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ORIGINAL RESEARCH

Shu-Feng-Jie-Biao Formula Ameliorates Influenza A Virus-Induced Acute Lung Injury by Inhibiting NF-κB and ERK MAPK Signaling Pathways

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Purpose: Shu-Feng-Jie-Biao formula (SFJBF) has been used to treat acute respiratory infections for a dozen years. This study aimed to explore its mechanisms and effects for the treatment of influenza.

Methods: Network pharmacology was used to explore the underlying mechanism of SFJBF against influenza. The protective effects of SFJBF in vivo were evaluated by lung indexes, body weight loss and pathological changes in lungs. The anti-inflammatory effects in vivo were evaluated by flow cytometry and ELISA. RAW264.7 cells stimulated with imiquimod (R837) were used to determine the anti-inflammatory effects of SFJBF. Neutrophils isolated from bone marrow were activated by phorbol 12-myristate 13-acetate (PMA) to validate the effects of the active components of SFJBF.

Results: SFJBF protected body weight loss, decreased lung indexes, reduced total protein content in lungs and mitigated pathological changes in mice. SFJBF inhibited the expression of chemokines (*Cxcl2* and *Ccl2*) and cytokines (*Il1b* and IL-6) accompanied by the decreased infiltration of neutrophils in lungs. SFJBF inhibited the expression of iNOS and MPO in lungs. The synergistic role of OSV and SFJBF was exhibited by suppressing virus-induced cytokine expression and reducing the infiltration of inflammatory monocytes in lungs. SFJBF inhibited the phosphorylation of ERK1/2 and NF-κBp65, thereby reducing the secretion of MIP-2, TNF-α, MCP-1 and CCL5 in vitro The active components of SFJBF, including baicalin and wogonin, reduced the production of reactive oxygen species (ROS), MIP-2, MCP-1, and IL-6 in vitro.

Conclusion: SFJBF ameliorated virus-induced lung injury by suppressing overactivated immune responses via NF-κB and ERK MAPK signaling pathways, thereby protecting mice from influenza virus infection. SFJBF could be considered a potent therapeutic agent for treating influenza.

Keywords: traditional Chinese medicine, TCM, anti-inflammatory, influenza virus, acute lung injury, NF-κB signaling pathway, ERK MAPK signaling pathway

Introduction

Influenza has become a constant threat to human health and is associated with substantial morbidity and mortality worldwide, causing an estimated 300,000 deaths annually.[1] According to data from the National Influenza-like Disease Surveillance Sentinel Hospitals, there are 3.4 million patients of influenza who receive clinical treatment annually in

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China.[2] Influenza is an acute respiratory infectious illness, caused by influenza A, B and C viruses.[1] Influenza A virus is the main circulating and highly mutated type of influenza virus, causing several pandemics over the past two centuries, including the Spanish flu, the Asian flu, the Hong Kong flu and the 2009 H1N1 pandemic.[1]

Vaccines are the primary preventative tools in the fight against influenza. However, due to inadequate vaccination coverage and mismatches with prevalent strains, vaccinations fail to provide full protection.[1] Oseltamivir, the first FDA-approved neuraminidase inhibitor (NAI), can inhibit viral neuraminidase, reduce the release of progeny viruses and has been recommended as the treatment of influenza for its effectiveness in decreasing the time to fever resolution, accelerating symptom recovery and reducing the risk of hospitalization, which was chosen as control medicine in our study.[3] Other antiviral agents, such as zanamivir, peramivir, and baloxavir, have been recommended for the treatment of influenza by the Center for Disease Control and Prevention (CDC). These drugs inhibit viral replication by targeting viral proteins. Antigenic shift and antigenic drift can lead to variations in influenza virus proteins, which may lead to the emergence of drug-resistant influenza viruses.[4,5] The occurrence of drug-resistant influenza viruses has reduced the susceptibility of influenza viruses to oseltamivir phosphate, zanamivir, peramivir, and baloxavir,[4,6] leading to epidemics.[4] Therefore, new strategies to prevent and control seasonal influenza epidemics or even pandemics are needed.

Traditional Chinese medicine (TCM) has a long history of being used to combat more than 500 outbreaks of pestilence, is often affordable and has been extensively used to treat diseases in China. During the treatment of acute respiratory viral infection, TCM is usually characterized by reducing disease severity, amelioration of clinical symptoms, protection against organ dysfunction, and suppressing inflammatory response.[7] In these years, TCM has been recommended to treat acute respiratory infectious diseases from mild to severe infection caused by COVID-19 or influenza.[8,9] Notably, compared with antiviral drugs, TCM mainly regulates host response and can be used to treat emerging infectious diseases.

Shu-Feng-Jie-Biao formula (SFJBF) originated from the classical Jing-Fang-Bai-Du formula, a famous prescription of Zhang Shiche, which is mainly employed to treat epidemic diseases, dysentery, common cold, influenza and other viral infections.[10] SFJBF, a hospital agreement formula of Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine with a history of over 10 years, is composed of 8 herbs, including *Schizonepeta tenuifolia* (Benth). Briq, *Saposhnikovia divaricata* (Turcz). Schisch, *Bupleurum chinensis* DC., *Radix Angelicae Dahuricae, Ligusticum chuanxiong* Hort., *Sojae Semen Praeparatum, Glycyrrhiza uralensis* Fisch. and *Notopterygium incisum Ting ex* H.T. Chang. Lianhua Qingwen can clear heat and toxins, disperse the lung and dispel heat. SFJBF is mainly used to dispel wind, relieve surface, and clear heat. *Schizonepeta tenuifolia, Saposhnikovia divaricata, Radix Angelicae Dahuricae, Ligusticum chuanxiong* and *Notopterygium incisum Ting ex* can expel wind and relieve the exterior, which possesses antiviral, anti-inflammatory, antipyretic, and immunomodulatory properties.[11–14] *Bupleurum chinensis* can remove pathogenic factors and release the exterior, which possess an antiviral effect against the influenza virus.[15] *Glycyrrhiza uralensis* could attenuate lipopolysaccharide-induced acute lung injury by decreasing COX-2 and inducible nitric oxide synthase (iNOS) expression.[16] In our previous study, SFJBF had antipyretic effects in a septic mouse model induced by peritoneal injection of lipopolysaccharide (LPS) from *Escherichia coli*. SFJBF gavage alleviated inflammatory exudation in mice induced by intraperitoneal injection of glacial acetic acid. However, the exact mechanism of this formula has not been demonstrated.[17] It has become a treatment option for the treatment of upper respiratory tract infections including influenza, in Longhua Hospital, with remarkable clinical effects for more than 10 years. However, a reliable basis for the treatment of acute influenza is lacking. Studying the mechanism of herbal medicines on influenza virus infection is tricky owing to their complex components. Network pharmacology has been recognized as a reasonable strategy to study active ingredient, target prediction and mechanism study via constructing compound-target-disease network and signaling pathway analysis.[18]

The exuberant immune responses known as "cytokine storm" can cause severe immunopathology in infectious diseases.[19] Unimpaired influenza viruses by innate immunity can result in persistent viral replication that triggers dysfunctional immune response including unrestrained inflammatory cell infiltration and uncontrolled release of cytokines, which can damage normal lung tissues and promote acute lung injury, even acute respiratory distress syndrome (ARDS). Massive inflammatory monocytes are recruited to lungs and they subsequently differentiate into macrophages. Neutrophils are the first intrinsic immune cells recruited to the lung in response to influenza virus infection.

Overactivated macrophages and neutrophils play an important role in driving the formation of cytokine storm and can aggravate the destruction of pulmonary endothelia and epithelia.[20] Therefore, it is vital to control virus-induced exuberant immune responses, particularly macrophages and neutrophil-related immune responses.

In this study, network pharmacology was employed to explore the underlying mechanism. The protective effects of SFJBF against influenza virus were validated by a flu mouse model. The inhibition of virus-induced overactivated inflammatory response by SFJBF and its key components was verified in Raw264.7 cells and neutrophils. Our results suggest that SFJBF can mitigate lung injury and alleviate excessive inflammatory responses by inhibiting the activation of the NF-κB and ERK MAPK signaling pathways, providing a research foundation for its clinical applications.

Materials and Methods

Potential Targets of SFJBF and Influenza

TCMSP databases were employed to collect the key compounds and drug targets of SFJBF as previously mentioned.[18] The components were retained for further analysis according to the criteria of oral bioavailability (OB) ≥30% and drug-likeness (DL) ≥0.18. The potential targets of influenza were obtained from Genecard, Drugbank, DisGeNET and CTD databases.[18]

Protein-Protein Interaction Network (PPI)

The common potential targets of SFJBF and influenza were analyzed by the STRING platform. The species was set as "Homo sapiens" and the genes were filtered by score > 0.9. The PPI network was exported and visualized by Cytoscape 3.7.2 software.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Based on Key Targets

Potential targets of SFJBF against influenza were uploaded into the Metascape platform (https://metascape.org/gp/index.html). GO and KEGG analysis were performed as previously described.[18] The species was set to "sapiens" and the p value was set as $p<0.05$.

Network Construction

Potential chemical compounds and core targets were used to construct herbs-compound-target network by Cytoscape 3.7.2 software. Network analyzer was utilized to analyze the network topology parameters for the components and targets, including degree, betweenness and closeness. The core targets are associated with the value of degree.

Preparation of SFJBF

SFJBF was provided by Longhua Hospital, affiliated with Shanghai University of Traditional Chinese Medicine. After weighing 8 herbs of SFJBF proportionally, they were mixed and soaked in water for 30 min. The samples were boiled twice for 1.5 h in 700 mL water. The decoctions were merged and filtered. The final decoction concentration was 2.336 g/mL. The decoction was stored at −80°C until use.

