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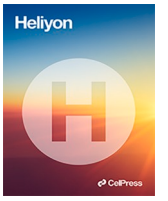

Research article

Investigating the active components and mechanistic effects of *Forsythia suspensa* Leaf against RSV via the PI3K/Akt-NLRP3 pathway

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ABSTRACT

Background: Pulmonary infections resulting from respiratory syncytial virus (RSV) continue to pose a significant threat to the well-being of infants and the elderly, but there is no safe, effective and specific treatment except symptomatic treatment. *Forsythia Suspensa* Leaf (FSL) is cold in nature and bitter in taste, and has the efficacy of clearing away heat and toxic materials. Previous research by our research group showed that the active components in FSL have the pharmacological effect of anti-RSV. Based on that, this study aims further to clarify the anti-RSV active components and mechanism of FSL.

Methods: Firstly, we established the BALB/c mouse model of RSV infection, assessed the *in vivo* anti-RSV efficacy, and determined the optimal dosage of FSL and its active components. Evaluation parameters included body weight changes, organ indices, lung tissue pathological sections, lung tissue viral load, and inflammatory factors. Additionally, we used RT-PCR, Western Blot and other molecular biology techniques to determine the expression changes of key factors such as Nrf2 and NLRP3 in PI3K/Akt-NLRP3 pathway, and revealed the anti-RSV mechanism of FSL and its active components.

Results: Pharmacodynamic experiments in animals showed that the FSL Low (0.4 g/kg·d), RosA Low (100 mg/kg·d) and Phillyrin Medium (100 mg/kg·d) groups could effectively improve the

Abbreviations: RSV, Respiratory syncytial virus; FSL, *Forsythia Suspensa* Leaf; RosA, Rosmarinic acid; PI3K, Phosphatidylinositol 3-kinase; Akt/PKB, Protein kinase B; Nrf2, Nuclear factor erythroid-2-related factor 2; NLRP3, Recombinant NLR Family, Pyrin Domain Containing Protein 3; ROS, Reactive Oxygen Species; UPLC, Ultra Performance Liquid Chromatography; MS, Mass Spectrometry; PCR, Polymerase Chain Reaction; Hep-2, Human laryngeal epidermoid carcinoma cell-2; BSL-2, Biosafety Laboratory II; IL-6, Interleukin-6; IL-1β, Interleukin-1β; RNA, Ribonucleic Acid; cDNA, Complementary Deoxyribonucleic Acid; IL-18, Interleukin-18; BSA, Bovine Serum Albumin; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium Dodecyl Sulfate; PVDF, Polyvinylidene Fluoride; TBST, Tris-Buffered Saline and Tween 20; IgG, Immunoglobulin G; H&E, Hematoxylin-eosin; ELISA, Enzyme Linked Immunosorbent Assay; IHC, Immunohistochemistry; ALI, Acute Lung Injury.

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pathological conditions of mice with RSV pneumonia, such as weight loss, the level of pulmonary inflammatory factors and the increase of viral load. In addition, oral administration of Phillyrin at a dose of 100 mg/kg d to RSV-infected mice can effectively control the trend that the expression of Nrf2 protein decreases and the expression of NLRP3 protein increases in RSV pneumonia mice. *Conclusion:* Phillyrin, the active component in FSL, can not only directly inhibit the replication of RSV, but also effectively control the inflammatory reaction caused by RSV infection and improve lung injury, which is expected to become a potential drug against RSV pneumonia.

1. Background

Respiratory syncytial virus (RSV) belongs to the *Orthopneumovirus* genus within the family *Pneumoviridae*, classified under the order *Mononegavirales* [1], and is a prevalent respiratory pathogen. At the initial stage of RSV infection, people typically exhibit symptoms indicative of upper respiratory tract infections, such as cough and sore throat. If not controlled in time, the disease will rapidly develop into a serious lower respiratory tract infection and even induce asthma. Survey results show that RSV is the main pathogen of early acute respiratory tract infection in children under 5 years old globally. Among 1-year-old infants, the number of children hospitalized with RSV infection is about 16 times that of children infected with influenza [2]. On a global scale, more than 30 million children are infected with RSV every year. Respiratory diseases caused by RSV infection are the main reason for infants to be hospitalized in the world, which has caused serious economic and social burdens to countries all over the world for many years [3]. Nevertheless, ideal anti-RSV drugs are still in short supply, and Ribavirin is the only broad-spectrum nucleoside antiviral drug approved for the treatment of RSV infection in clinic [4]. Its anti-RSV effect may be related to the inhibition of inosine monophosphate dehydrogenase, RNA-dependent RNA polymerase, RNA blocking and increasing the mutation rate of RSV [5]. However, the inconvenience of atomization treatment and the hidden danger to medical staff greatly limit the use of Ribavirin [6]. In addition, recent studies have also questioned the efficacy of Ribavirin, and it seems that the use of Ribavirin will not effectively reduce clinical mortality, hospital stay and the use of supportive treatment [7].

In contrast, natural medicines, particularly traditional Chinese medicine, present a vast and accessible resource with a longstanding history of application in antiviral disease treatment. In vitro studies show that the aqueous extract of Radix Glycyrrhizae can inhibit RSV from attaching and entering the host cell, and it is speculated that 18β-glycyrrhetinic acid may be one of its main active components [8]. Moreover, the latest research shows that Herba Ephedrae and Cinnamomi Cortex interact with the central conserved domain (CCD) of RSV-G protein specifically, thus blocking the attachment of the virus to the cell receptor CX3CR1, affecting the virus invasion, significantly reducing the viral load in the lung tissue of RSV-infected mice, and playing a direct antiviral role [9]. *Forsythia suspensa* (Thunb.) Vahl, a plant belonging to the *Oleaceae* family, has been extensively utilized in traditional Chinese medicine. Nowadays, it is commonly consumed as tea, boasting various health-related functions, including blood lipid regulation, blood sugar reduction, antioxidation, anti-inflammation, and antivirus properties [10]. For example, Qu et al. [11] observed the inhibitory effect of Phillyrin, the main active ingredient in FSL, on influenza A virus *in vivo*, and found that the administration of Phillyrin could obviously prolong the survival time of mice infected with influenza A virus and reduce lung tissue damage, indicating that Phillyrin has a certain protective effect on influenza A virus infection. In the early stage, we explored the anti-RSV active components and their effects of *Forsythia Suspensa* Leaf (FSL) using liquid chromatography-mass spectrometry, network pharmacology, and *in vitro* pharmacodynamics. Results demonstrated that RosA and Phillyrin, active components in FSL, significantly enhanced the survival rate of Hep-2 cells infected by RSV, exerting antiviral effects, potentially through the modulation of the PI3K/Akt signaling pathway [12].

