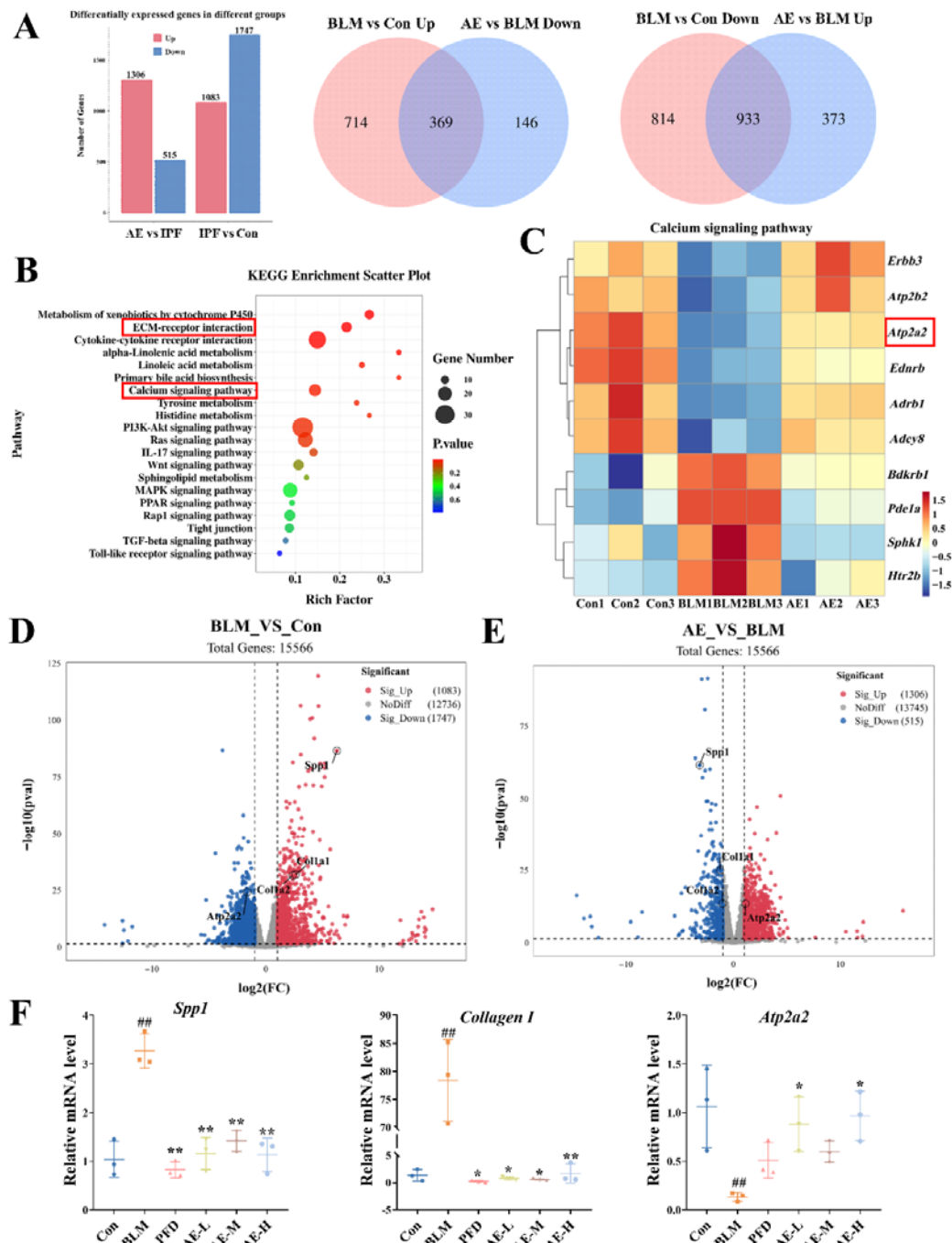


Fig. 6. Transcriptomics research on the intervention mechanism of AE in PF. (A) DEGs among groups and Venn diagram of DEGs. (B) KEGG. (C) Heatmap of DEGs in the calcium signaling pathway. (D) Volcano plot of DEGs between BLM and Con. (E) Volcano plot of DEGs between AE and BLM. (F) Validation of *Spp1*, *collagen I*, and *Atp2a2* using q-PCR.

Data are presented as the mean $\pm$ SD. For assays with limited sample size ($n=3$), representative data from independent experiments with consistent trends are shown. $\#P < 0.05$, $\#\#P < 0.01$ vs. Con. $*P < 0.05$, $**P < 0.01$ vs. BLM. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AE: *Alisma orientate* group.

3.6 Validation of SERCA2 as a Direct Target of AliA

Triterpenoids are considered to be the main substance responsible for the pharmacological effects of AE, as AliA is a protopine-type tetrahydro-triterpenoid with high content and significant activity, we selected it for further investigation of its mechanism^[9, 24, 25]. To further confirm whether SERCA2 is the direct therapeutic target of AliA (the primary bioactive component of AE), we performed molecular docking and CETSA. As shown in Fig. 7A and 7B, AutoDock Vina docking based on the SERCA2 structure (PDB ID: 6LN5) predicted that AliA is well accommodated within the SERCA2 binding pocket, yielding a favorable binding energy of -14.1 kcal/mol. The docked pose suggests that AliA is stabilized by a network of polar interactions/hydrogen bonds involving Arg-489, Lys-514, and Gly-625 (with nearby Lys-492 and Arg-559 providing additional electrostatic complementarity), together with hydrophobic packing against Phe-487 and Met-494.

Furthermore, to validate this interaction in a physiological environment, CETSA was conducted. As shown in Fig. 7C and 7D, AliA treatment significantly increased the thermal stability of SERCA2 protein compared to the vehicle group, indicating distinct physical engagement between the drug and the protein. These results, combined with the RNA-seq screening, solidly demonstrate that AliA exerts its effects by directly targeting SERCA2.

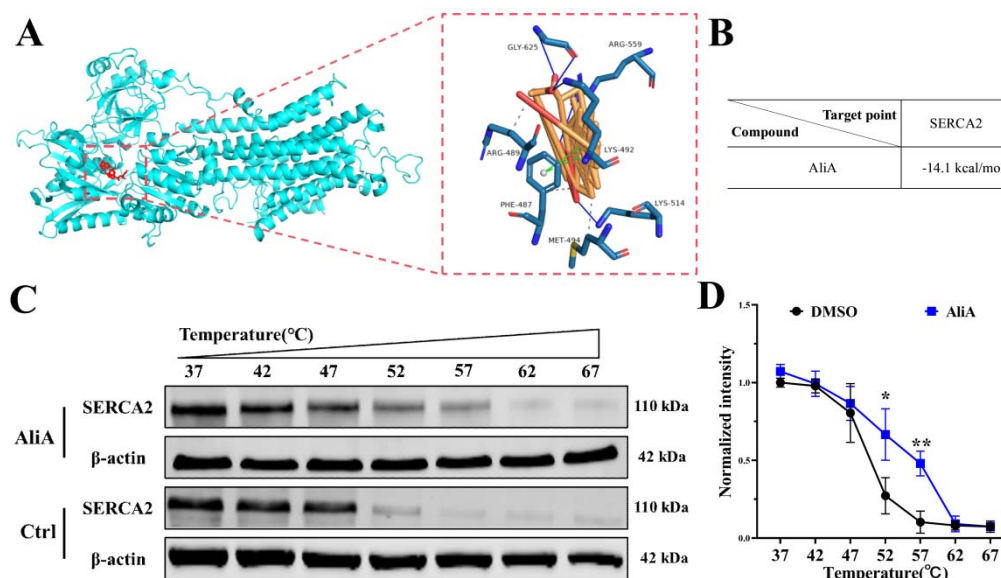


Fig. 7. AliA binds to SERCA2 as predicted by molecular docking and validated by CETSA. (A) Docking pose of AliA in SERCA2 (PDB ID: 6LN5). (B) AutoDock Vina docking score. (C) CETSA showing the thermal stabilization of SERCA2 upon AliA treatment. (D) Quantification of CETSA results (n=3). Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs. DMSO at the corresponding temperature.

3.7 Investigating the effects of AliA on-survival rate and lung pulmonary function in mice with PF by targeting SERCA

In the PF model, high-dose AliA (AliA-H) significantly improved mouse survival rates (Fig. 8A), reduced the lung index (Fig. 8B), and enhanced pulmonary arterial blood flow velocity (Fig. 8C). Pulmonary function tests demonstrated that AliA-H effectively reversed BLM-induced respiratory impairment, as evidenced by increased tidal volume, elevated peak inspiratory and expiratory flow rates, shortened

abnormally prolonged expiratory time, and restored mid-expiratory flow (Fig. 8D-I). However, co-treatment with TG, a selective inhibitor of SERCA, largely abolished the protective effects of AliA-H. Most physiological and functional parameters in the AliA-H+TG group reverted to levels comparable to those in the BLM group. In contrast, TG treatment alone in control mice (Con+TG group) did not affect normal lung function, indicating that the anti-fibrotic effects of AliA are critically dependent on the activation of the SERCA signaling pathway.