The criteria for the quality of 8 herbs in SFJBF were consistent with 2020 Chinese pharmacopoeia.[21] The compounds including pulegone (Sunny Biotech, Shanghai, China), prim-O-glucosylcimifugin, 5-O-Methylvisammioside, genistein, daidzein, imperatorin, ferulic acid, notopterol, saikosaponins D, liquiritin, glycyrrhizic acid and saikosaponin A (Tauto Biotech, Shanghai, China), were examined by ultra performance liquid chromatography-triple quadrupole tandem mass spectrometry (UPLC-MS/MS). The mobile phase consisted of A (0.1% formic acid) and B (acetonitrile). The gradient elution was performed as below: 0–8.0 min at 25% – 45% B, 8.0–13.5 min at 45% – 95% B, 13.5–15.5 min at 95% B, 15.5–17.0 min at 95% – 25% B, which were conducted at a flow rate of 0.3 mL/min and a volume of 2 μL. Chromatographic separations were carried out on a Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) at 40°C. The mass spectrometer was performed as follow: ion spray voltage, +5500 /-4500 V; curtain gas, 241.3 kPa; CAD, 48.3 kPa; source temperature, 550°C; nebulizer gas (Gas 1), 413.7 kPa; heater gas (gas 2), 413.7 kPa. These instrumentations were controlled and synchronized by Analyst software (versions 1.6.3; Applied Biosystems/MDS Sciex).

The components in SFJBF were analyzed by ultra-high-performance liquid chromatography-quadrupole-electrostatic field orbitrap mass spectrometry (UHPLC-QE-MS) and conducted on a UHPLC system (Vanquish, Thermo Fisher Scientific) with a Waters UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm), which was performed at a flow rate of 0.4 mL/min and a volume of 5 μL. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% in acetonitrile (B). The multi-step linear elution gradient program was as follows: 0–3.5 min, 95–85% A; 3.5–6 min, 85–70% A; 6–6.5 min, 70–70% A; 6.5–12 min, 70–30% A; 12–12.5 min, 30–30% A; 12.5–18 min, 30–0% A; 18–25 min, 0–0% A; 25–26 min, 0–95% A; 26–30 min, 95–95% A.

SD male rats were gavaged with SFJBF (19.5 g/kg) or ultrapure water once daily for 7 days to obtain SFJBF-containing sera and control sera, which were prepared as previously described.[22] The blood samples obtained from abdominal aorta were centrifuged at 3500 rpm for 25 min after being placed at room temperature for 2 h. The sera were collected and centrifuged for 5 min at 12,000 rpm to remove the remnant erythrocytes. After being inactivated at 56 ◦C for 0.5 h, the sera were filtered through a microporous membrane, mixed, divided into Eppendorf tubes and stored at −80 ◦C for use.

Cell Experiment

Mouse macrophage cell line, RAW264.7, was purchased from ATCC (Manassas, VA, USA). Cells were grown in DMEM containing 10% FBS and treated with 10% SFJBF-containing sera or 10% control sera with the stimulation of 5 μg/mL imiquimod (R837, cat: tlrl-imqs).

Animals and Virus

Six-week-old SPF C57BL/6J female mice (17–19 g) were obtained from the Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). They were adaptively housed in the barrier environment of the Laboratory Animal Center for one week. The room was alternated between light and dark every 12 h/12 h at 20–26°C. The relative humidity was 40–70%, and the mice were allowed to drink freely. All in vivo experiments related to animals were conducted following the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethics Committee of Guangzhou Medical University (No. 20230309). All animal studies were performed in accordance with ARRIVE guidelines.

Influenza virus A/Puerto Rico/8/1934 (H1N1) (PR8) was reproduced in MDCK cells. The virus was inoculated and incubated in a viral growth medium containing TPCK trypsin (2 μg/mL). The cell culture supernatant was collected when most cells showed cytopathic effects. After centrifugation, the supernatants were stored in aliquots and frozen in liquid nitrogen.

The Establishment of an Influenza Virus-Infected Mouse Model

As a final concentration of 3.52×10^4 PFU/mL, the virus suspension was diluted with cold sterile PBS. For animal experiments, the clinical dose is chosen to be the low dose in this study. The clinical dose of SFJBF = 73 g/60 kg/day × 12 = 14.6 g/kg/day (The clinical dosage of SFJBF for an adult is 73 g/person/day, the equivalent dose ratio of mouse to human is 12, and the average weight of an adult is supposed to 60 kg). The mice were randomly assigned into 7 groups, including normal control group (NC), PR8 infection group (PR8), oseltamivir (OSV, 30 mg/kg) treatment group (PR8 + OSV), high dose of SFJBF (43.8 g/kg)-treated group [PR8 + SFJBF(H)], middle dose of SFJBF (29.2 g/kg)-treated group [PR8 + SFJBF(M)], low dose of SFJBF (14.6 g/kg)-treated group [PR8 + SFJBF(L)] and the combined treatment group [PR8 + OSV + SFJBF(M)]. After anesthesia by isoflurane, mice in PR8 group, PR8 + OSV group, PR8 + SFJBF(H), PR8 + SFJBF(M), PR8 + SFJBF(L) and PR8 + OSV + SFJBF(M) group were slowly administrated with 50 μL diluted PR8 into the left nasal cavity, while mice in the NC group were administrated with 50 μL PBS. The treatment of OSV or SFJBF was conducted by gavage for 6 consecutive days and the body weights of mice were recorded at the same time point every morning during the experiment. In addition, lung samples were collected for Flow cytometric analysis, pathological observation and biochemical analysis on the 6^{th} day of infection.

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR)

After mice were sacrificed on the 6^{th} day after infection, lung tissues were collected and homogenized. Firstly, total RNA from mouse lung tissues was extracted using TRIzol and reverse transcribed into cDNA based on the instructions of PrimeScript^{TM} RT Master Mix (Takara, Dalian, China) reverse transcription kit. The cDNA obtained by reverse

transcription as described above was quantified with SYBR Premix Ex Taq kit (Takara, Dalian, China) and LightCycler (Roche Diagnostics, Indianapolis, IN, USA). The sequences of primers are shown in Supplementary Table 1. Eventually, the quantitative comparison was obtained through the $2^{-\Delta\Delta Ct}$ method.

Flow Cytometric Analysis

Lung tissues were minced and digested in PBS containing collagenase and DNase I. The tissue suspension was filtered through a strainer and centrifuged. To obtain lung single cell suspension, the supernatant was discarded and red blood cells were lysed with red blood cell lysis buffer. The populations of immune cells were detected by Flow cytometry (CytoFLEX LX, Beckman Coulter, Inc., CA, USA) after staining cells with antibodies purchased from BioLegend, Inc. (San Diego, CA, USA), including Zombie Aqua™ Fixable Viability Kit (cat: 423101, BioLegend, USA), FITC anti-mouse CD45 antibody (cat: 103108, BioLegend, USA), Alexa Fluor® 700 anti-mouse CD3 antibody (cat: 100216, BioLegend, USA), Brilliant Violet 650™ anti-mouse Ly-6G antibody (cat: 127641, BioLegend, USA), Brilliant Violet 421™ anti-mouse Ly-6C antibody (cat: 128032, BioLegend, USA), and PE/Cyanine7 anti-mouse F4/80 antibody (cat: 123114, BioLegend, USA).

Neutrophil Isolation

Neutrophils were isolated from mouse bone marrow using neutrophil isolation kits (BEAVER, Suzhou China). The purity of the neutrophils was analyzed by FACS.

The Detection of Reactive Oxygen Species (ROS) and Cytokines

Isolated neutrophils (1×10^5) were plated in a 96-well plate and treated with PMA or compound (wogonin, liquiritin, glycyrrhizic acid or baicalin). ROS production was detected by ROS detection assay kits (cat# S0033S, Beyotime, China). After stimulation for 3 h, neutrophils were incubated with DCFH-DA for 30 min at 37°C in 5% CO_2. ROS were detected by EnSight® Multimode Plate Reader (EnSight, Revvity, America) within 30 min.

Isolated neutrophils (2×10^6) were plated in a 24-well plate and treated with phorbol 12-myristate 13-acetate (PMA) or compounds (wogonin, liquiritin, glycyrrhizic acid or baicalin) for 3 h. After stimulation for 3 h, cell culture supernatants were collected and detected by ELISA kits.

ELISA

Lung tissues were prepared in 1mL PBS using a tissue grinder (SCIENTZ-48, China) at 60 Hz for 90s. Myeloperoxidase (MPO), iNOS, intercellular adhesion molecule 1 (ICAM-1) and SOD in lung homogenates were measured using ELISA kits (YEPCOME, Shanghai, China). The ELISA kits for IL-6, MCP-1, TNF-α, CCL5, and MIP-2 were purchased from R&D Systems (Minneapolis, MN, USA). Assays were conducted according to the manufacturer's instructions.

Western Blot

Protein concentrations were measured by a BCA protein assay kit (Pierce). Western blots were carried out as previously described.[23] The primary antibodies including phospho-p44/42 MAPK (p-ERK1/2, cat# 4370, CST, USA), p44/42 MAPK (ERK1/2, cat# 4695, CST, USA), NF-κBp65 (cat# 8242, CST, USA), phospho-NF-κBp65 (cat# 3033, CST, USA) or β-Actin (cat# 3700, CST, USA) were incubated overnight at 4°C, followed by the incubation with corresponding secondary antibodies (anti-rabbit IgG, cat# 7074, CST, USA or anti-mouse IgG cat# 7076, CST, USA) for 1 h.

Statistical Analysis

The experimental data were independently analyzed and quantified using Prism GraphPad 8 software. Group comparisons were conducted using one-way analysis of variance (one-way ANOVA) with Bonferroni or Dunnett test according to homogeneity of variance test. The statistical significance was denoted as follows: $*p < 0.05$; $**p < 0.01$ or $***p < 0.001$. The Student's *t*-test was employed for comparison between PR8 + OSV group and PR8 + OSV + SFJBF group. The statistical significance was determined as: #, $p < 0.05$; ##, $p < 0.01$ or ###, $p < 0.001$.

Results

Potential Targets of SFJBF for Treating Influenza

A total of 961 influenza-related targets and 247 SFJBF-related targets were obtained. There were 61 targets that might account for the therapeutic effects of SFJBF against influenza (Figure 1A). According to the PPI network, STAT3, IL-6, TNF, TP53, AKT1, IL1B, ESR1, EGFR, RELA, MAPK1, IFNG, IL-10, CCL2 and BCL2 were key genes regulated by SFJBF (Figure 1B).