Building upon this foundation, this paper conducted a pharmacodynamic evaluation of the anti-RSV effect of FSL and its active components *in vivo*, assessing parameters such as body weight, organ index, lung tissue pathology, viral load, and inflammatory factors. Additionally, various molecular biological methods were employed to elucidate the active components and mechanisms underlying the anti-RSV properties of FSL, providing experimental support for the development of novel anti-RSV drugs.

2. Materials and methods

2.1. Viruses and cells

Respiratory syncytial virus (Long strain) and Hep-2 cells were provided by the Institute of Basic Medicine, Shandong First Medical University. The virus amplification experiment was carried out in the BSL-2 Laboratory of Traditional Chinese Medicine Antivirus Platform of Shandong University of Traditional Chinese Medicine, where virus virulence was determined meticulously.

2.2. Drugs and materials

The FSL was procured in Changqing District, Jinan City, Shandong Province, in accordance with local food safety standards in Shanxi Province, and identified by researcher Lin Huibin from the Institute of Traditional Chinese Medicine Resources, Shandong Academy of Traditional Chinese Medicine. The FSL specimens underwent natural drying under well-ventilated conditions and were subsequently archived at the Shandong Institute of Traditional Chinese Medicine. To prepare the extraction, 20 g of FSL was weighed and placed in a flask, followed by the addition of 10 times the volume of water. After soaking for 2 h, the mixture underwent two

rounds of heat and reflux extraction. The resulting extracts were combined, filtered, and concentrated to obtain an extract containing 1 g/mL of the original medicinal materials, which was then stored in a refrigerator at −20 °C for subsequent use.

Considering the drug loss of Ribavirin tablets in the process of solution preparation, and as a solvent for unified drug group, the injection dosage form with high purity and convenient preparation was selected. Ribavirin injection was purchased from Chenxin Pharmaceutical Co., Ltd. (Lot: H19993512). Both Phillyrin and Rosmarinic acid were purchased from Lemeitian Pharmaceutical Technology Co., Ltd. (Lot: LL0048BD, DM0027). Mouse IL-6 and IL-1β ELISA Kit were purchased from Hangzhou Lianke Biotechnology Co., Ltd. (Lot: EK206/3–96, EK201B/3–96)

2.3. Experimental animals and grouping

The experiment was conducted under the supervision and evaluation of the Experimental Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (batch number: SDUTCM20230509001). Seventy-two SPF BALB/c mice, healthy females, aged 4 weeks and weighing 12–14g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (The animal license number SCXK (Beijing) 2021-0006). The experimental procedures took place in the ABSL-2 laboratory of the Animal Experimental Center of Shandong University of Traditional Chinese Medicine. The mice were housed under controlled conditions, maintaining a temperature of 23 ± 3 °C, relative humidity ranging from 40 % to 70 %, and a 12-h light-dark cycle. They were provided with sterilized feed and water ad libitum, without dietary restrictions. The BALB/c mice underwent a 3-day adaptive rearing period, and on the 4th day, they were randomly allocated into 12 groups (n = 6): normal group, model group, positive drug (Ribavirin) group, and low, medium, and high dose groups of FSL, Phillyrin, and RosA, respectively.

2.4. Experimental model establishment

After undergoing isoflurane inhalation anesthesia, mice in each experimental group received 60 μL RSV virus solution daily for three consecutive days, while mice in the normal group were administered an equivalent volume of maintenance medium. The treatment group received 0.2 mL/10g via gavage 4 h after the initial nasal infection, whereas the normal and model groups were given the same volume of normal saline. By consulting the literature, It was determined that the dosages for FSL were 0.4, 0.8, and 1.6 g/kg·d [13]. Phillyrin was administered at dosages of 50, 100, and 200 mg/kg d [14]; RosA at dosages of 100, 200, and 400 mg/kg·d [15], and Ribavirin at a dosage of 46 mg/kg·d [16]. Administration occurred once a day for four days, with a daily recording of mouse weights.

2.5. Organ index

The weights of mice were consistently recorded daily, and their behavioral characteristics were meticulously observed. Following dissection, the lung, spleen, and thymus tissues underwent two washes with normal saline, after which the surface moisture was absorbed using filter paper. Subsequently, the tissues were weighed, and the measurements were recorded. The computation of the lung index, spleen index, and thymus index was carried out according to the formula outlined in Ref. [17], as follows:

Lung index = (lung weight/body weight) × 100 %

Spleen index = (spleen weight/body weight) × 100 %

Thymus index = (thymus weight/body weight) × 100 %

2.6. Pathological tissue section

Fresh lung tissue samples were meticulously preserved using 4 % paraformaldehyde fixation, followed by paraffin embedding and slicing into approximately 5 μm thick sections. Subsequently, two random paraffin sections were extracted from each tissue sample and subjected to hematoxylin and eosin staining. The morphological alterations in the lung tissue of mice within each experimental group were meticulously examined under an optical microscope, and comprehensive photographic documentation was undertaken to capture and analyze the observed changes.

2.7. Inflammatory factor

The centrifuge tube, containing lung tissue soaked in phosphate buffered saline (PBS), was subjected to mechanical disruption using a grinding bead at a frequency of 50 Hz for 90 s. Subsequently, the disrupted tissue was centrifuged at 3000 rpm at 4 °C for 15 min, and the resulting supernatant was carefully collected, packed into a new centrifuge tube, and then stored in a freezer at −80 °C for subsequent analysis of inflammatory factors. The quantification of IL-6 and IL-1β levels in the lung tissue was performed according to the provided kit instructions.

2.8. Quantitative reverse transcription polymerase chain reaction [18,19]

Following the guidelines of the TianGen total RNA extraction kit designed for animal tissues, RNA was isolated from mouse lung tissues, and subsequent assessments were conducted to determine its concentration and purity. Subsequently, in accordance with the FastKing cDNA first-strand synthesis kit instructions, the reaction system was prepared, genomic DNA was removed, and the mixture was heated to generate cDNA. Further, adhering to the SuperReal fluorescent quantitative premixed reagent enhanced kit instructions,

a 20 μL loading system was assembled with a loading amount of 50 ng. The prepared sample underwent loading onto the Quantum Studio 5 real-time fluorescent quantitative PCR instrument, followed by amplification. The $2^{-\Delta\Delta Ct}$ method was employed for data calculation and analysis, with β-actin serving as the internal reference for standardization. The primer sequences for the relevant genes are presented in Table 1, and the primer synthesis was conducted by Sangon Biotech (Shanghai) Co., Ltd.