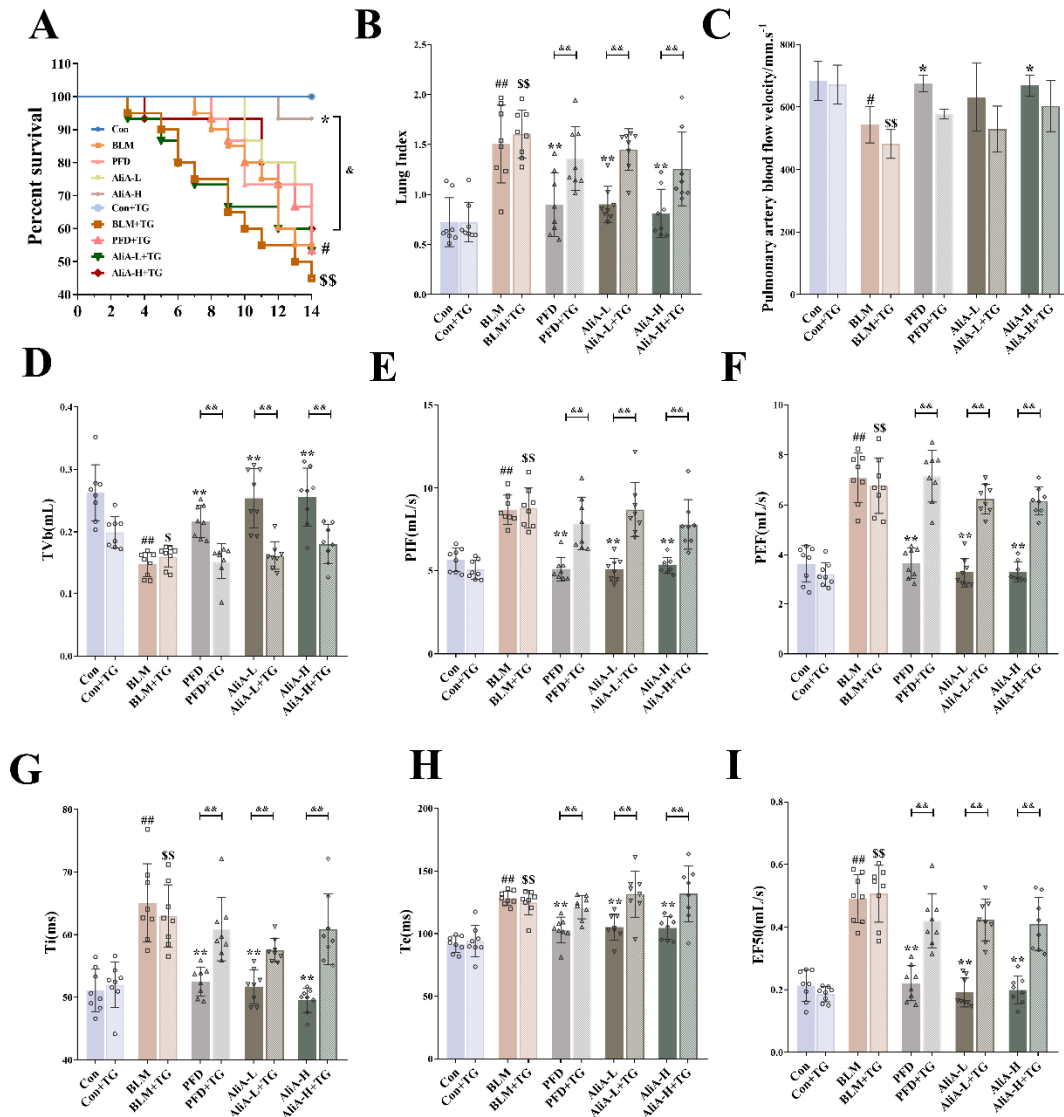


Fig. 8. Investigating the effects of AliA on survival rate and pulmonary function in mice with PF by targeting SERCA. (A) Kaplan-Meier survival curves of mice in each group (n=15). (B) Lung coefficient (n=8). (C) Pulmonary artery blood flow velocity (n=3). (D) Tidal Volume (box) (n=8). (E) Peak Inspiratory Flow (box) (n=8). (F) Peak Expiratory Flow (box) (n=8). (G) Inspiratory Time (n=8). (H) Expiratory Time (n=8). (I) Expiratory flow at the point 50% of TV is expired (Uncompensated) (n=8). Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AliA-L: AliA low dose group, AliA-H: AliA high dose group. Groups labeled with a “+” suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean $\pm$ SD. # $P < 0.05$, ## $P < 0.01$ vs. Con. * $P < 0.05$, ** $P < 0.01$ vs. BLM. \$ $P < 0.05$, \$\$ $P < 0.01$ vs. Con+TG. & $P < 0.05$, && $P < 0.01$ vs. treated with the inhibitor.

3.8 Exploring the effect of AliA on pathological injury in mice with PF by targeting SERCA

In the BLM-induced PF model, mice exhibited significant pathological lung injury. H&E staining revealed disrupted alveolar architecture accompanied by pronounced inflammatory cell infiltration (Fig.

9A), while Masson's trichrome staining showed extensive collagen deposition (Fig. 9B). Gross observation indicated pulmonary edema (Fig. 9E). Treatment with AliA-H significantly alleviated these pathological changes, as evidenced by reduced Szapiel inflammatory scores (Fig. 9C) and Ashcroft fibrosis scores (Fig. 9D), along with suppressed TGF- β 1 expression in both serum and BALF (Fig. 9F-G). However, co-administration of the SERCA inhibitor TG effectively counteracted abolished the protective effects of AliA-H, with pathological scores and TGF- β 1 levels closely mirroring those in the BLM group. Notably, TG alone did not induce any pathological alterations in normal mice (Con+TG group), confirming that the anti-fibrotic and anti-inflammatory effects of AliA are mediated through the SERCA pathway and downregulating TGF- β 1 expression.

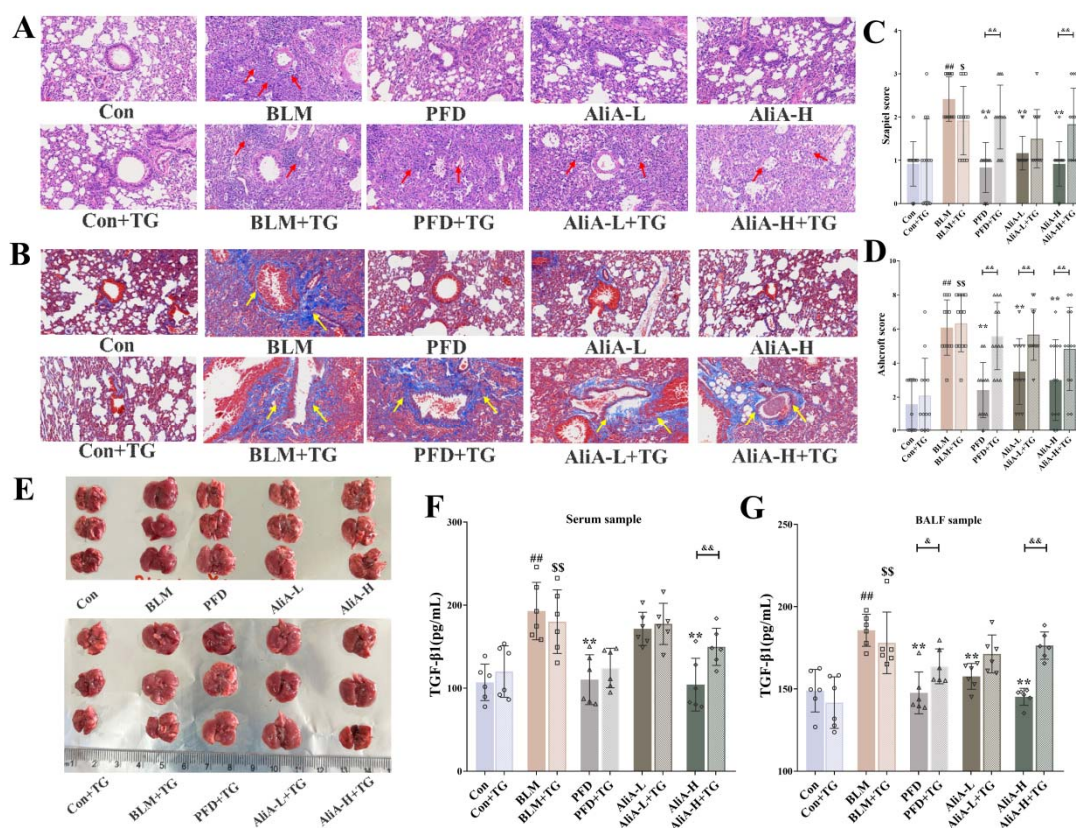


Fig. 9. Exploring the effect of AliA on pathological injury in mice with PF by targeting SERCA. (A) HE histopathological staining of lung tissue (n=3). (B) Masson's trichrome staining of lung tissue (n=3). (C) Szapiel Score. (D) Ashcroft Score. (E) Gross Morphology of Lung Tissue (n=3). (F) Levels of TGF- β 1 in serum (n=6). (G) Levels of TGF- β 1 in BALF (n=6). Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AliA-L: AliA low dose group, AliA-H: AliA high dose group. Groups labeled with a "+" suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean $\pm$ SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. # P < 0.05, ## P < 0.01 vs. Con. * P < 0.05, ** P < 0.01 vs. BLM. \$ P < 0.05, \$\$ P < 0.01 vs. Con+TG. & P < 0.05, && P < 0.01 vs. treated with the inhibitor.