GO and KEGG Enrichment Analysis

A total of 494 BP terms, 39 CC terms and 99 MF terms were obtained using GO enrichment analysis. The top 10 terms were shown in Figure 2. Inflammatory response, positive regulation of nitric oxide biosynthetic process, cytokine activity and protein kinase activity were key process regulated by SFJBF. Results indicated that SFJBF probably could inhibit influenza virus-induced overactivated inflammatory response.

A total of 149 terms were obtained by KEGG signaling pathway analysis. The top 20 terms were shown in Figure 3. TNF signaling pathway, PI3K-Akt signaling pathway and MAPK signaling pathway are key signaling pathways among them. These signaling pathways are associated with influenza virus-induced overactivated inflammatory response and are in accordance with the BP terms mentioned above. These results indicated that SFJBF regulated influenza virus-induced inflammatory response.

Targets–Active Ingredients Network

A total of 142 active components and 61 influenza-related targets were employed to construct targets–active ingredients network (Figure 4). Results showed that 9 key active components, including quercetin, beta-sitosterol, kaempferol, stigmasterol, isorhamnetin, luteolin, wogonin, 7-methoxy-2-methyl isoflavone and naringenin, were identified with a degree value exceeding 35. These active components probably formed the foundational materials for therapeutic effects of SFJBF in the treatment of influenza.

Identification of the Components of SFJBF

According to the results of UPLC-MS/MS, 1 mL SFJBF (10 mg/mL) contained 531.00 ng prim-o-glucosylcimifugin, 379.00 ng 4'-o-beta-glucopyranosyl-5-o-methylvisamminol, 3.93 ng genistein, 5.79 ng daidzein, 0.02 ng imperatorin, 92.88 ng ferulic acid, 0.70 ng notopterol, 1.26 ng saikosaponin D, 88.40 ng liquiritin, and 104.50 ng saikosaponin A (Figure 5).

To explore the pharmacological compounds of SFJBF, UHPLC-QE-MS analysis was used to clarify the chemical composition profiling. Overall, 453 compounds were identified, including 138 kinds of flavonoids, 58 kinds of alkaloids, 11 kinds of quinones, 146 kinds of terpenoids, 70 kinds of coumarins, 10 kinds of organic acids, 20 kinds of lignans (Supplementary Table 2 and Figure 6).

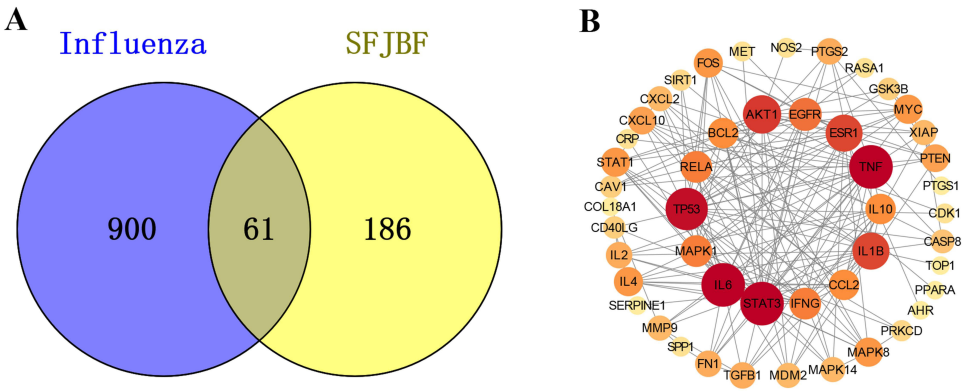

Figure 1 Potential targets of SFJBF against influenza virus infection. (**A**) The venn diagram of targets between active compounds-related targets and influenza-related targets. The overlapping shape represented 61 influenza-related targets regulated by SFJBF. (**B**) The protein–protein interactions of 61 targets. The darker and larger circles represent the more important targets regulated by SFJBF.

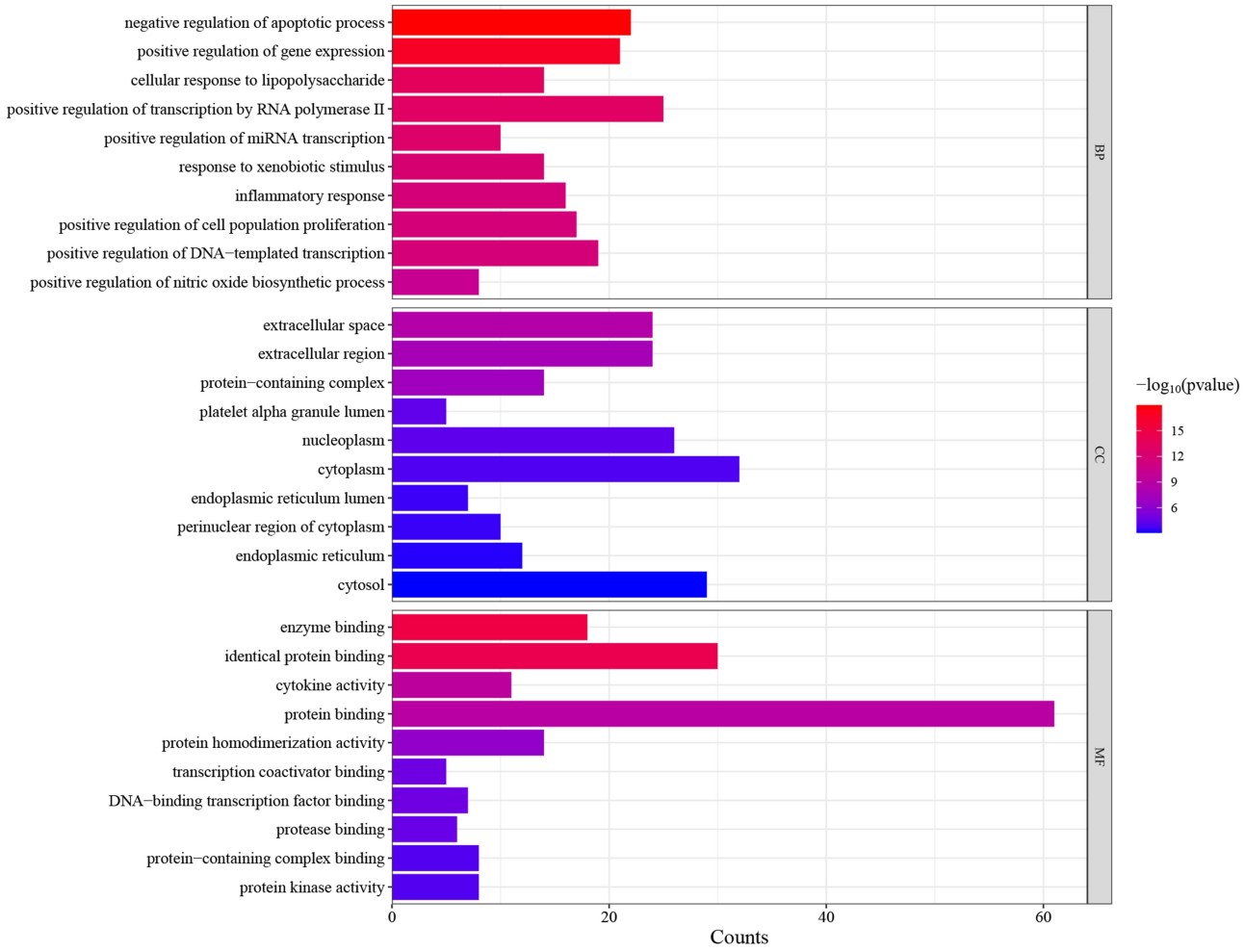

Figure 2 Top 10 GO terms regulated by SFJBF.

SFJBF Exerted Therapeutic Effects in Flu Mice

An in vivo influenza model was created by intranasal administration of influenza A virus (PR8) to C57BL/6J mice (Figure 7A). OSV was also used as a positive control for the flu mice. Compared with the NC group, PR8 infection led to a significant loss in body weight ($p < 0.001$). SFJBF(H), SFJBF(M), SFJBF(L), and OSV treatment significantly protected against body weight loss in mice (Figure 7B) ($p < 0.01$ or 0.001). The combination of OSV and SFJBF(M) had an advantage over OSV treatment alone in protecting the body weight loss of the mice ($p < 0.05$) (Figure 7B).

The total protein content in lungs and lung indexes were used to evaluate the severity of inflammatory exudates and the infiltration of inflammatory cells in this infective mouse model. The indexes and total protein content of the lungs in the PR8 group were markedly higher than those in the NC group ($p < 0.001$) (Figure 7C and D, which were significantly reduced with the treatment of SFJBF(L), SFJBF(M), SFJBF(H) and OSV ($p < 0.05$, 0.01, or 0.001) (Figure 7C and D). However, the combination of OSV and SFJBF(M) had an advantage over OSV treatment alone in reducing lung indexes and total protein content, suggesting that the combination of OSV and SFJBF provided better protection against lung injury than OSV treatment alone ($p < 0.01$) (Figure 7C and D).

To further determine the effects of SFJBF on PR8-infected mice, H&E staining was performed to assess pathological changes in the lungs. After infection with PR8, the lungs showed interstitial edema, alveolar hemorrhage, and thickened alveolar walls (Figure 7E and F). There was a massive infiltration of inflammatory cells into the alveolar walls. However, treatment with SFJBF(M), SFJBF(H) and OSV significantly alleviated the pulmonary histopathological changes ($p < 0.01$, or 0.001) (Figure 7E and F). These results showed that SFJBF treatment could protect against lung injury caused by influenza virus.