2.9. Immunohistochemistry (IHC) staining

The antigen extraction process involved microwave heating, followed by cooling, and washing with PBS thrice. Subsequently, the specimen was immersed in a 3 % H_2O_2 solution, incubated in darkness at room temperature for 25 min as pretreatment, and subsequently rinsed with PBS. A 3 % bovine serum albumin (BSA) solution was meticulously applied to the histological section, ensuring even coverage, and then sealed for 30 min. Following the removal of the solution, the sections underwent an overnight incubation at 4 °C in the primary antibody solution. After the secondary antibody incubation, cells were subjected to coloration using a DAB chromogenic solution. The stained slices were subsequently examined under an optical microscope post-counterstaining and dehydration.

2.10. Western Blot detects the protein expression of key factors in the PI3K/Akt-NLRP3 signaling pathway

An appropriate quantity of lung tissue was weighed, cut into pieces, and subsequently subjected to the addition of 200 μL of a pre-prepared lysis solution (formulated under provided instructions, prepared contemporaneously, and pre-cooled on ice). The tissue was then placed into grinding beads for homogenization, followed by incubation on ice for pyrolysis and subsequent centrifugation at 12,000 rpm at 4 °C for 10 min (precooled centrifuge). The protein concentration in the resulting supernatant was determined as per the BCA kit instructions, and the loading amount was ascertained. The protein sample was uniformly mixed with loading buffer and subjected to denaturation on a metal bath at 100 °C for 10 min.

Based on the distinct molecular weights of proteins, 7.5%–12.5 % PAGE-SDS gel electrophoresis was conducted, followed by the transfer of proteins to a polyvinylidene fluoride (PVDF) membrane post-electrophoresis. Subsequently, the PVDF membrane underwent sealing with 5 % skimmed milk powder at room temperature for 3 h, and the specific primary antibody of each target molecule was incubated overnight at 4 °C. The primary antibodies utilized included P-PI3K (1:1000), p-Akt (1:1500), Nrf2 (1:1000), NLRP3 (1:1000), and β-actin (1:1000). Following a 1 × TBST wash, anti-rabbit IgG labeled with horseradish peroxidase (Zhongshan Jinqiao, China) was added and incubated at room temperature for 1 h. Protein bands were visualized using an enhanced electrochemiluminescence kit, and the obtained results were subjected to analysis through Image J software.

2.11. Statistical analysis

Statistical analysis was conducted with GraphPad Prism software (version 8.0). The results were presented as $\bar{x} \pm s$ deviation. One-way analysis of variance (ANOVA) was employed to compare multiple data sets, A p-value lesser than 0.05 was considered signiffcant.

3. Results

3.1. Weight change

After 2 days of RSV infection, all mouse groups (excluding the normal group) exhibited symptoms indicative of viral infection, such as reduced food intake, weight loss, fatigue, and shortness of breath. In the normal group, no discernible changes were observed in

Table 1
Primer sequence.

Gene	Primer sequence (5′- 3’)
RSV-F	F: TGAAAGTCCACCTCCTTACAGA
	R: CCGGATAAAAAGAGTACGCTGG [20]
RSV	F: GGAAGATGGAAAGCCCGTT
	R: CATTGTGGAGGAGTGTGGCTT [21]
PI3K	F: CTCCGTGCAGGGACAAAGAG
	R: CCTCCGAACAGACTGCATCA
Akt	F: GTTTTGTTTCTCGGATGCGCT
	R: CATGGTCGCGTCAGTCCTTA [22]
Nrf2	F: AAGCACAGCCAGCACATTCTCC
	R: TGACCAGGACTCACGGGAACTTC [23]
NLRP3	F: TGGGTTCTGGTCAGACACGAG
	R: GGCGGGTAATCTTCCAAATGC [24]
IL-18	F: ACAGGCCTGACATCTTCTGC
	R: CCTTGAAGTTGACGCAAGAGT [25]
β-actin	F: GTGACGTTGACATCCGTAAAGA
	R: GCCGGACTCATCGTACTCC

demeanor, fur quality, or activity, and body weight showed a steady increase. The alterations in the body weight of mice following RSV modeling were monitored. As shown in Fig. 1a-c, substantial decreases in body weight were evident in all groups, except the normal group, affirming the successful modeling of RSV infection in mice. Notably, while the administration of Ribavirin, the FSL Low, RosA Low, and Phillyrin Medium groups mitigated the weight loss symptoms in mice, the Ribavirin group displayed prolonged weight loss, implying inherent toxicity. Furthermore, the mental state and activity levels of RSV-infected mice exhibited varying degrees of restoration across all administration groups, with the Phillyrin Medium group (Fig. 1c) demonstrating the most pronounced efficacy. The average weight of mice in the normal group after five days was 15.00g, and that in the Phillyrin Medium group was 14.91g. On the other hand, we speculate that Ribavirin itself may exert a degree of toxicity in mice.