3.9 Exploring the ameliorative effect of AliA on fibrosis in mice with PF by targeting SERCA

In the BLM-induced PF model, the hydroxyproline content in lung tissues was significantly increased (Fig. 10A), along with upregulated expression of fibrotic markers α -SMA, collagen I, and *Spp1* at both mRNA (Fig. 10B) and protein levels (Fig. 10C-D). Moreover, the rate of pulmonary cell apoptosis was markedly elevated (Fig. 10E). AliA-H treatment effectively reversed these pathological alterations.

However, co-treatment with the SERCA inhibitor TG substantially diminished the protective effects of AliA-H, resulting in hydroxyproline levels, collagen I and α -SMA expression, and apoptosis rates returning to levels similar to those in the BLM group. In contrast, TG administration in normal mice (Con+TG group) did not produce any significant changes. These findings confirm that AliA mitigates collagen deposition and apoptosis in a SERCA-dependent manner.

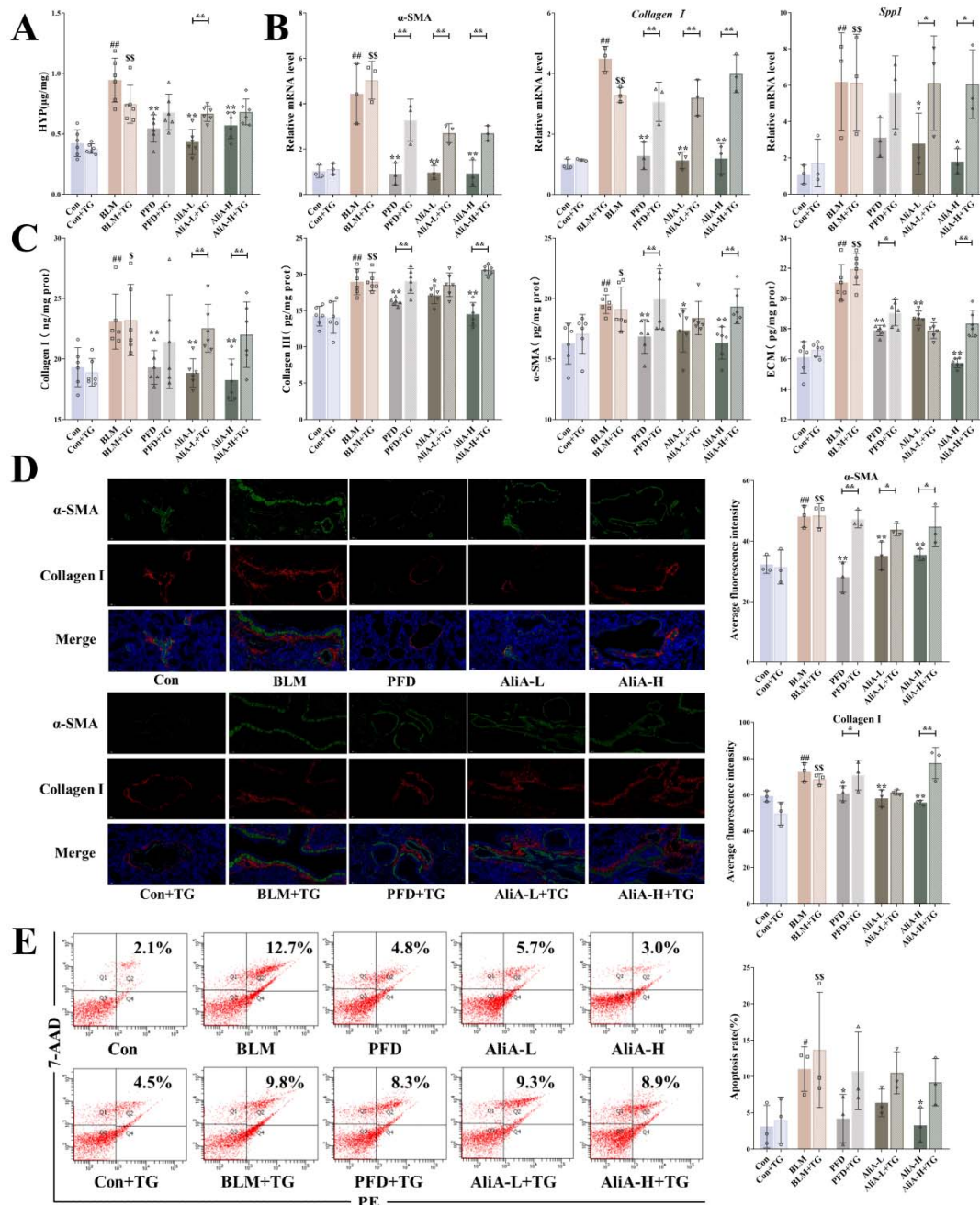
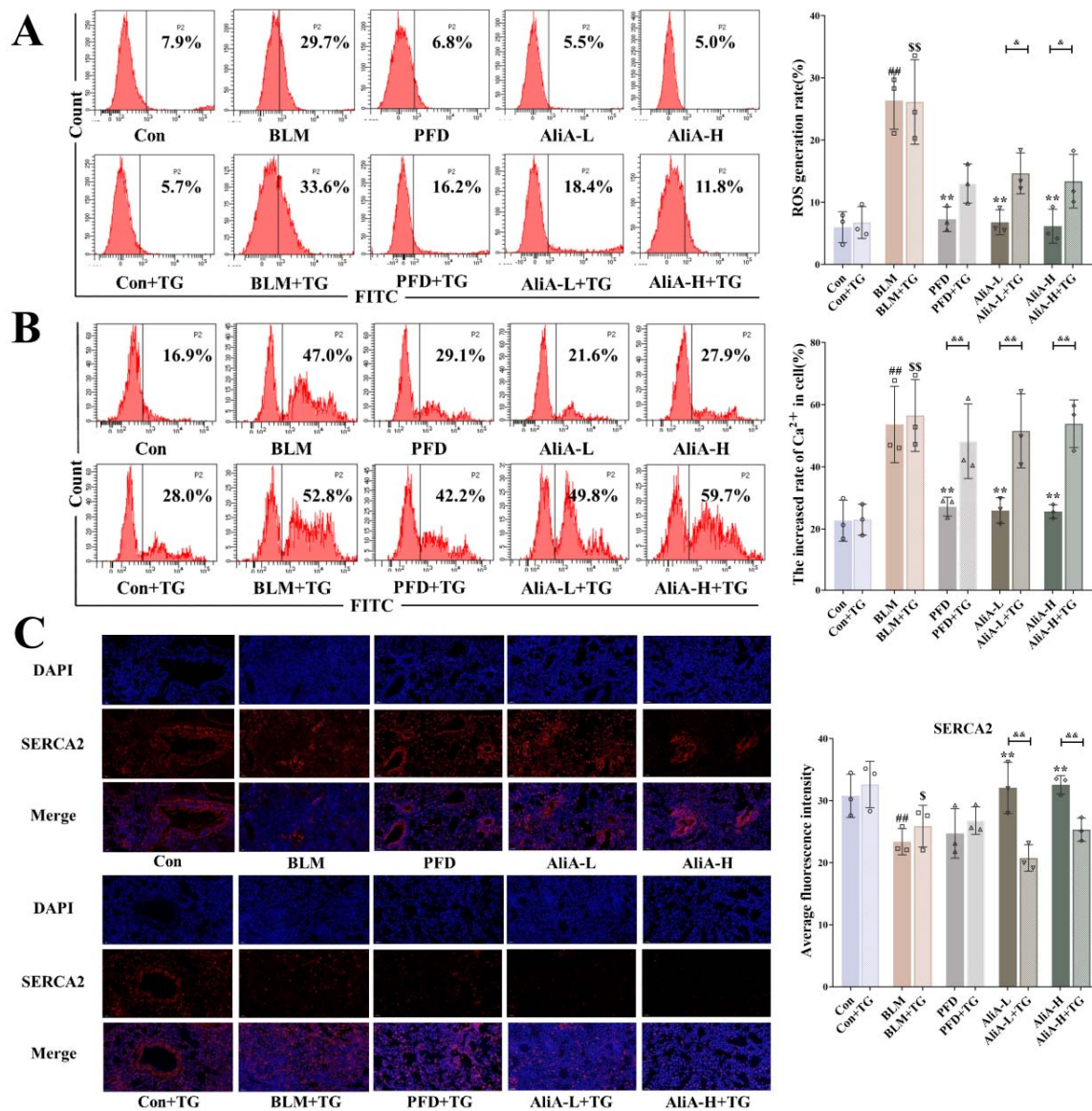