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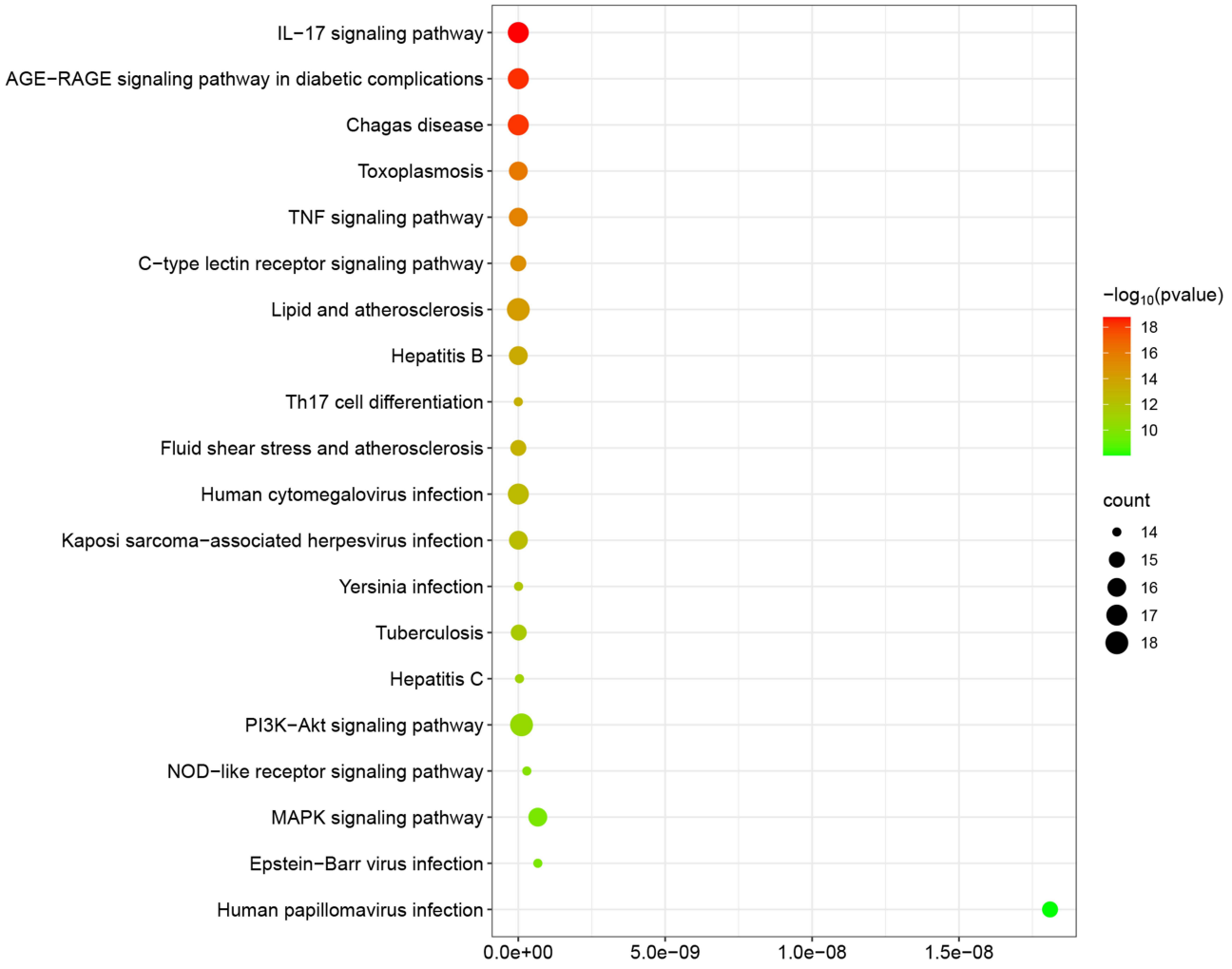

Figure 3 Top 20 signaling pathways regulated by SFJBF.

SFJBF Inhibited the Infiltration of Neutrophils in Lungs of Flu Mice

Uncontrolled overactivation of immune response is the main etiology of organ injury, which is orchestrated through multiple immune molecules and immune cells.[18] FACS analysis of lung digestive samples was further utilized to analyze the proportions of different immune cells of flu mice. According to the analytical strategy, immune cells in lung were separated into neutrophils ($CD45^+Ly6G^+$), T cells ($CD45^+Ly6G^-CD3^+$), inflammatory monocytes ($CD45^+Ly6G^-CD3^-Ly6C^{+\text{ to hi}}$ $F4/80^{-\text{ to mid}}$) and macrophages ($CD45^+Ly6G^-CD3^-Ly6C^{-\text{ to }+}$ $F4/80^+$) (Figure 8A). Compared with those of the lungs in NC group, a significant increase in the proportion of neutrophils and inflammatory monocytes, and a marked decrease in the proportion of T cells were observed in PR8-infected lungs ($p < 0.001$) (Figure 8B–G). However, the administration of OSV, different doses of SFJBF or their combination showed a significant reduction in the proportion of neutrophils ($p < 0.01$ or 0.001) (Figure 8B and E). Intriguingly, the combined treatment group led to a considerable decrease in the proportion of inflammatory monocytes and maintained the T cell proportion in lungs of flu mice ($p < 0.001$) (Figure 8C, D, F and G), which were more efficient than OSV treatment alone. These results implied that SFJBF exerted protective effects in PR8-induced lung injury, possibly via alleviating the overactivated inflammatory response, especially via decreasing the infiltration of neutrophils.

SFJBF Decreased the Expression of Inflammatory Mediators in Lungs of Flu Mice

Due to the pivotal role of cytokine and chemokine expression in the chemotaxis of inflammatory cells in situ during viral pneumonia,[18] the impacts of SFJBF on the expression of inflammatory cytokines and chemokines were examined by

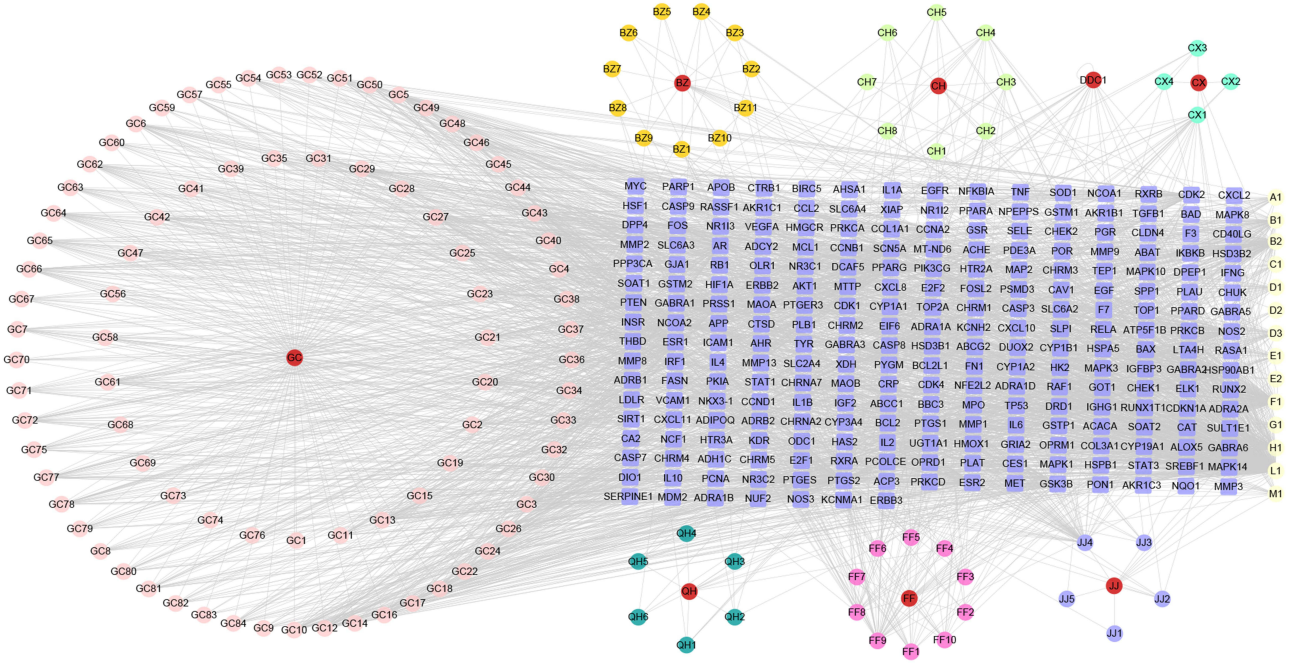

Figure 4 Components-target network. Purple nodes represented the influenza-related targets, circles represented active ingredients and red nodes represented eight herbs including *Schizonepeta tenuifolia* (Jingjie, JJ), *Saposhnikovia divaricata* (Fangfeng, FF), *Bupleurum chinensis* (Chaihu), *Radix Angelicae Dahuricae* (Baizhi, BZ), *Ligusticum chuanxiong* (Chuanxiong, CX), *Sojae Semen Praeparatum* (Dandouchi, DDC), *Glycyrrhiza uralensis* (Gancao, GC) and *Notopterygium incisum Ting ex* (Qianghuo, QH).