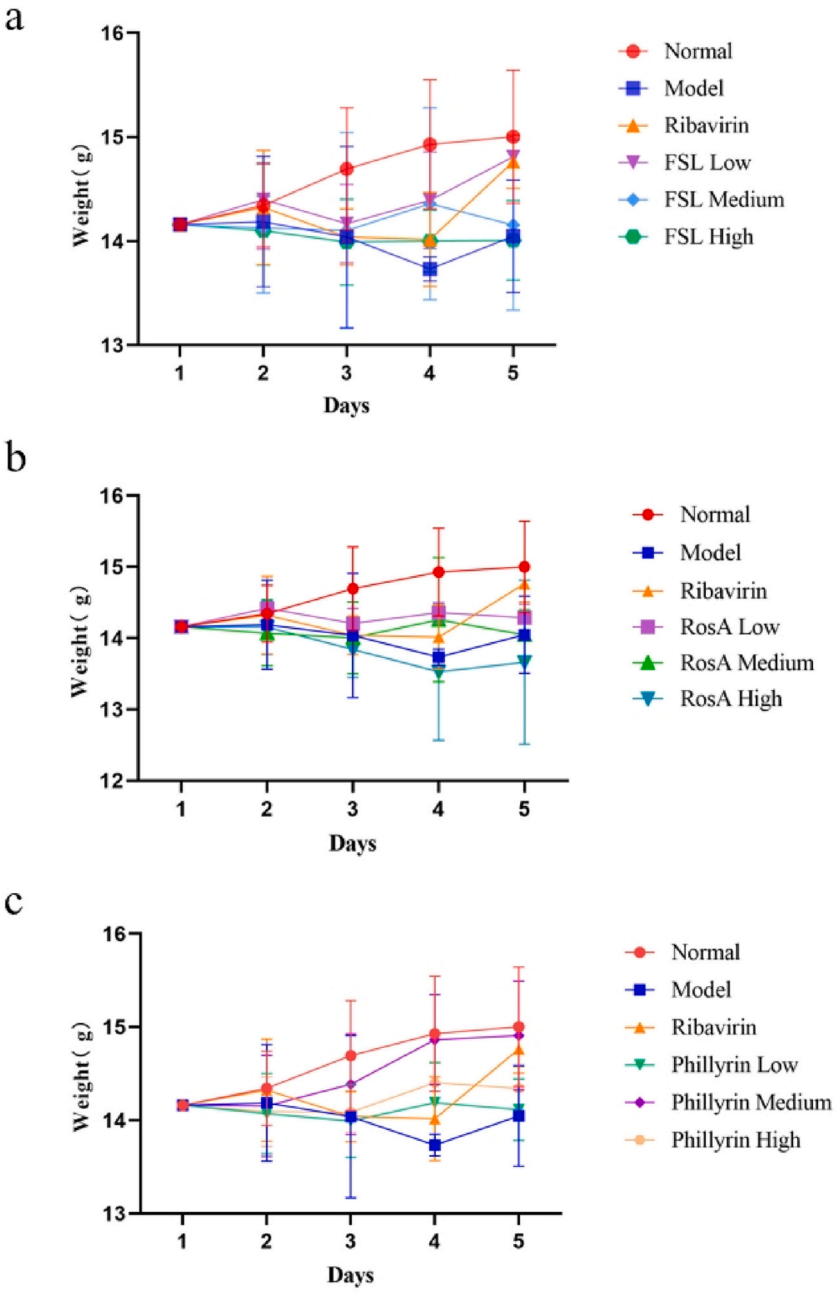

Fig. 1. Effects of different dosage groups of FSL, RosA, and Phillyrin on the body mass of mice infected with RSV ($x \pm s$, $n = 6$).

3.2. Pathological analysis of lung tissue in mice

H&E staining was used to evaluate the effect of FSL and its active components on the pathological changes of lung tissue in mice infected with RSV. As shown in Fig. 2A-L, the alveolar wall in the control group appears thin, with no discernible inflammatory cell infiltration. In contrast, RSV-infected mice (Fig. 2B) exhibited thickening of the alveolar wall, capillary blood stasis, and obvious infiltration of inflammatory cells compared to the control group. Notably, the administration of FSL (Fig. 2D-F) mitigated the thickening of the alveolar wall and the infiltration of inflammatory cells in the treated groups relative to the model group. Furthermore, among the various dosage groups, the middle-dose group within each administration group demonstrated a notably superior effect compared to the other dose groups.

3.3. Organ index

As depicted in Fig. 3, when compared with the normal group, mice infected with RSV exhibited elevated lung index ($P < 0.01$) (Fig. 3a), thymus index (Fig. 3c), and spleen index ($P < 0.05$) (Fig. 3b), signifying the successful establishment of the RSV-infected mouse model. Following the administration of the drug to RSV-infected mice, the lung index in each treatment group

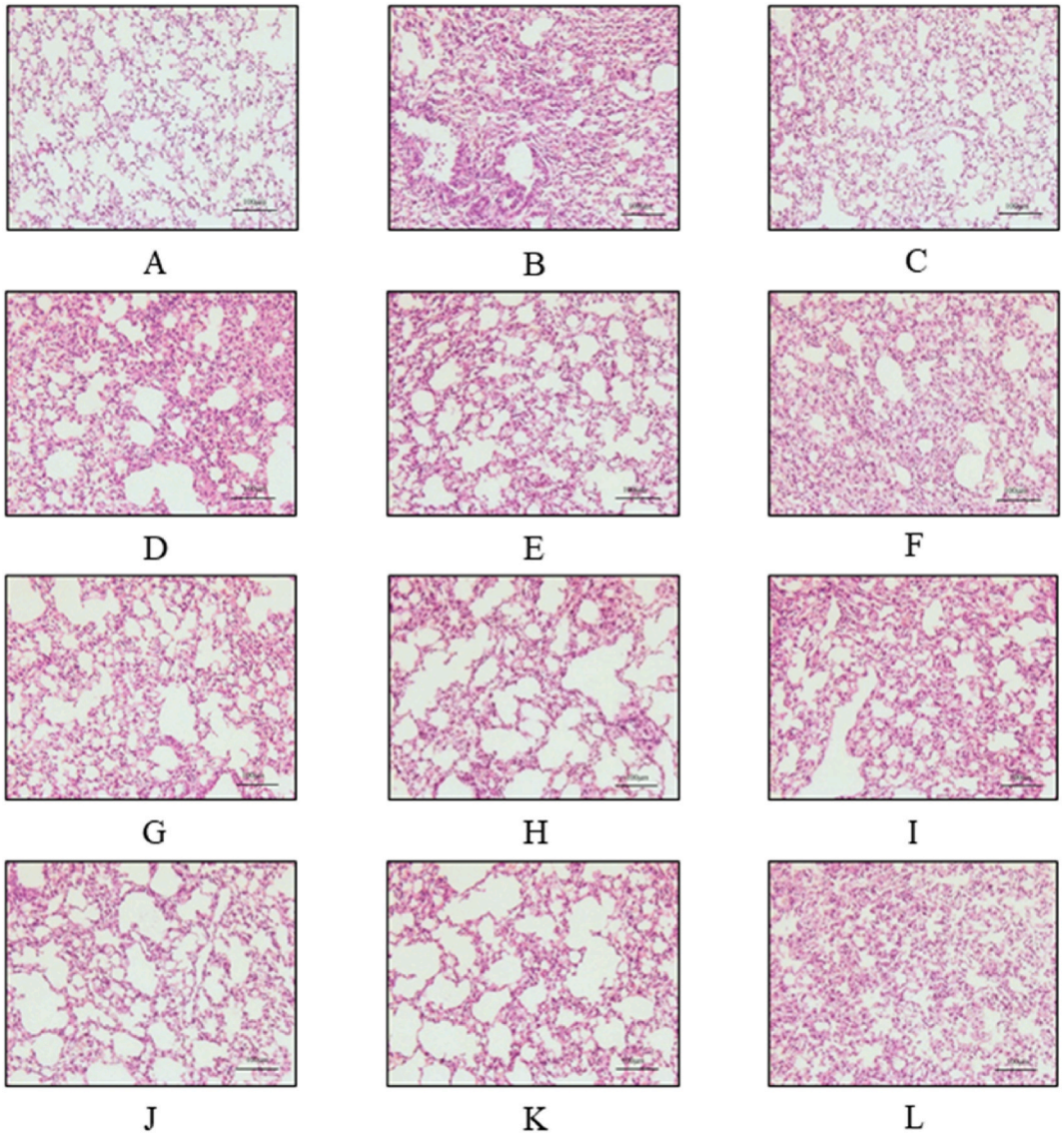

Fig. 2. Effect of FSL and its active components on pathological change of lung tissue in mice infected with RSV (× 200). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. FSL Medium; F. FSL High; G. RosA Low; H. RosA Medium; I. RosA High; J. Phillyrin Low; K. Phillyrin Medium; L. Phillyrin High.

demonstrated varying degrees of reduction. Notably, the FSL Medium and Phillyrin Medium groups exhibited the most significant effects ($P < 0.01$), indicating that FSL and its active components effectively ameliorate RSV-induced lung injury. Thymus index and spleen index, crucial indicators of immune function, demonstrated recovery in each treatment group, suggesting that FSL and its active components may exert a positive influence on immune function.