Fig. 10. Exploring the ameliorative effect of AliA on fibrosis in mice with PF by targeting SERCA. (A) Hydroxyproline content in lung tissue (n=6). (B) PCR detection of mRNA levels of the α -SMA, *Spp1* and *collagen I* genes (n=3). (C) Levels of α -SMA, ECM, Collagen I, and Collagen III in lung tissue (n=6). (D) Immunofluorescence detection of α -SMA and collagen I levels in lung tissue (n=3). (E) Flow cytometry analysis of apoptosis levels in mice with PF (n=3). Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AliA-L: AliA low dose group, AliA-H: AliA high dose group. Groups labeled with a “+” suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean $\pm$ SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. # P < 0.05, ## P < 0.01 vs. Con. * P < 0.05, ** P < 0.01 vs. BLM. \$ P < 0.05, \$\$ P < 0.01 vs. Con+TG. & P < 0.05, && P < 0.01 vs. treated with the inhibitor.

3.10 AliA improves PF in mice by targeting SERCA2

In the BLM-induced PF model, both ROS and intracellular Ca^{2+} levels were markedly elevated in lung tissues (Fig. 11A-B), while the expression of SERCA2-encoding gene *Atp2a2* was significantly downregulated at both the mRNA and protein levels (Fig. 11C-D), the gene encoding the SERCA2 pump, which resulted in a corresponding decrease in Ca^{2+} -ATPase activity (Fig. 11E). AliA-H treatment effectively reversed these abnormalities, reducing ROS and Ca^{2+} accumulation and restoring the expression of *Atp2a2*. However, co-treatment with the SERCA inhibitor TG largely negated these protective effects of AliA-H, with ROS/ Ca^{2+} levels and gene expression returning to levels resembling those observed in the BLM group. These results indicate that the anti-fibrotic function of AliA relies on the activation of SERCA2 (*Atp2a2*), thereby restoring calcium homeostasis and suppressing ROS accumulation.



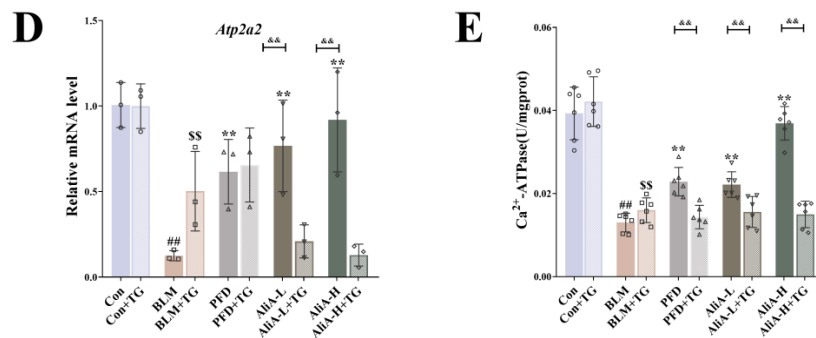


Fig. 11. AliA improves PF in mice by targeting SERCA2. (A) Flow cytometry analysis of ROS levels in mice with PF (n=3). (B) Flow cytometry analysis of Ca^{2+} levels in mice with PF (n=3). (C) Immunofluorescence detection of SERCA2 levels in lung tissue and quantitative results of SERCA2 immunofluorescence (n=3). (D) PCR detection of mRNA levels of the *Atp2a2* gene (n=3). (E) The levels of Ca^{2+} -ATPase. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AliA-L: AliA low dose group, AliA-H: AliA high dose group. Groups labeled with a “+” suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean $\pm$ SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Con. $^*P < 0.05$, $^{**}P < 0.01$ vs. BLM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. Con+TG. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. treated with the inhibitor.

3.11 AliA regulates the levels of immune cells in mice with PF by targeting SERCA

Systemic immune imbalance was observed in BLM-induced PF mice, characterized by significant elevations in pro-inflammatory macrophages, neutrophils, and Tregs in peripheral blood (Fig. S4). In the lung tissue, there was marked infiltration of macrophages and neutrophils, along with increased Treg levels (Fig. 12), indicating a pronounced local inflammatory response at the lesion site. AliA-H treatment significantly reversed these immune abnormalities in both peripheral circulation and the pulmonary microenvironment, notably reducing the levels of macrophages, neutrophils, and Tregs. However, co-administration of the SERCA inhibitor TG substantially reversed the immunomodulatory effects of AliA-H, with immune cell populations rebounding to levels similar to the BLM group. In contrast, the Con+TG group (healthy mice + TG) showed no changes in immune parameters, excluding the possibility of TG-mediated immunotoxicity. These findings suggest that AliA ameliorates PF by activating the SERCA pathway, thereby orchestrating the regulation of systemic immune responses and the local inflammatory microenvironment in the lung.

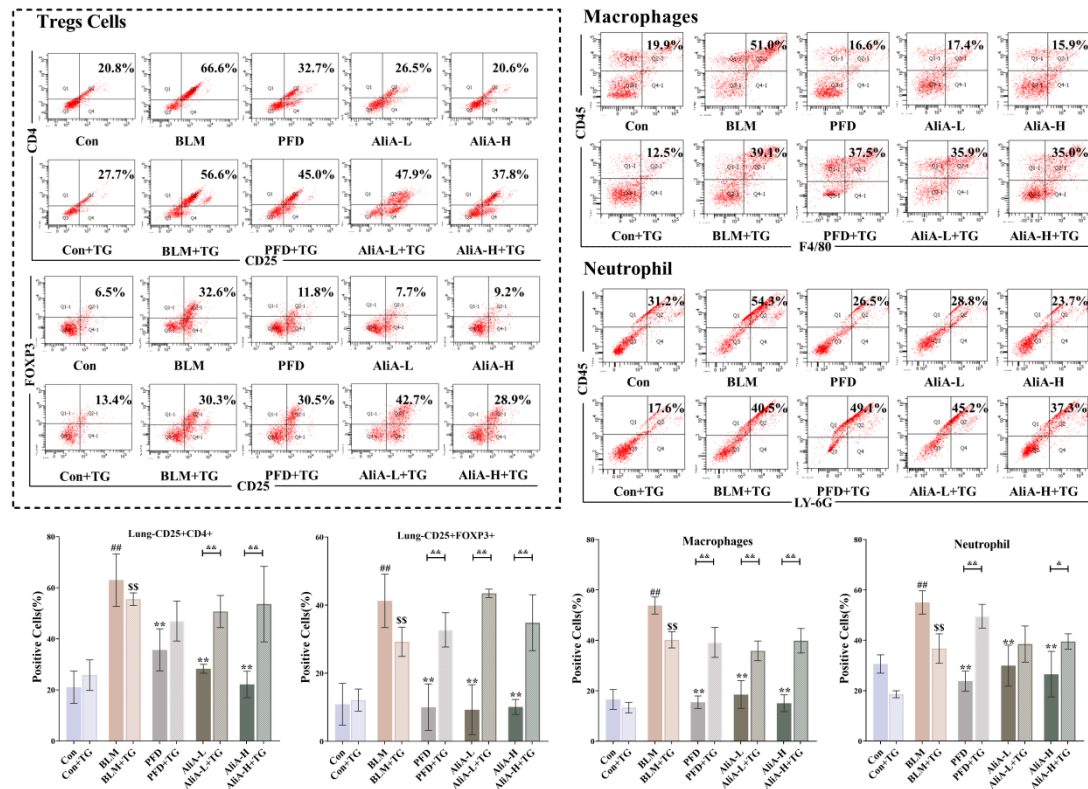


Fig. 12. AliA regulates immune cell levels in the lung tissue of mice with PF by targeting SERCA2. Con: Control, BLM: Model, PFD: Pirfenidone Capsules, AliA-L: AliA low dose group, AliA-H: AliA high dose group. Groups labeled with a “+” suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean $\pm$ SD. For assays with limited sample size ($n=3$), representative data from independent experiments with consistent trends are shown. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Con. $^*P < 0.05$, $^{**}P < 0.01$ vs. BLM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. Con+TG. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. treated with the inhibitor. Macrophages: Macrophages, Neutrophil: Neutrophil, Tregs Cells: regulatory T cells.