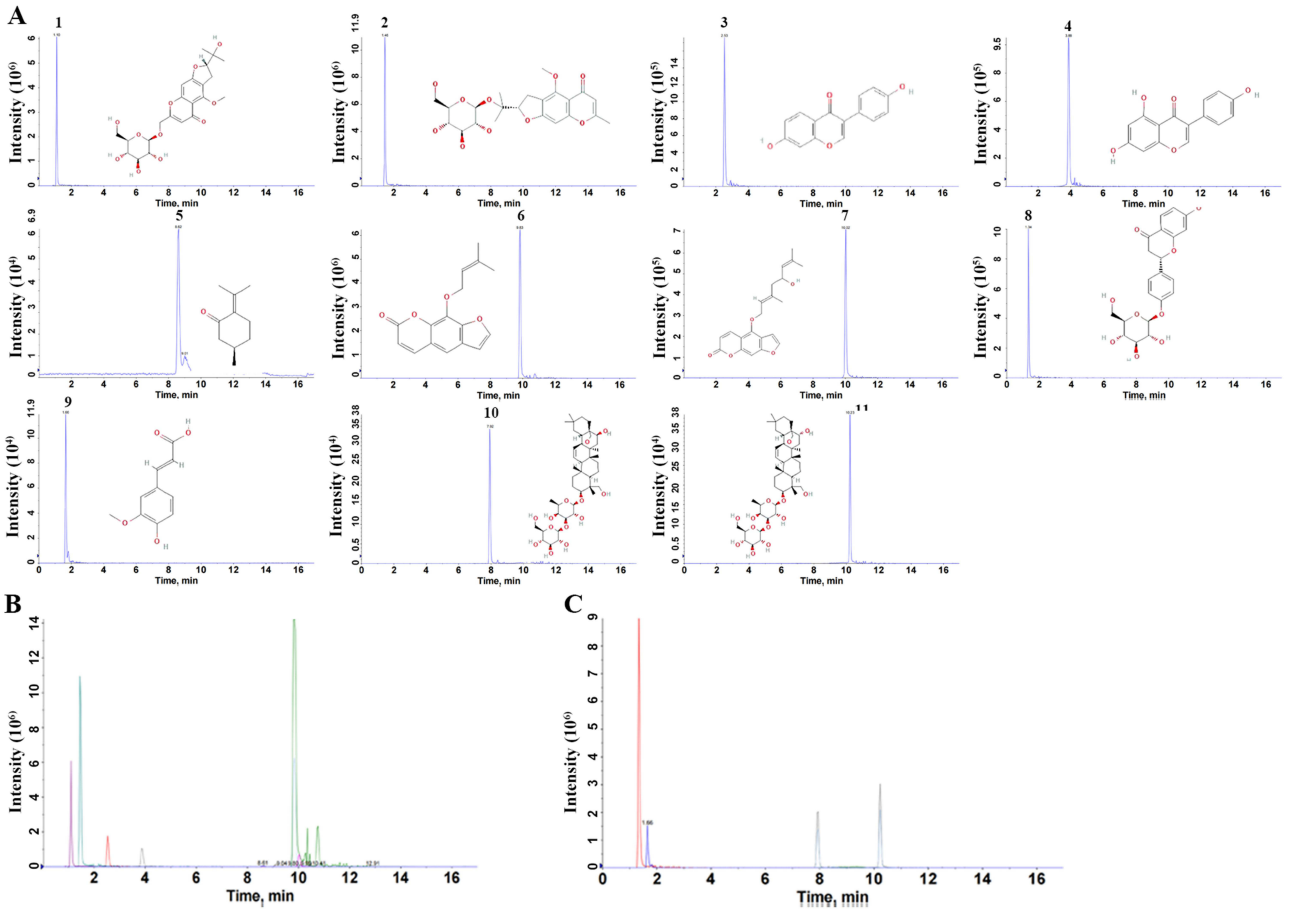

Figure 5 UPLC-MS/MS analysis of SFJBF. (**A**) Positive ion mode; (**B**) Negative ion mode. (**C**) The 11 representative compounds in positive or negative ion modes of SFJBF and their chemical structures.

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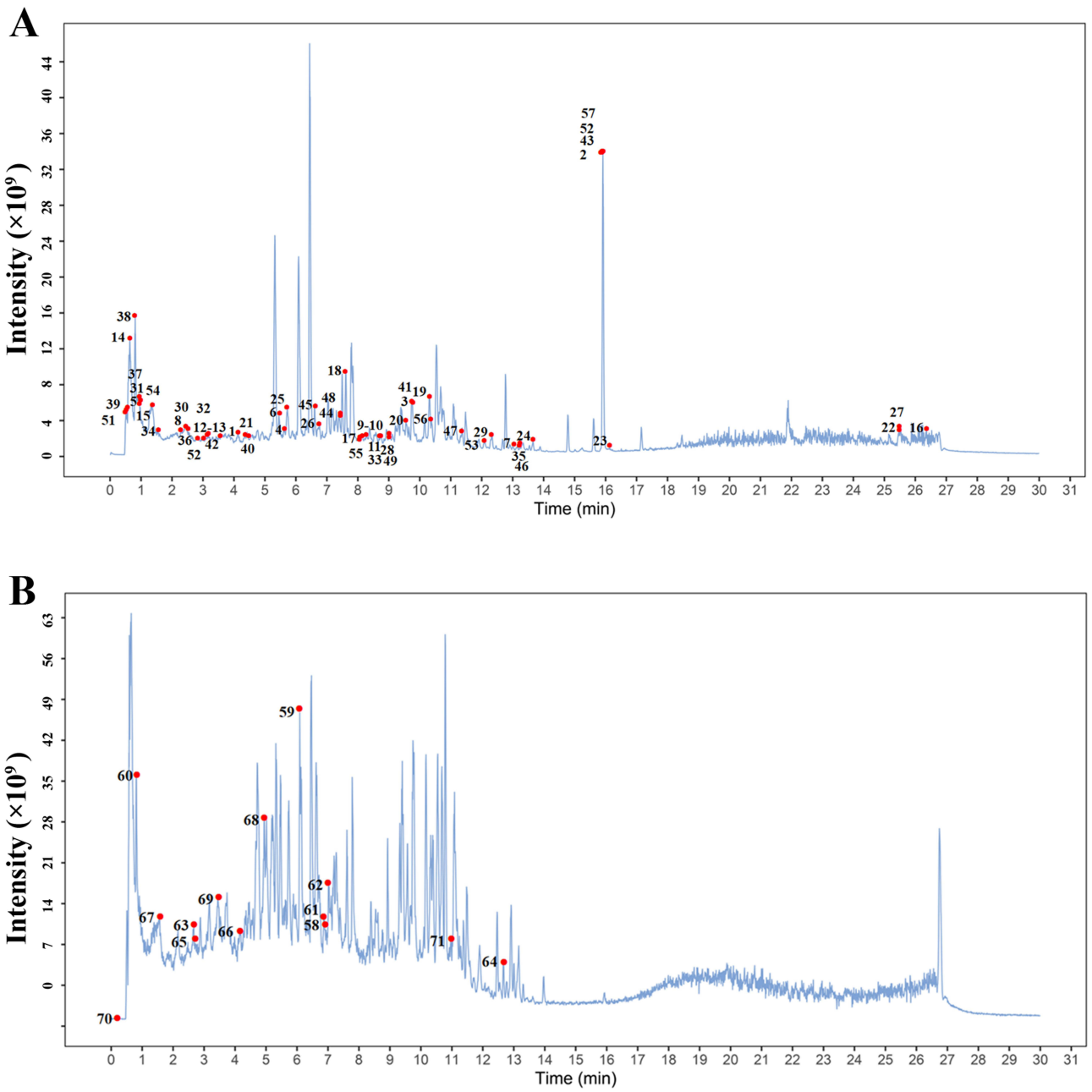

Figure 6 Base peak intensity chromatograms of 71 compounds with a score of 1 in positive ion mode (**A**) and negative ion mode (**B**) using UHPLC-QE-MS analysis.

qRT-PCR or ELISA in lungs. Results showed that PR8 infection induced a robust increase in *Cxcl2, Ccl2, Il1b, Tnf, Ccl3, Ccl4, Cxcl10* and IL-6 levels in lungs ($p < 0.05$, 0.01, or 0.001) (Figure 9). OSV administration reduced the expression of *Cxcl2* and *Tnf* ($p < 0.05$) (Figure 9A and F). In line with network pharmacology (Figure 1B), SFJBF(M) treatment significantly reduced the expression of *Cxcl2, Ccl2, Il1b* and IL-6 ($p < 0.05$ or 0.01) (Figure 9A–C and 9H). Moreover, the combination of OSV and SFJBF(M) showed better downregulatory effects on the expression of *Cxcl2, Ccl2, Tnf, Ccl3, Ccl4* and IL-6 vs OSV treatment alone ($p < 0.05$, 0.01, or 0.001) (Figure 9).

As we found that SFJBF decreased the infiltration of neutrophils and cytokines in lungs, we further determined the expression of inflammatory mediators and neutrophil-related mediators in lungs. The results showed that SFJBF(M), OSV, and the combined treatment decreased the overexpression of iNOS and MPO in lungs ($p < 0.05$ or 0.01) (Figure 10A and B). The combined treatment could also inhibit the expression of ICAM-1 and reverse the decrease in SOD levels in lungs ($p < 0.05$