3.4. Inflammatory factor

The standard curves of IL-1β and IL-6 measured by ELISA Kit were $y = -1E\text{-}05x^2+0.0125x+0.0814$, $R^2 = 0.9993$; $y = -5E\text{-}06x^2+0.0085x\text{-}0.0086$, $R^2 = 0.9999$. As shown in Fig. 4, compared with the normal group, the inflammatory factors IL-1β (130.60 pg/mL) and IL-6 (52.62 pg/mL) in the lung tissue of the model group were significantly increased ($P < 0.01$). Compared with the model

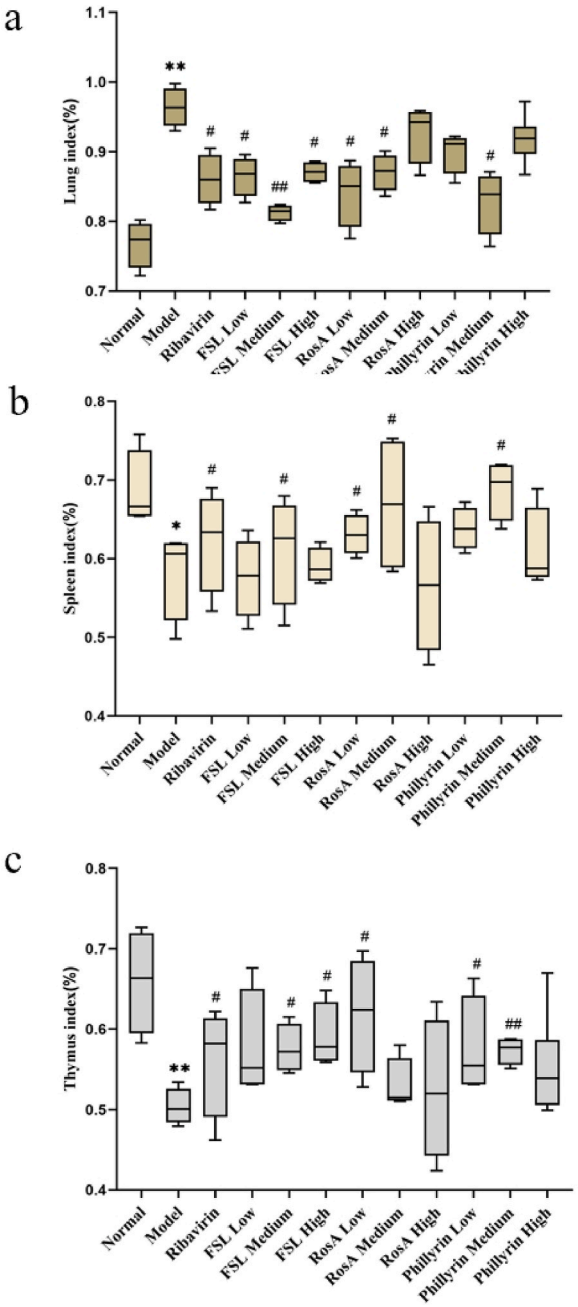

Fig. 3. Comparison of lung, spleen, and thymus index of mice in each group ($\bar{x} \pm s$, $n = 6$). Data were subjected to one-way ANOVA to determine if changes in data between groups were significant.Compared with the normal group, $^{*}P < 0.05$; Compared with the model group, $^{\#}P < 0.05$.

group, the administration group can significantly adjust the contents of IL-1β and IL-6 in the lung tissue of mice, among which the Ribavirin, RosA Low and Phillyrin Medium groups exerted better effects ($P < 0.05$ or $P < 0.01$).

3.5. Replication of the RSV in the mouse lungs

After RSV infection, the existence of RSV-specific nucleic acid in mouse lung tissue was assessed through fluorescence quantitative RT-PCR to determine the relative expression of RSV and RSV-F related protein mRNA. As illustrated in Fig. 5, the administration group demonstrated a significant reduction in the expression of RSV mRNA compared with the model group. Notably, the FSL Low (The relative expression level was 0.65), RosA Low (The relative expression level was 0.79) and Phillyrin High (The relative expression level was 0.66) groups exhibited superior effects ($P < 0.05$ or $P < 0.01$). Furthermore, FSL and its active components were found to markedly down-regulate the expression of F protein mRNA, a mediator of RSV fusion with the cell membrane ($P < 0.05$). It is noteworthy that, although Ribavirin could down-regulate the expression of RSV mRNA, it failed to inhibit the expression of F protein mRNA.

3.6. Relative mRNA expression of key factors in the PI3K/Akt-NLRP3 signaling pathway

The mRNA expression of PI3K/Akt-NLRP3 pathway related proteins was also detected. As shown in Fig. 6, the relative expressions of PI3K, NLRP3, and IL-18 mRNA in the lung tissue of the model group were significantly higher than those in the normal group ($P < 0.05$ or $P < 0.005$), while the relative expressions of Akt and Nrf2 mRNA were significantly lower ($P < 0.05$). Compared with the model group, the administration group can down-regulate the mRNA expressions of PI3K, NLRP3, and IL-18 in the lung tissue of RSV-infected mice, and the effects are better in the FSL Low and Phillyrin Medium groups ($P < 0.05$). In addition, the administration group demonstrated regulatory effects on the mRNA expressions of Akt and Nrf2 in the mice's lung tissue, with a more pronounced effect noted in the FSL Low group ($P < 0.05$).

3.7. Immunohistochemistry staining

The immunohistochemical results of each index of mouse lung tissue are shown in Figs. 7–10. Examination of the figures reveals that following RSV infection, the expression levels of p-PI3K, p-Akt, and Nrf2 proteins in the lung tissue of mice were significantly diminished compared with those in the normal group, with a noticeable reduction in the number of positive cells. Conversely, the number of positive cells expressing NLRP3 protein increased, indicative of elevated expression levels compared with the normal group. Following drug intervention, each drug group exhibited a varying degree of recovery trend, with the Ribavirin and Phillyrin groups demonstrating more pronounced positive effects compared with the model group.