3.12 Investigating the effect of AliA on TGF- β 1-induced PF in BEAS-2B cells *in vitro* by blocking the SERCA target

Using an *in vitro* model of PF in BEAS-2B cells, we further confirmed that AliA exerts anti-fibrotic effects by targeting the SERCA protein. In TGF- β 1-stimulated bronchial epithelial cells, the SERCA inhibitor TG exhibited no cytotoxicity within the 1–20 μ M concentration range (Fig. 13A), while the effective concentration range of AliA was determined to be 10–20 μ M (Fig. 13B). TGF- β 1 stimulation significantly increased intracellular Ca^{2+} levels and apoptosis (Fig. 13C–D). Treatment with 10 μ M AliA reduced Ca^{2+} accumulation and attenuated apoptosis. Furthermore, TGF- β 1 markedly upregulated the expression of fibrotic markers including α -SMA, collagen I, and SPP1 (Fig. 13E), while AliA significantly suppressed their expression (Fig. 13F–H). Mechanistic investigations revealed that TGF- β 1 suppressed SERCA2 protein expression, whereas AliA restored its expression (Fig. 13I). Critically, the protective effects of AliA—including reduction in Ca^{2+} overload, inhibition of apoptosis, and downregulation of fibrosis-related proteins—were effectively neutralized when co-administered with TG. In addition, SERCA2 activation was selectively blocked by TG, confirming that the anti-fibrotic effects of AliA are contingent upon SERCA2 activation.

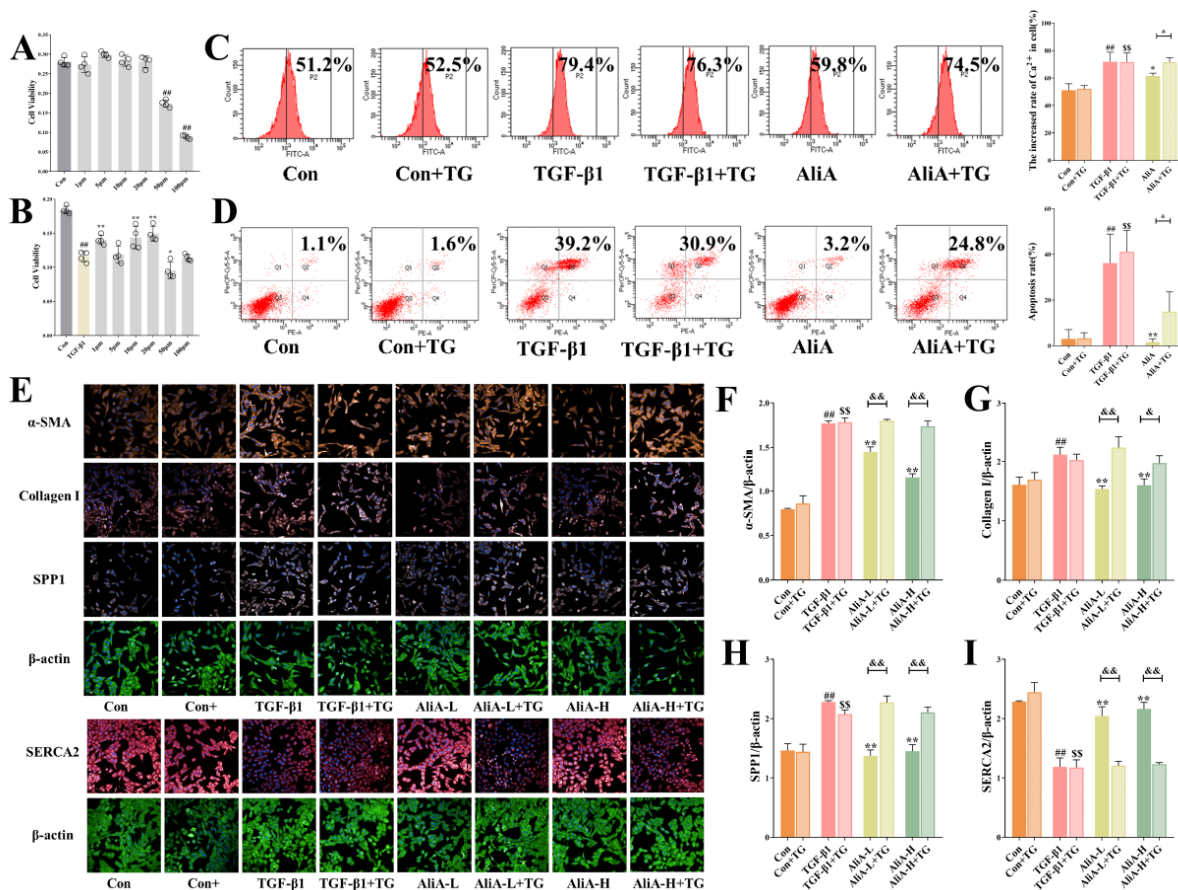


Fig. 13. Investigating the effect of AliA on TGF-β1-induced PF in BEAS-2B cells in vitro by blocking the SERCA target. (A) MTT assay for detecting the toxicity of TG (n=4). (B) MTT assay to determine the effective concentration of AliA against PF in vitro (n=4). (C) Flow cytometry analysis of Ca²⁺ levels in BEAS-2B treated with TGF-β1 and TG (n=3). (D) Flow cytometry analysis of apoptosis levels in BEAS-2B treated with TGF-β1 and TG (n=3). (E) Immunofluorescence detection of α-SMA, collagen I, SPP1 and SERCA2 levels in BEAS-2B cell treated with TGF-β1 and TG. (F) Quantitative results of α-SMA immunofluorescence (n=3). (G) Quantitative results of Collagen I immunofluorescence (n=3). (H) Quantitative results of SPP1 immunofluorescence (n=3). (I) Quantitative results of SERCA2 immunofluorescence (n=3). Con: Control, TGF-β1: Model, AliA: AliA group. Groups labeled with a “+” suffix (e.g., Con+TG, BLM+TG, PFD+TG, AliA-L+TG, AliA-H+TG) represent the respective groups co-treated with TG, a SERCA inhibitor. Data are presented as the mean ±SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. #*P* < 0.05, ##*P* < 0.01 vs. Con. **P* < 0.05, ***P* < 0.01 vs. TGF-β1. \$*P* < 0.05, \$\$*P* < 0.01 vs. Con+TG. &*P* < 0.05, &&*P* < 0.01 vs. treated with the inhibitor.

3.13 Investigating the effect of AliA on TGF-β1-induced PF in vitro using BEAS-2B cells through overexpression of the SERCA2 target

To further elucidate the mechanism of AliA, we overexpressed the SERCA2 target gene (*Atp2a2*) in the TGF-β1-induced BEAS-2B PF model. Overexpression of SERCA2 directly inhibited fibrotic marker expression: transfection with *Atp2a2* overexpression plasmids (Con-OE group) significantly suppressed the TGF-β1-induced upregulation of fibrosis-related genes, including α-SMA, collagen I, and *Spp1* mRNA (Fig. 14A). Restoration of calcium homeostasis and attenuation of apoptosis were also observed: TGF-β1 stimulation elevated intracellular Ca²⁺ levels and increased apoptosis (Fig. 14B-C), whereas SERCA2 overexpression reduced Ca²⁺ accumulation and lowered the apoptotic rate, comparable to the effects of 10 μM AliA. AliA exerted a synergistic anti-fibrotic effect through SERCA2 activation: co-treatment with AliA and SERCA2 overexpression further enhanced the suppression of fibrotic protein markers (α-SMA,

Collagen I, and SPP1) compared to SERCA2 overexpression alone (Fig. 14D-G), and significantly upregulated SERCA2 protein levels beyond that achieved by either intervention alone (Fig. 14H). Importantly, SERCA2 overexpression closely phenocopied the protective effects of AliA, with no additive benefit observed when the two were combined, confirming that SERCA2 is the primary molecular mediator of the anti-fibrotic effects of AliA.