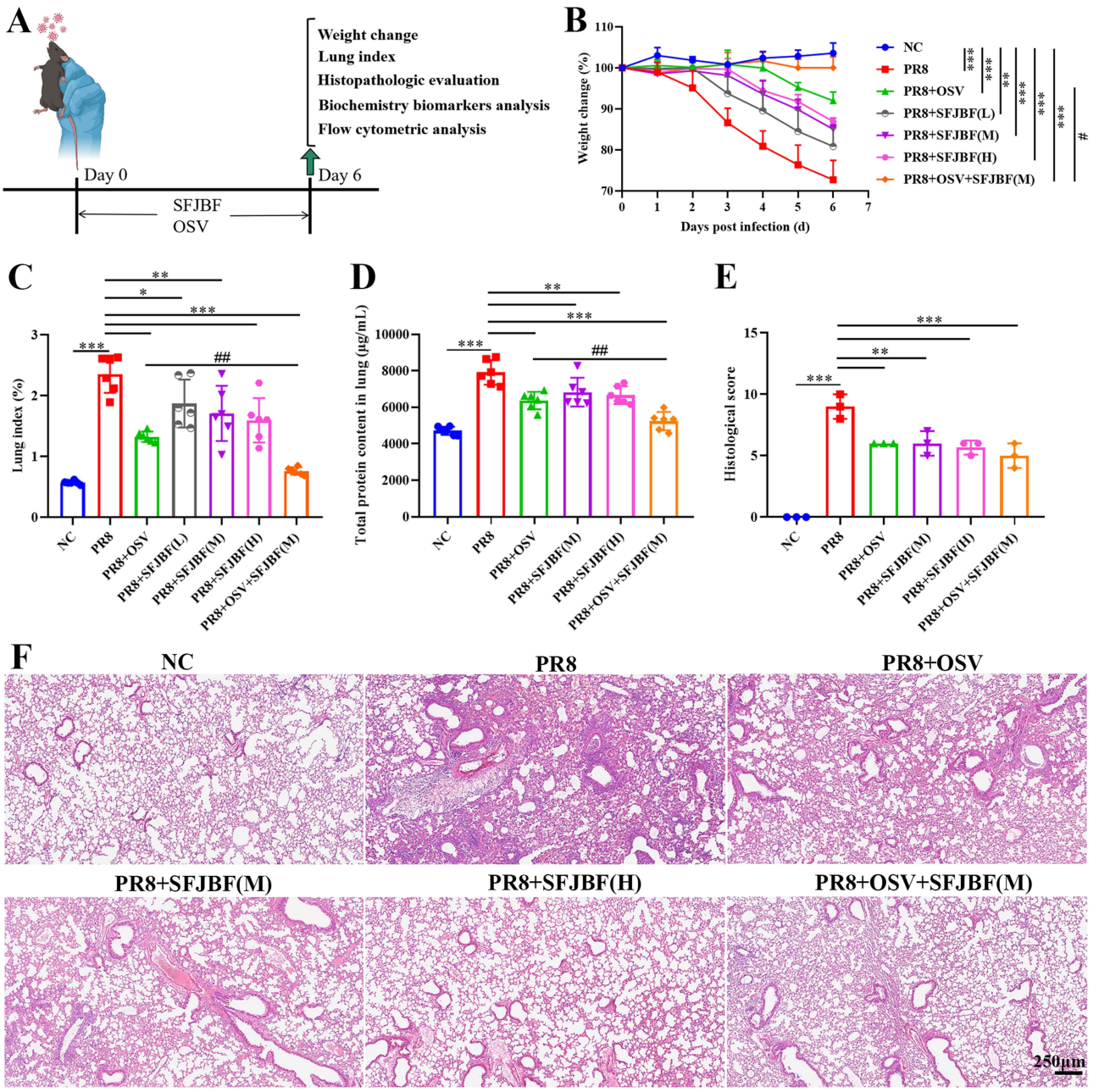

Figure 7 The protective effect of SFJBF(L), SFJBF(M), SFJBF(L), OSV, or the combination OSV and SFJBF(M) on PR8-infected mice. (**A**) The scheme of exploring the protective mechanisms of SFJBF against influenza in vivo. (**B**) Weight changes of mice in different groups at 6 dpi (n=6). (**C**) Lung indexes of mice in different groups at 6 dpi (n=6). (**D**) Total protein content in lungs in different groups at 6 dpi (n=6). (**E** and **F**) Histopathological score of lung tissues and the statistic histopathological changes of lung tissues at 6 dpi (n=3). scale bar = 250 μm. Data were shown as mean ± SD and analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. *, $p < 0.05$; **, $p < 0.01$ or ***, $p < 0.001$. vs PR8 group. #, $p < 0.05$ or ##, $p < 0.01$ vs PR8 + OSV group.

or 0.001) (Figure 10C and D). SFJBF could not decrease virus titre in lungs on day 6 (Figure S1). These results indicated that SFJBF protected mice from influenza virus infection probably by inhibiting overactivated inflammatory response, and the synergistic role of SFJBF(M) and OSV in suppressing cytokine expression was demonstrated.

SFJBF Decreased the Secretion of Inflammatory Mediators by Inhibiting NF-κB and ERK MAPK Signaling Pathways in RAW264.7 Cells

Influenza virus infection can induce an overactivated inflammatory response by triggering activation of the TLR7 signaling pathway. R837 is a synthetic TLR7 agonist that simulates influenza virus-induced inflammatory responses.

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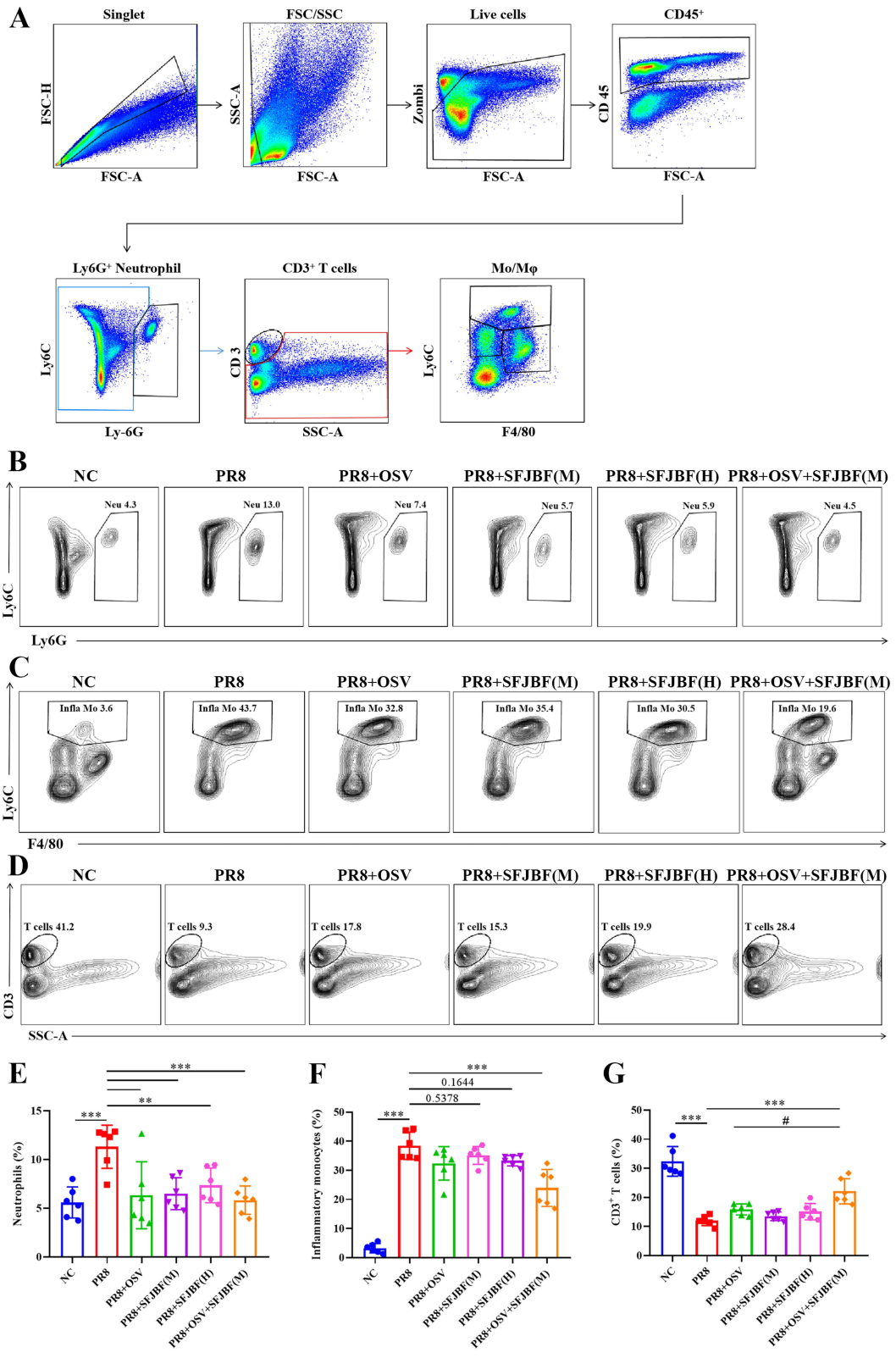

Figure 8 SFJBF(H), SFJBF(M), OSV, or the combination of OSV and SFJBF(M) decreased the excessive innate immune cells recruitment in lung tissues of flu mice. (**A**) Gating strategy. (**B–D**) Typical diagrams of neutrophils, inflammatory monocytes and T cells in each group. (E-G) The proportion of neutrophils, inflammatory monocytes and T cells (n=6). The values were presented as mean ± SD and analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. **, $p < 0.01$ or ***, $p < 0.001$. vs PR8 group. #, $p < 0.05$ vs PR8 + OSV group.