3.8. Relative protein expression of key factors in the PI3K/Akt-NLRP3 signaling pathway

The relative expressions of p-PI3K, p-Akt and Nrf2 proteins in the lung tissue of the model group were significantly lower than those in the normal group ($P < 0.05$), while the relative expression of NLRP3 protein was significantly higher than that in the normal group ($P < 0.05$) (Fig. 11). In comparison with the model group, the drug administration group can significantly reduce the protein expression of p-PI3K, p-Akt, and Nrf2 in the lung tissue of RSV-infected mice ($P < 0.05$ or $P < 0.01$), and effectively inhibit the protein expression of NLRP3 ($P < 0.05$). The Ribavirin and Phillyrin demonstrated better effects, which are consistent with the immunohistochemical results. Inflammatory corpuscle-related protein NLRP3 can reflect the inflammatory response of the body, indicating

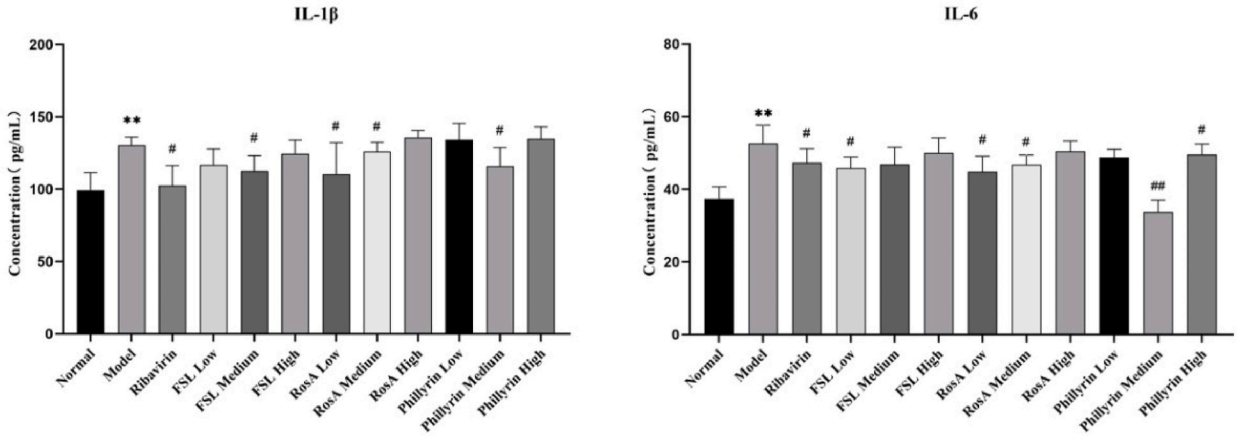

Fig. 4. Comparison of IL-1β and IL-6 contents in lung tissue of mice in each group ($\bar{x} \pm s$, $n = 6$). Data were subjected to one-way ANOVA to determine if changes in data between groups were significant. Compared with the normal group, $^{*}P < 0.05$, $^{**}P < 0.01$; Compared with the model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

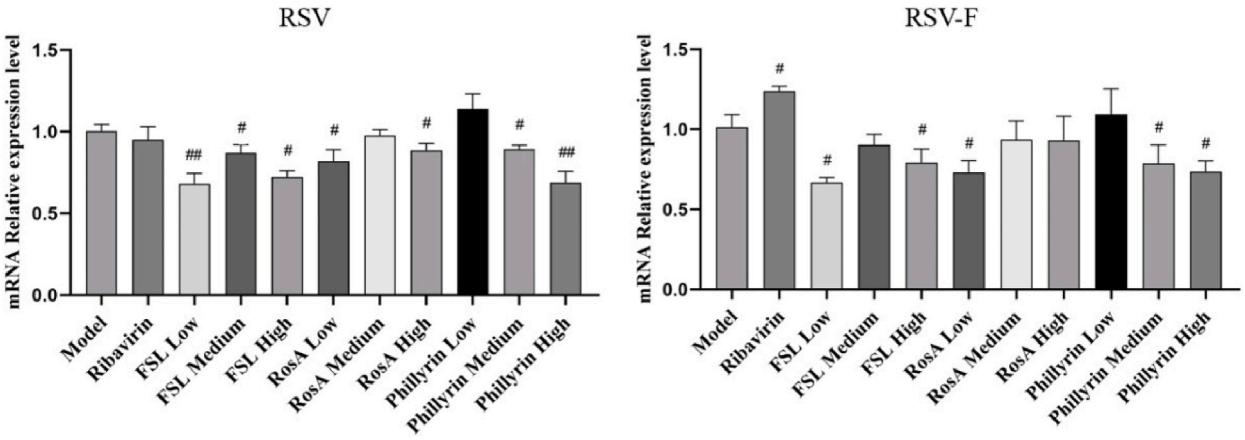

Fig. 5. Effects of FSL and its active components on the expression of RSV and RSV-F mRNA in lung tissue of mice infected with RSV ($\bar{x} \pm s$, $n = 6$). Data were subjected to one-way ANOVA to determine if changes in data between groups were significant. Compared with the model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