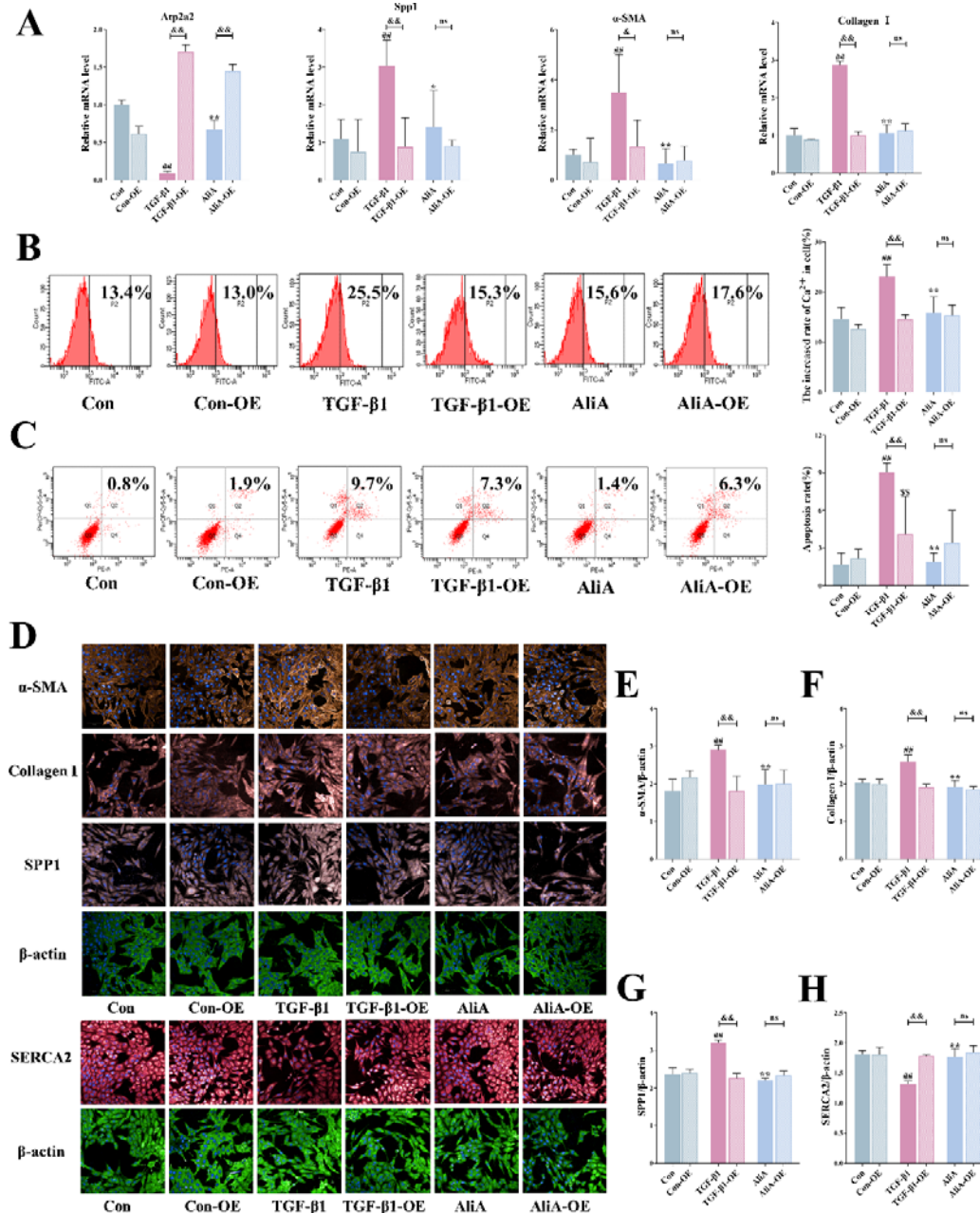


Fig. 14. Investigating the effect of AliA on TGF- β 1-induced PF *in vitro* using BEAS-2B cells through overexpression of the SERCA2 target. (A) PCR detection of mRNA levels of *Atp2a2*, *Spp1*, α -SMA, and collagen I genes in BEAS-2B cells treated with TGF- β 1 and *Atp2a2* overexpression plasmids (n=3). (B) Flow cytometry analysis of Ca^{2+} levels in BEAS-2B with TGF- β 1 and *Atp2a2* overexpression plasmid (n=3). (C) Flow cytometry analysis of apoptosis levels in BEAS-2B treated with TGF- β 1 and *Atp2a2* overexpression plasmid (n=3). (D) Immunofluorescence detection of α -SMA, collagen I, SPP1 and SERCA2 levels in BEAS-2B cell treated with TGF- β 1 and *Atp2a2* overexpression plasmid (n=3). (E) Quantitative results of α -SMA immunofluorescence (n=3). (F) Quantitative results of collagen I immunofluorescence (n=3). (G) Quantitative results of SPP1 immunofluorescence (n=3). (H) Quantitative results of SERCA2 immunofluorescence (n=3). Con: Control, TGF- β 1: Model, AliA: AliA group. Con-OE: Control treated with *Atp2a2* overexpression plasmid, all other groups were treated with the overexpression plasmid. Data are presented as the mean $\pm$ SD. For assays with limited sample size (n=3), representative data from independent experiments with consistent trends are shown. # P < 0.05, ## P < 0.01 vs. Con. * P < 0.05, ** P < 0.01 vs. TGF- β 1, & P < 0.05, && P < 0.01 vs. treated with the overexpression plasmid, ns: no significance.

3.14 In vivo safety profile of AliA in zebrafish model

To further evaluate the potential toxicity of AliA *in vivo*, we conducted an acute toxicity assay using a zebrafish model. Zebrafish larvae (48 hpf) were exposed to varying concentrations of AliA ranging from 0.1 to 100 μ M for 72 hours. As shown in Fig. 15, no significant increase in mortality or gross morphological abnormalities was observed across the entire concentration range tested when compared to the vehicle control group. These findings indicate that AliA possesses a favorable safety profile within the effective dose range.

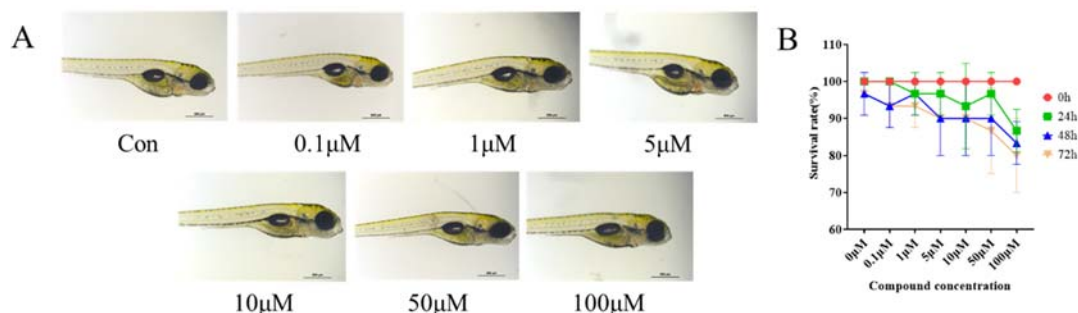


Fig. 15. Safety assessment of AliA in a zebrafish developmental toxicity model. Zebrafish larvae were exposed to a series of AliA concentrations (0.1–100 μ M) or a vehicle control (0.1% DMSO) for 72 hours, starting at 48 hours post-fertilization (hpf). (A) Representative brightfield microscopy images of larvae at 72 h. (A) Survival rate of larvae at 72 h (n=3).

4. Discussion

The search for novel therapeutic strategies against PF has intensified interest in nutritional interventions and bioactive compounds derived from medicine food homology plants. AE is a widely cultivated resource valued for both its medicinal properties and its traditional use in health-promoting diets, yet the specific chemical basis for its dual applications remains under investigation. In this study, we identified that the AE extract, and notably its major bioactive dietary component, AliA, exhibit robust anti-fibrotic activities in BLM-induced PF models. Our findings demonstrate that these effects are mediated through a tightly orchestrated network, culminating in the modulation of the SERCA2-dependent calcium signaling pathway, thus highlighting the potential of Alisol A as a functional food ingredient for fibrotic disease management.

Our initial *in vivo* results revealed that the crude AE extract correlated the amelioration of fibrosis with the modulation of the immune microenvironment and suppression of oxidative stress. AE significantly reduced lung injury and collagen deposition, which coincided with a restored immune profile—marked by reduced infiltration of macrophages, neutrophils, and Treg cells. This suggests the anti-fibrotic effects of *A. orientale* are, at least in part, mediated by the complex phytochemical mixture within the extract rebalancing the immune microenvironment. Given that macrophages and neutrophils secrete profibrotic cytokines (Chen et al., 2025; Xu et al., 2023), their suppression by AE likely contributes to the attenuation of fibrotic remodeling^[26, 27].