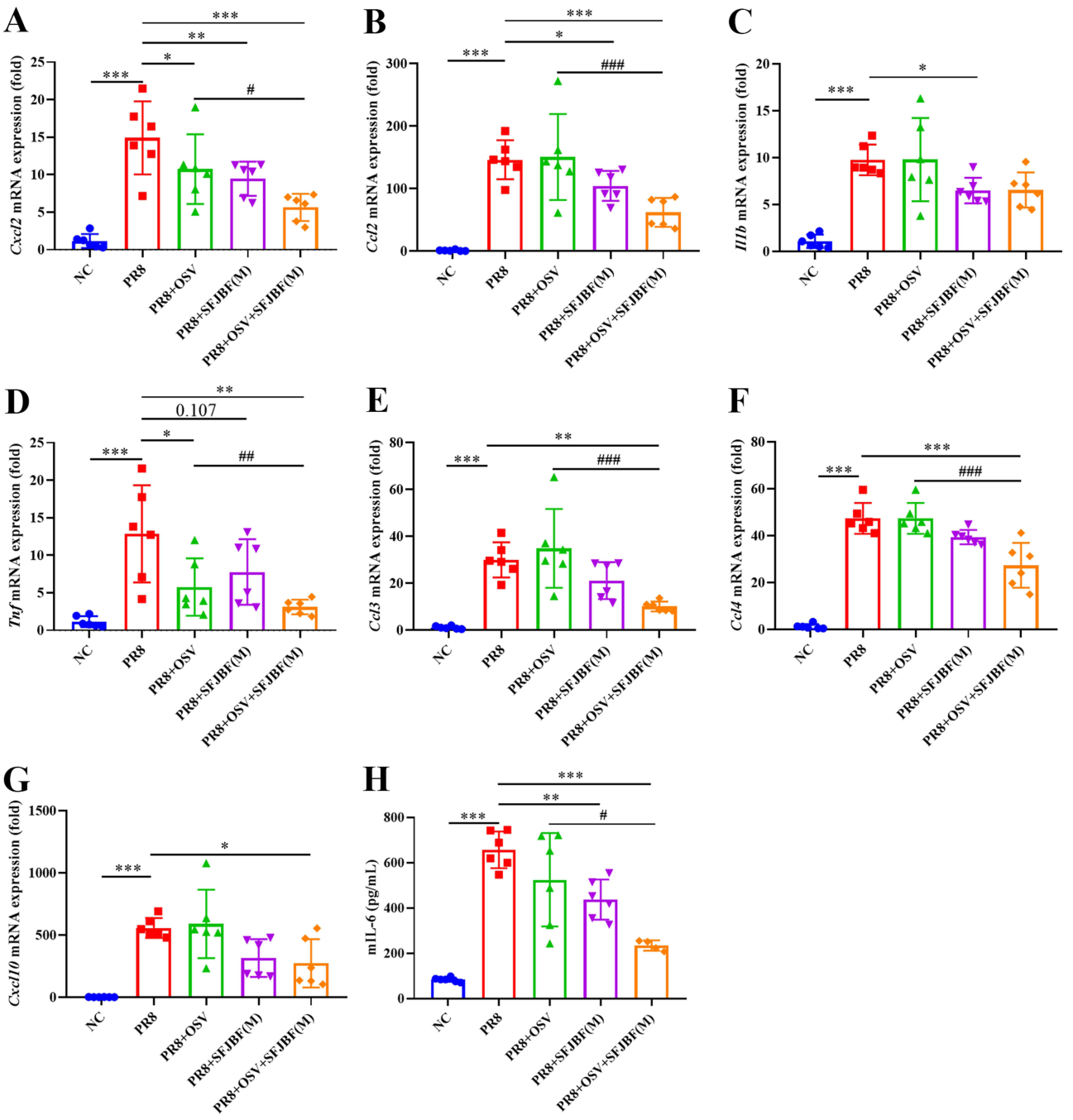

Figure 9 SFJBF, OSV, or the combination of OSV and SFJBF(M) inhibited the overexpression of cytokines and chemokines in lung tissues of flu mice at 6 dpi. (**A–G**) The mRNA expression of *Cxcl2, Ccl2, Il1b, Ccl4, Ccl3, Tnf* and *Cxcl10* (n=6). (**H**) The expression of IL-6 in lung homogenates by ELISA assays (n=6). The data were shown as mean ± SD and analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. *, $p < 0.05$; **, $p < 0.01$ or ***, $p < 0.001$. vs PR8 group. #, $p < 0.05$; ##, $p < 0.01$ or ###, $p < 0.001$ vs PR8 + OSV group.

R837 induced increased secretion of MIP-2, MCP-1, CCL5 and TNF-α in RAW264.7 cells compared with NC group ($p < 0.001$) (Figure 11A–D). SFJBF-containing sera (SFJBF) could significantly reduce the expression of these inflammatory mediators ($p < 0.05$) (Figure 11A–D). According to network pharmacology, RELA and MAPK1 are key genes regulated by SFJBF (Figure 1B). RELA and MAPK1 encode p65 and ERK1/2 proteins, respectively. R837 induced increased phosphorylation of NF-κBp65 and ERK1/2 ($p < 0.05$) (Figure 11E–G), which was reduced by SFJBF ($p < 0.05$) (Figure 11E–G). These results indicated that SFJBF inhibited the overactivated inflammatory response via the NF-κB and ERK MAPK signaling pathways.

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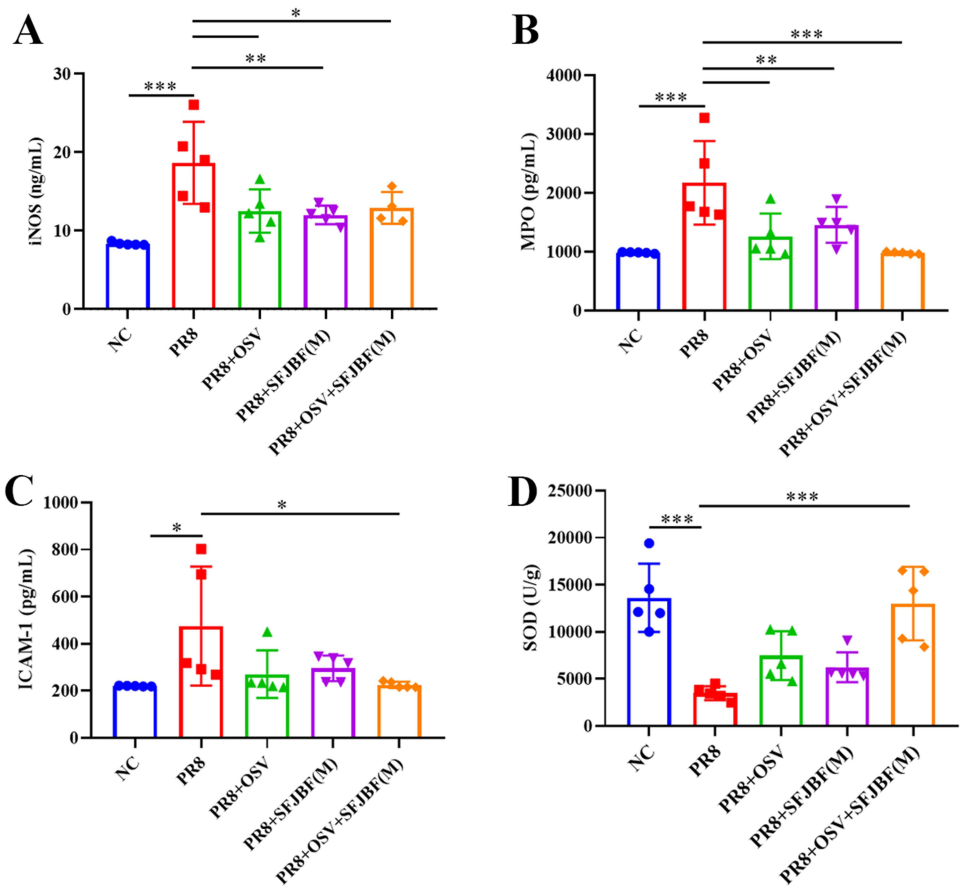

Figure 10 SFJBF, OSV, or the combination of OSV and SFJBF(M) inhibited the overexpression of mediators in lung tissues of flu mice at 6 dpi. (**A–D**) The expression of iNOS, MPO, SOD and ICAM-1 in lung homogenates by ELISA assays (n=5). The values were presented as mean ± SD and analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. *, $p < 0.05$; **, $p < 0.01$ or ***, $p < 0.001$. vs PR8 group.

In vitro Validation of Bioactive Components in SFJBF

Not only the number of innate immune cells, such as neutrophils or macrophages, but also their overactivated bioactivity contributes to the severity of viral pneumonia or sepsis.[19] R837 (5 μg/mL) did not induce an overactivated inflammatory response in neutrophils (data not shown). To evaluate the effects of the active components on inhibiting neutrophil-related inflammatory responses, PMA was used to activate neutrophils in vitro. The purity of neutrophils was typically > 75%, which was determined as $CD11b^{+}Ly6G^{+}$ cells by Flow cytometry (Figure S2). Wogonin, liquiritin, baicalin, and glycyrrhizic acid were the key components of SFJBF (Figure 4 and Supplementary Table 2). The production of ROS, MIP-2, MCP-1 and IL-6 was increased in PMA-treated neutrophils ($p < 0.001$) (Figure 12). Wogonin and baicalin significantly reduced the production of ROS, MIP-2 and MCP-1 in neutrophils ($p < 0.05$, 0.01 or 0.001) (Figure 12A–C). Baicalin significantly decreased the expression of IL-6 ($p < 0.001$) (Figure 12D). These results indicated that wogonin and baicalin could significantly downregulate neutrophil activation in vitro, which might account for the anti-inflammatory protective role of SFJBF.

Discussion

Influenza virus remains a leading contributor to lower respiratory viral infection, accounting for more than 9,000,000 hospitalizations annually.[24] Therefore, effective therapeutic methods are urgently required to prevent and control influenza epidemics. Currently, TCM is relatively safe, effective, and widely used to treat respiratory virus infections with a long history. Many TCM formulas have been proven to reduce the duration of symptom resolution and shorten the course of diseases.[25] TCM may be a potentially effective source for research and development of drug candidates. SFJBF has been used

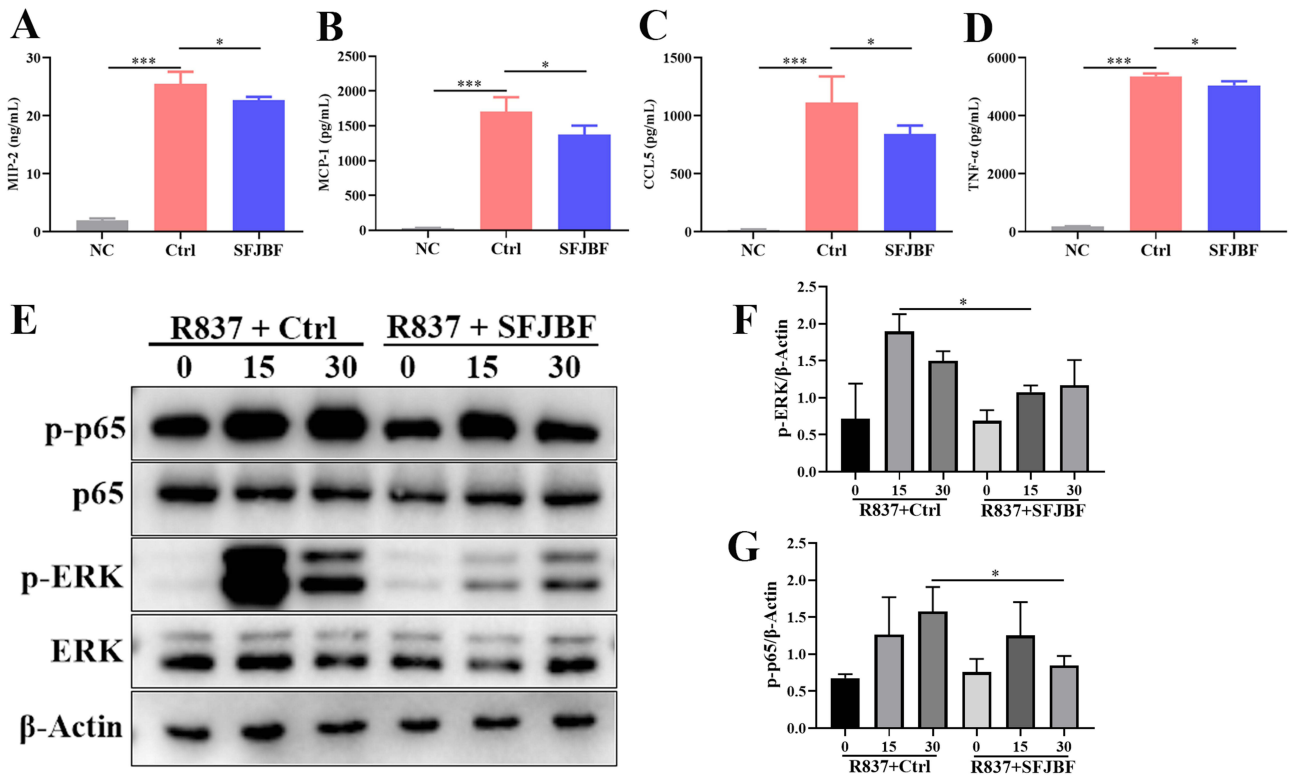

Figure 11 SFJBF-containing sera inhibited the expression of mediators in RAW264.7 cells. (**A**–**D**) The expression of MIP-2, MCP-1, CCL5 and TNF-α in RAW264.7 cells stimulated with R837 (n=3). (**E**) The expression of p-p65, p65, p-ERK1/2, ERK1/2 and β-Actin in RAW264.7 cells stimulated with R837 (n=3). (**F** and **G**) The relative expression of p-p65 and p-ERK1/2 analyzed by Image J. The values were analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. *, $p < 0.05$; ***, $p < 0.001$. vs ctrl group.

to treat respiratory infections for more than 10 years. However, evidence for the treatment of influenza is lacking. Our results show that SFJBF protects mice from influenza virus infection by alleviating immunopathological damage to lungs.