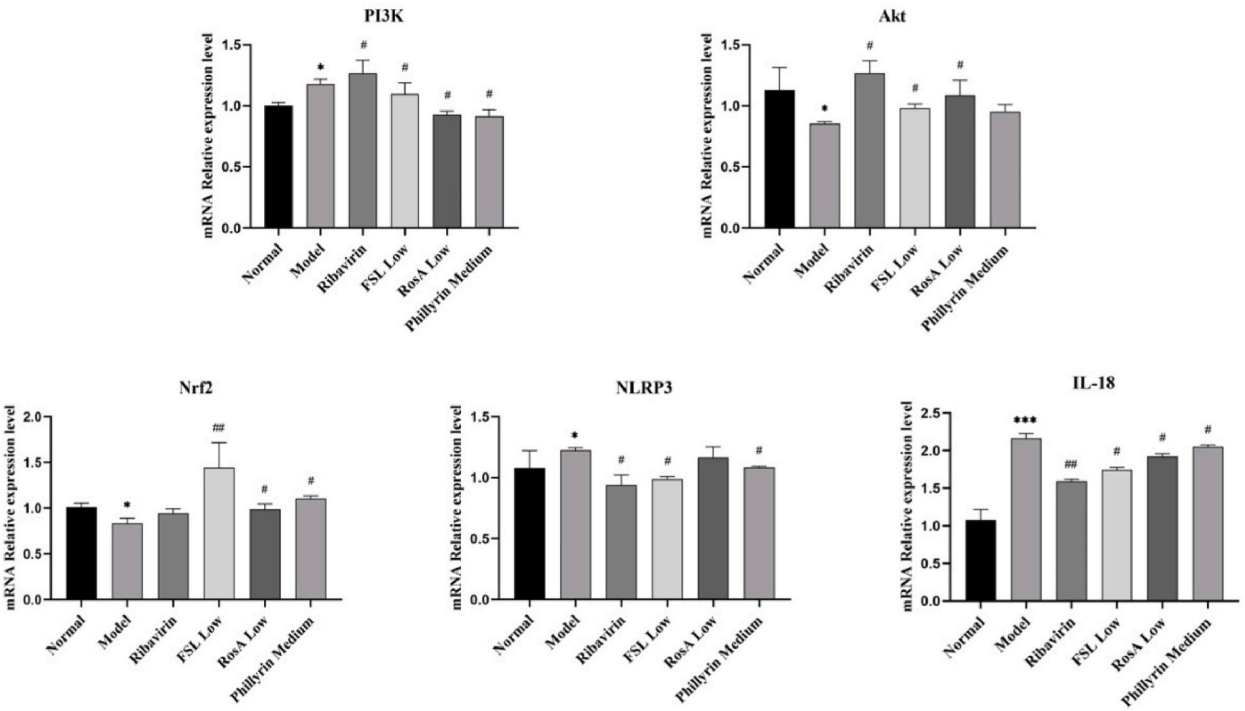

Fig. 6. Effects of FSL and its active components on the mRNA expression of PI3K/AKT-NLRP3-related proteins in lung tissue of RSV-infected mice ($\bar{x} \pm s$, $n = 6$). Data were subjected to one-way ANOVA to determine if changes in data between groups were significant. Compared with the normal group, $^{*}P < 0.05$, $^{***}P < 0.005$; Compared with the model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

that Ribavirin and Phillyrin can effectively reduce the inflammatory response and lung injury in RSV-infected mice.

4. Discussion

The model research of RSV is very extensive. At present, the animal models that can be used for experimental research include orangutans, baboons, ferrets, cotton mice, sheep, guinea pigs and mice, and each model has its advantages and limitations. Mouse model is widely used in the study of RSV infection *in vivo* because of its easy availability, clear genetic background, mature genetic modification technology and a variety of commercial reagents, among which BALB/c mice are the most widely used [26]. Therefore, in this experiment, BALB/c mice were used to prepare RSV infection model, and the anti-RSV effect of FSL and its active components *in*

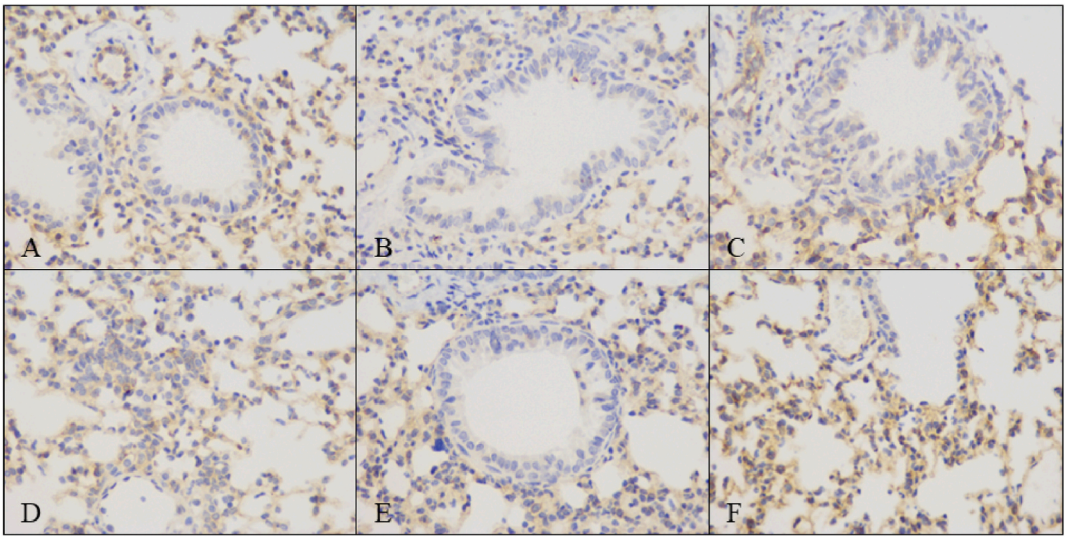

Fig. 7. IHC staining of p-PI3K in mouse lung tissue (× 400). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. RosA Low; F. Phillyrin Medium.

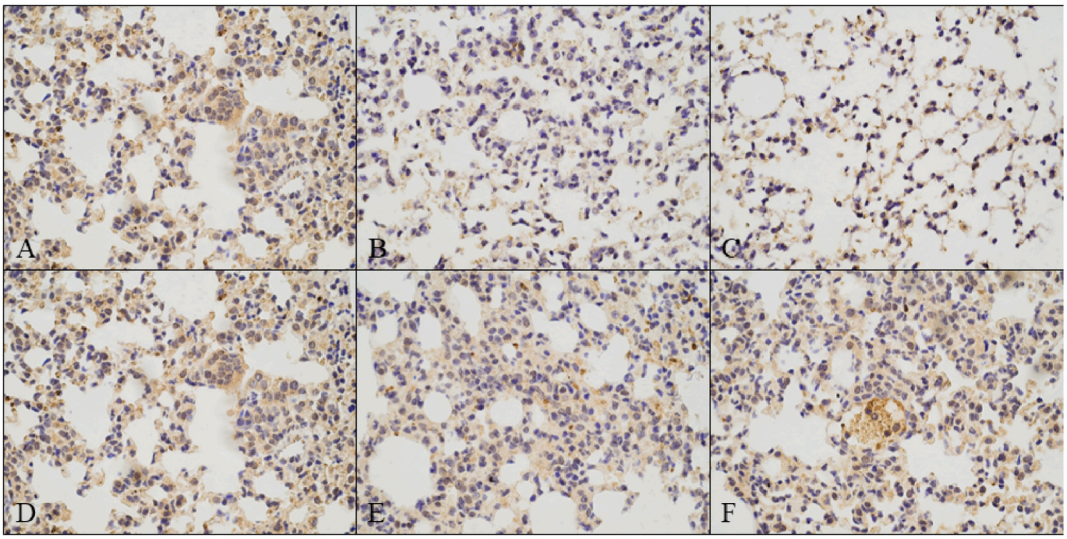

Fig. 8. IHC staining of p-Akt in mouse lung tissue (× 400). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. RosA Low; F. Phillyrin Medium.