Oxidative stress is a known upstream driver of epithelial injury and fibroblast activation in PF^[28–31]. Our data showed that the AE extract markedly reduced ROS levels, lipid peroxidation (MDA), and apoptosis while enhancing antioxidant enzymes (SOD, GSH-Px). This suggests a cascade wherein the extract's antioxidant phytochemicals attenuate oxidative stress, leading to dampened inflammation, which in

turn suppresses fibroblast activation and ECM accumulation. The convergence of these pathways highlights the broad-spectrum protective effects of the *A. orientale* extract against PF pathology.

Mechanistically, transcriptomic analysis provided the critical link, identifying calcium signaling and ECM-receptor interaction as key pathways modulated by AE. This led us to investigate sarco/endoplasmic reticulum SERCA2, a critical regulator of intracellular calcium homeostasis^[32, 33]. Dysregulation of SERCA2, which we observed in fibrotic tissue, impairs ER calcium storage, induces ER stress, promotes mitochondrial Ca^{2+} overload, and triggers excessive ROS production^[34, 35]-all central to fibrotic remodeling. Reduced SERCA2 expression is documented in both IPF patients and animal models^[36, 37]. The reactivation of SERCA2 by the AE extract not only restored calcium equilibrium but also interrupted this pathological cascade, reinforcing SERCA2 as a pivotal molecular hub for the extract's anti-fibrotic effects.

A key objective in food and agricultural chemistry is to identify the specific active principles from crude extracts. We therefore investigated AliA, a major protostane-type triterpenoid characteristic of the *A. orientale* plant. AliA treatment recapitulated the potent anti-fibrotic effects of the whole extract, including improved lung function and reduced ECM accumulation. This is a significant finding from a natural product chemistry perspective, as it identifies a specific, purified phytochemical as the chemical entity responsible for the extract's primary mechanism. Crucially, the anti-fibrotic benefits of AliA were largely abolished by TG, a specific SERCA inhibitor, confirming that SERCA activation is central to AliA-mediated lung protection.

In vitro studies using TGF- β 1-stimulated BEAS-2B cells further validated this SERCA2-dependent mechanism. AliA significantly reduced fibrotic markers and restored SERCA2 levels, while SERCA2 inhibition significantly reversed these effects. Conversely, overexpression of SERCA2 mimicked the anti-fibrotic actions of AliA, and their combined use showed no synergistic benefit. This strongly indicates that SERCA2 is the principal molecular target of the natural product AliA. PF is inherently a multicellular pathology, and we recognize that our focus on BEAS-2B captures only part of this complex interplay. While the rescue of SERCA2 function in epithelial cells is evident, the direct architects of ECM deposition-lung fibroblasts and their myofibroblast differentiation-demand equal scrutiny. Consequently, validating whether the SERCA2-dependent protective mechanism of AliA is conserved within human lung fibroblasts (e.g., MRC-5) or primary isolates remains an imperative next step.

Moreover, identifying SERCA2 as a downstream target elucidates the effect but not the cause; the upstream molecular events by which AliA restores SERCA2 expression are still opaque. Whether AliA intervenes by engaging transcriptional machinery, influencing epigenetic modifiers, or physically stabilizing the SERCA2 protein against degradation is a question that defines the trajectory for future mechanistic inquiry.

Beyond direct cellular targets, the pharmacokinetics of orally administered AliA warrants a broader systemic view. As a triterpenoid, AliA inevitably engages the gut microbiota, a crucial node in the gut-lung axis. It is plausible that the immunomodulation observed in our data-specifically the suppression of

macrophages and neutrophils-relies partially on microbiota-derived signals or metabolites, such as short-chain fatty acids. Integrating nutritional immunology to profile the gut microbiome in AliA-treated subjects will therefore be essential to fully decode its pharmacological footprint.

Regarding experimental design, our cohort was restricted to male mice to mitigate hormonal variability and mirror the clinical male predominance of PF [38, 39], yet this leaves the impact of sexual dimorphism unexplored. While our zebrafish assays suggest a favorable safety profile, the leap to clinical application faces practical hurdles. Future translational efforts must prioritize optimizing AliA's formulation to overcome bioavailability barriers inherent to oral delivery.

Ultimately, this study establishes that AE and its bioactive constituent, AliA, do not merely suppress fibrosis but actively restore SERCA2-dependent calcium homeostasis while modulating immune and oxidative stress responses. These data provide the empirical bedrock necessary to advance AliA from a phytochemical candidate to a viable nutraceutical strategy for compromising fibrotic lung disease.

Financial support

This study was supported by the National Key Research and Development Project (2019YFC1708802, 2017YFC1702800), the Major Science and Technology Projects in Henan Province (171100310500), National Natural Science Foundation of China Project (32200322), Henan Science and Technology Research and Development Plan Joint Fund (242301420082), Henan Province "Science and Technology Vice General" Three-Year Action Plan (2024-2026), Henan Postdoctoral Fund (HN2025059).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We are grateful for bioinformatics support from (LC-Bio Technology co.,ltd.).

Declaration of generative AI and AI-assisted technologies in the writing process.

In this study, the author utilized ChatGPT to optimize and polish the language expressions. Following the use of this artificial intelligence tool, the author conducted a systematic review and professional revision of the generated content, and assumes full responsibility for the academic content and language of the manuscript.

References

- [1] L. Chen, J.W. Hou, X. Fu, et al., tPA promotes the proliferation of lung fibroblasts and activates the Wnt/ β -catenin signaling pathway in idiopathic pulmonary fibrosis, *Cell Cycle*. 18 (2019) 3137-3146. <https://doi.org/10.1080/15384101.2019.1669997>.
- [2] Y. Fang, J.Y. Tian, Y.M. Fan, et al., Latest progress on the molecular mechanisms of idiopathic pulmonary fibrosis, *Molecular Biology Reports*. 47 (2020) 9811-9820. <https://doi.org/10.1007/s11033-020-06000-6>.
- [3] M. Kreuter, F. Bonella, M. Wijsenbeek, et al., Pharmacological Treatment of Idiopathic Pulmonary Fibrosis: Current Approaches, Unsolved Issues, and Future Perspectives, *BioMed research international*. 2015 (2015) 329481. <https://doi.org/10.1155/2015/329481>.