Influenza virus infection can lead to intracellular signaling cascades by activating pattern recognition receptors (PRRs) such as Toll-like receptor 3/7 (TLR3/7) and retinoic acid-inducible gene I (RIG-I), resulting in the production of various inflammatory factors.[26] The overwhelming inflammatory response induced by influenza viruses can cause lung damage, viral pneumonia, ARDS, and even death.[18] Activated macrophages and neutrophils are the key drivers of overactivated inflammatory response.[27] Macrophages can secrete high levels of TNF-α, iNOS and matrix metalloproteinases, leading to pulmonary damage during infection.[19] In addition, they secrete massive amounts of chemokines to recruit more inflammatory cells into the lungs, exacerbating the immunopathology. Chemokine MCP-1 and pro-inflammatory cytokines IL-1β and IL-6 are associated with disease severity during influenza.[28] MCP-1 and MIP-2 are vital chemokines that recruit neutrophils to the locally infected organs.[19] The NF-κB and ERK MAPK signaling pathways are essential for the regulation of cytokine expression and the production of iNOS and matrix metalloproteinases in macrophages. In this study, SFJBF inhibits the overactivated inflammatory response by suppressing the activation of the NF-κB and ERK MAPK signaling pathways, accompanied by a marked decrease in neutrophil infiltration in the lungs. Neutrophil infiltration and activation can impair the epithelial-endothelial barrier by releasing massive amounts of proteases, ROS and neutrophil extracellular traps (NETs).[29] ROS can activate MPO and contribute to NET formation.[30] NET formation impairs the glycocalyx of endothelial cells and increases endothelial permeability, resulting in the leakage of inflammatory mediators.[30] In addition, NET formation can amplify coagulopathy by releasing S100A8/A9 and histones.[31,32] SFJBF inhibits the expression of MPO and reduces the leakage of inflammatory mediators in the lung, indicating that SFJBF ameliorates lung injury by inhibiting neutrophil activation. The active components of SFJBF, including wogonin and baicalin, effectively inhibits the PMA-induced neutrophil activation. These findings demonstrate that SFJBF protects against influenza virus-induced lung injury possibly by inhibiting the activation of macrophages and neutrophils.

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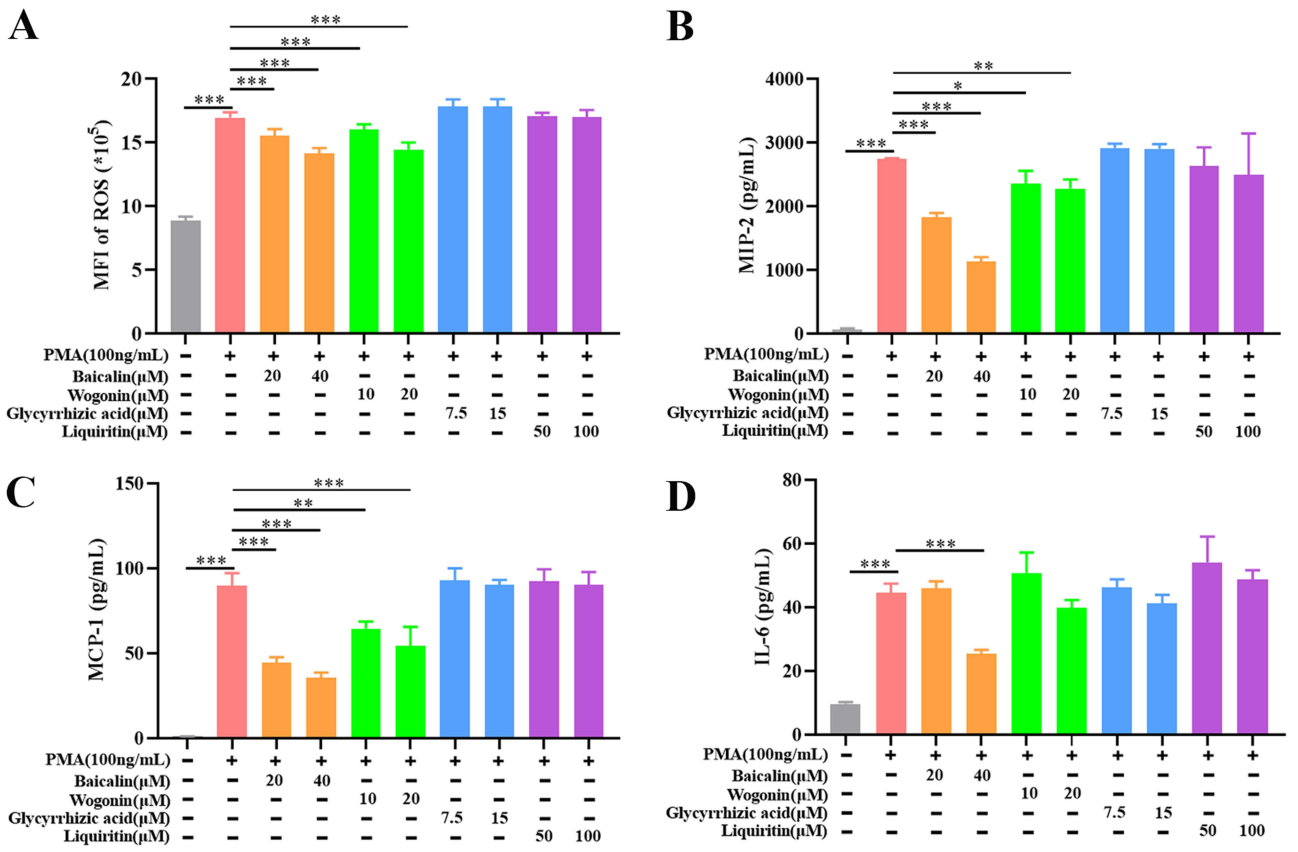

Figure 12 The active components of SFJBF including wogonin and baicalin inhibited neutrophil activation induced by PMA. (**A**–**D**) The production of ROS, MIP-2, MCP-1 and IL-6 with the stimulation of PMA in different groups (n=3). The values were analyzed by one-way ANOVA Bonferroni or Dunnett's multiple comparisons tests according to homogeneity of variance test. *, $p < 0.05$; **, $p < 0.01$ or ***, $p < 0.001$. vs PMA group.

NAIs, such as oseltamivir, an FDA-approved drug, have been recommended for the treatment of influenza worldwide for a long time. A variety of drug classes in development have been studied in combination with NAIs, polymerase inhibitors, monoclonal antibodies, or adjunctive therapies, respectively.[33,34] In severe influenza, the pathological changes and prognosis of infectious diseases are determined by two aspects: the host and pathogen. An overactivated host immune response and uncontrolled viral replication are highly associated with the poor prognosis of influenza.[27] OSV is a well-known agent for the treatment of influenza because of its inhibitory role on the release of virions. Different from OSV, many Chinese medicines including SFJBF can inhibit overactivated inflammatory response during influenza virus infection.[18,22,35] The combination of OSV and SFJBF plays a synergistic anti-inflammatory immunoregulatory role. Thus, SFJBF is an effective adjuvant drug candidate for treating influenza from the perspective of the host, which is complementary to the inhibitory role of influenza virus replication by OSV. However, further clinical trials are required to verify its safety and efficacy in the treatment of uncomplicated influenza.

In this study, a synthetic TLR7 agonist was used to simulate influenza virus-induced inflammatory responses. Whether SFJBF could inhibit TLR3, RIG-I or other signaling pathways remains unknown. In addition, further validation is required to explore whether SFJBF targets the upstream of NF-κB and ERK MAPK signaling pathways.

Conclusion

In summary, SFJBF inhibits the activation of the NF-κB and ERK MAPK signaling pathways, accompanied by reduced neutrophil infiltration and cytokine secretion, thereby mitigating immunopathological damage. These findings reveal that SFJBF shows potential as an adjunctive therapy, pending clinical validation. Further clinical trials are required to validate the safety and efficacy of SFJBF in the treatment of influenza. The combination of OSV and SFJBF shows synergistic effects against influenza and is expected to provide a new therapeutic option for patients with influenza.

Abbreviations

SFJBF, Shu-Feng-Jie-Biao formula; OSV, oseltamivir; TCM, traditional Chinese medicine; CDC, Center for Disease Control and Prevention; UPLC-MS/MS, ultra performance liquid chromatography-triple quadrupole tandem mass spectrometry; PR8, A/Puerto Rico/8/1934 (H1N1); NC, normal control group; NAI, neuraminidase inhibitor; ROS, reactive oxygen species; ARDS, acute respiratory distress syndrome; iNOS, inducible nitric oxide synthase; PMA, phorbol 12-myristate 13-acetate.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Approval and Consent to Participate

This animal experiment was approved by the Ethics Committee of Guangzhou Medical University (No. 20230309).

Consent for Publication

The manuscript was approved by all authors for publication.

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Disclosure

The author(s) report no conflicts of interest in this work.

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