- [4] Q. Zhang, M. Zeng, B. Zhang, et al., Evening primrose and its compounds of 1-Oxohederagenin and remangilone C ameliorate bleomycin-induced pulmonary fibrosis by regulating β -catenin signaling, *Phytomedicine*. 133 (2024) <https://doi.org/10.1016/j.phymed.2024.155933>.
- [5] M. Zeng, A. Feng, Y. Ren, et al., *Salvia miltiorrhiza* Bunge extract and Przewalskin ameliorate Bleomycin-induced pulmonary fibrosis by inhibition of apoptosis, oxidative stress and collagen deposition via the TGF- β 1 pathway, *Phytomedicine*. 125 (2024) <https://doi.org/10.1016/j.phymed.2024.155339>.
- [6] S.K. Law, D.C.T. Au, A review of medicine and food homology on traditional Chinese medicine as functional food, *Food & Medicine Homology*. 3 (2026) 9420091. <https://doi.org/10.26599/FMH.2026.9420091>.
- [7] Y. Zhang, B. Cong, The development road of Chinese characteristic food & medicine homology, *Food & Medicine Homology*. 3 (2026) 9420104. <https://doi.org/10.26599/FMH.2026.9420104>.
- [8] P.L. Wang, T.X. Song, R. Shi, et al., Triterpenoids From *Alisma* Species: Phytochemistry, Structure Modification, and Bioactivities, *Frontiers in Chemistry*. 8 (2020) 363. <https://doi.org/10.3389/fchem.2020.00363>.
- [9] H.H. Li, C.Y. Zhang, Y.J. Zhou, et al., Neurovascular protection of alisol A on cerebral ischemia mice through activating the AKT/GSK3 β pathway, *Aging-Us*. 15 (2023) 11639-11653. <Go to ISI>://WOS:001120064000006
- [10] C.J. Yue, W.W. Dai, X.H. Song, et al., Alisol A inhibits the circ_0001831/miR-346/LIN28B pathway to ameliorate high glucose-induced injury of human renal mesangial cells, *Nephrology*. 29 (2024) 154-163. <https://doi.org/10.1111/nep.14258>.
- [11] Y. Ma, D.Z. Song, J. Yuan, et al., Alisol A inhibits and stabilizes atherosclerotic plaques by protecting vascular endothelial cells, *Frontiers in Pharmacology*. 15 (2024) 1493948. <https://doi.org/10.3389/fphar.2024.1493948>.
- [12] P. Cameli, A. Carleo, L. Bergantini, et al., Oxidant/Antioxidant Disequilibrium in Idiopathic Pulmonary Fibrosis Pathogenesis, *Inflammation*. 43 (2020) 1-7. <https://doi.org/10.1007/s10753-019-01059-1>.
- [13] Y. Gong, N. Wang, N. Liu, et al., Lipid Peroxidation and GPX4 Inhibition Are Common Causes for Myofibroblast Differentiation and Ferroptosis, *DNA and cell biology*. 38 (2019) 725-733. <https://doi.org/10.1089/dna.2018.4541>.
- [14] Y. Motomura, W.I. Khan, R.T. El-Sharkawy, et al., Induction of a fibrogenic response in mouse colon by overexpression of monocyte chemoattractant protein 1, *Gut*. 55 (2006) 662-670. <https://doi.org/10.1136/gut.2005.068429>.
- [15] R. Herro, R. Da Silva Antunes, A.R. Aguilera, et al., Tumor necrosis factor superfamily 14 (LIGHT) controls thymic stromal lymphopoietin to drive pulmonary fibrosis, *The Journal of allergy and clinical immunology*. 136 (2015) 757-768. <https://doi.org/10.1016/j.jaci.2014.12.1936>.
- [16] R.M. Strieter, B. Mehrad, New Mechanisms of Pulmonary Fibrosis, *Chest*. 136 (2009) 1364-1370. <https://doi.org/10.1378/chest.09-0510>.
- [17] B. Strauss, Y. Sassi, C. Bueno-Beti, et al., Intra-tracheal gene delivery of aerosolized SERCA2a to the lung suppresses ventricular arrhythmias in a model of pulmonary arterial hypertension, *Journal of Molecular and Cellular Cardiology*. 127 (2019) 20-30. <https://doi.org/10.1016/j.yjmcc.2018.11.017>.
- [18] W.S. Chen, L.J. Liu, M. Tang, et al., Type I collagen-targeted liposome delivery of Serca2a modulates myocardium calcium homeostasis and reduces cardiac fibrosis induced by myocardial infarction, *Materials Today Bio*. 28 (2024) 101162. <https://doi.org/10.1016/j.mtbio.2024.101162>.
- [19] K. Wang, H. Zhu, J. Kang, et al., Theaflavin-3,3'-digallate alleviates glucolipotoxicity-induced insulin secretion dysfunction in pancreatic β -TC-6 cells and zebrafish via anti-ferroptotic mechanisms by activating SERCA2, *Food Science and Human Wellness*. 14 (2025) 9250096. <https://doi.org/10.26599/FSHW.2024.9250096>.
- [20] Y.Q. Li, Z.H. Zhang, J.F. Pan, et al., Study on the anticancer activities and chemical constituents of the *Grateloupia filicina* C.Ag., *Chinese Journal of Marine Drugs*. 29 (2010) 29-33. <https://doi.org/10.13400/j.cnki.cjmd.2010.06.006>.
- [21] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, *Faseb Journal*. 22 (2008) 659-661. <https://doi.org/10.1096/fj.07-9574LSF>.
- [22] P. Xu, W. Zhou, S. Wang, et al., Alisol A ameliorates vascular cognitive impairment via AMPK/NAMPT/SIRT1-mediated regulation of cholesterol and autophagy, *Theranostics*. 15 (2025) 9415-9446. <https://doi.org/10.7150/thno.112661>.
- [23] K. Wang, B. Zhang, D. Song, et al., Alisol A Alleviates Arterial Plaque by Activating AMPK/SIRT1 Signaling Pathway in apoE-Deficient Mice, *Frontiers in Pharmacology*. 11 (2020) <https://doi.org/10.3389/fphar.2020.580073>.

- [24] R. Shen, K.B. Cheng, G.Y. Li, et al., Alisol A, the Eye-Entering Ingredient of *Alisma orientale*, Relieves Macular Edema Through TNF- α as Revealed by UPLC-Triple-TOF/MS, Network Pharmacology, and Zebrafish Verification, Drug Design Development and Therapy. 18 (2024) 3361-3382. <https://doi.org/10.2147/dddt.S468119>.
- [25] X.H. Chen, H.Q. Liu, Alisol A Inhibited the Proliferation, Migration, and Invasion of Nasopharyngeal Carcinoma Cells by Inhibiting the Hippo Signaling Pathway, Yonsei Medical Journal. 62 (2021) 895-902. <https://doi.org/10.3349/ymj.2021.62.10.895>.
- [26] Y.H. Xu, P.X. Lan, T. Wang, The Role of Immune Cells in the Pathogenesis of Idiopathic Pulmonary Fibrosis, Medicina-Lithuania. 59 (2023) 1984. <https://doi.org/10.3390/medicina59111984>.
- [27] S.J. Chen, X.D. Song, C.J. Lv, Macrophages and Pulmonary Fibrosis, Current Molecular Medicine. 25 (2025) 416-430. <https://doi.org/10.2174/0115665240286046240112112310>.
- [28] S.Q. Lv, Y. Li, X.Y. Li, et al., Silica nanoparticles triggered epithelial ferroptosis via miR-21-5p/GCLM signaling to contribute to fibrogenesis in the lungs, Chemico-Biological Interactions. 399 (2024) 111121. <https://doi.org/10.1016/j.cbi.2024.111121>.
- [29] L.Q. Wang, S. Li, Y.Q. Yao, et al., The role of natural products in the prevention and treatment of pulmonary fibrosis: a review, Food & Function. 12 (2021) 990-1007. <https://doi.org/10.1039/d0fo03001e>.
- [30] M.A. Abdel-Wahhab, A.A. El-Nekeety, A.S. Salman, et al., Modulation of oxidative damage and genotoxicity of biosynthesized zinc oxide nanoparticles via supplementation with modified cinnamaldehyde-loaded chitosan nanoparticles in mice, Food Science and Human Wellness. 14 (2025) 9250374. <https://doi.org/10.26599/FSHW.2024.9250374>.
- [31] X. Liu, X. Meng, D. Liu, et al., A rapid analysis method to discover antioxidant active components and mechanisms in soybean: untargeted metabolomics combined with network pharmacology and spectrum-effect relationship-component knockout-identification techniques, Food Science and Human Wellness. 14 (2025) 9250620. <https://doi.org/10.26599/FSHW.2025.9250620>.
- [32] W.Q. Song, Q.J. Zhang, Z.Y. Cao, et al., Targeting SERCA2 in Anti-Tumor Drug Discovery, Current Drug Targets. 26 (2025) 1-16. <https://doi.org/10.2174/0113894501325497240918042654>.
- [33] L. Lipskaia, E.R. Chemaly, L. Hadri, et al., Sarcoplasmic reticulum Ca(2+) ATPase as a therapeutic target for heart failure, Expert opinion on biological therapy. 10 (2010) 29-41. <https://doi.org/10.1517/14712590903321462>.
- [34] H.Q. Wang, B. Han, N.L. Wang, et al., Oxymatrine attenuates arsenic-induced endoplasmic reticulum stress and calcium dyshomeostasis in hepatic stellate cells, Annals of Translational Medicine. 8 (2020) 1171. <https://doi.org/10.21037/atm-20-5881>.
- [35] K.D. Li, C.Z. Guo, J.C. Ruan, et al., Cadmium Disrupted ER Ca²⁺ Homeostasis by Inhibiting SERCA2 Expression and Activity to Induce Apoptosis in Renal Proximal Tubular Cells, International Journal of Molecular Sciences. 24 (2023) 5979. <https://doi.org/10.3390/ijms24065979>.
- [36] M. Bissierier, L. Hadri, Lung-targeted SERCA2a Gene Therapy: From Discovery to Therapeutic Application in Bleomycin-Induced Pulmonary Fibrosis, Journal of cellular immunology. 2 (2020) 149-156. <Go to ISI>://MEDLINE:32587955
- [37] M. Bissierier, J. Milara, Y. Abdeldjebbar, et al., AAV1.SERCA2a Gene Therapy Reverses Pulmonary Fibrosis by Blocking the STAT3/FOXO1 Pathway and Promoting the SNON/SKI Axis, Molecular therapy : the journal of the American Society of Gene Therapy. 28 (2020) 394-410. <https://doi.org/10.1016/j.ymthe.2019.11.027>.
- [38] G. Raghu, H.R. Collard, J.J. Egan, et al., An Official ATS/ERS/ALAT Statement: Idiopathic Pulmonary Fibrosis: Evidence-based Guidelines for Diagnosis and Management, American Journal of Respiratory and Critical Care Medicine. 183 (2011) 788-824. <https://doi.org/10.1164/rccm.2009-040GL>.
- [39] J. Hutchinson, A. Fogarty, R. Hubbard, et al., Global incidence and mortality of idiopathic pulmonary fibrosis: a systematic review, European Respiratory Journal. 46 (2015) 795-806. <https://doi.org/10.1183/09031936.00185114>.