vivo was evaluated by investigating the changes in animal body weight, organ index, pathological tissue, inflammatory factors, and virus load. The results showed that FSL and its active components, Phillyrin and RosA, demonstrated certain anti-RSV activity, with the combined treatment of the FSL Low, RosA Low and Phillyrin Medium groups exhibiting superior therapeutic efficacy. Cytokine analysis demonstrated that the positive drugs Ribavirin and Phillyrin effectively reduced the levels of IL-1β and IL-6 in the lung tissue of RSV-infected mice. IL-1β, a crucial proinflammatory cytokine, serves as a key mediator of the inflammatory response, reflecting the host's resistance to pathogens [27]. At the same time, IL-1β production often signifies the activation of NLRP3 inflammatory corpuscles, inducing the secretion of various chemokines by lung epithelial cells and recruiting additional inflammatory factors into alveoli and lung tissues, thus aggravating lung tissue inflammation [28]. IL-6, an important inflammatory mediator activated by IL-1β and tumor necrosis factor (TNF-α), plays a vital role in immune response, blood activation, and acute reaction during infection or tissue damage [29]. Based on the above results, Phillyrin, the active ingredient in FSL, may play an anti-RSV role by regulating immune response and controlling inflammatory process. In addition, we also used RTPCR technology to detect the viral load in the lung tissue of mice. The results indicated that both FSL and Phillyrin significantly reduced RSV mRNA expression, with FSL exhibiting a more pronounced effect, presumably due to the synergistic antiviral action of multiple components in FSL. Moreover, FSL and Phillyrin can also reduce the mRNA expression of RSV-F protein. Studies show that F protein can mediate the fusion process between virus and cell membrane [30], and it is speculated that FSL and its active components play an anti-RSV role by inhibiting the fusion process between virus and host cells, thus inhibiting virus replication. However, in this experiment, we found that the high-dose drug group did not

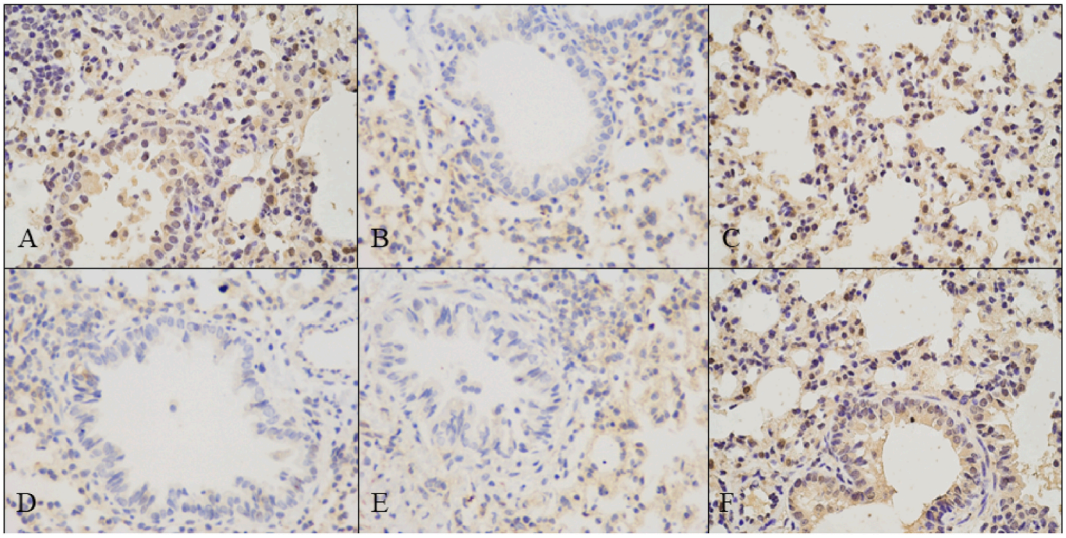

Fig. 9. IHC staining of Nrf2 in mouse lung tissue (× 400). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. RosA Low; F. Phillyrin Medium.

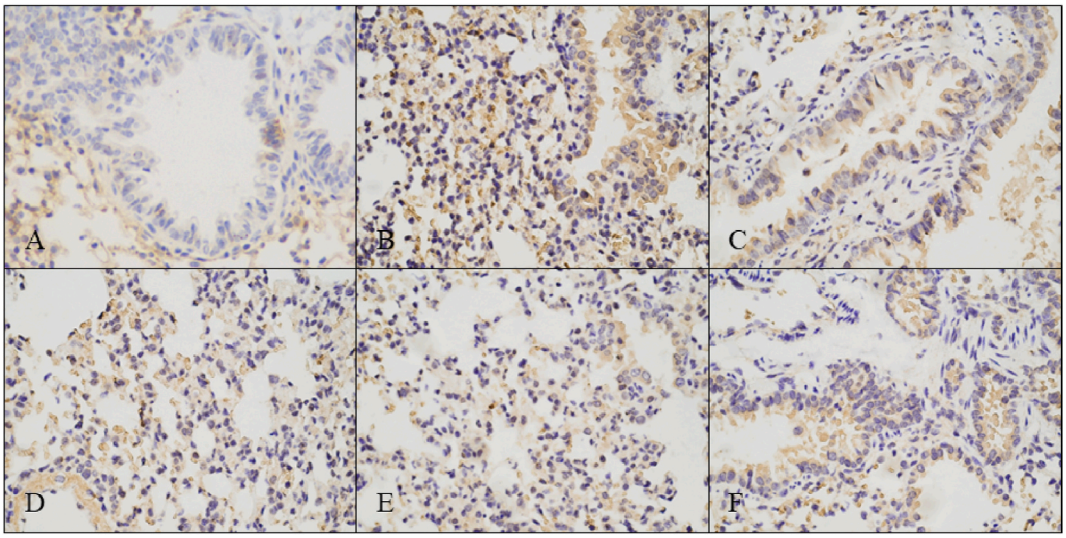

Fig. 10. IHC staining of NLRP3 in mouse lung tissue (× 400). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. RosA Low; F. Phillyrin Medium.

achieve good therapeutic effect. We speculated that high-concentration drugs often had strong taste, which greatly reduced the compliance of animals in the process of administration, thus resulting in drug loss and reduced curative effect.

Through pharmacodynamic experiments *in vivo*, we comprehensively evaluated the efficacy of three doses of FSL, RosA and Phillyrin with reference to animal body mass, organ index, pathological tissue, inflammatory factors and virus load, and selected the best dose. We further explored the anti-RSV mechanism of FSL and its active components by fluorescence quantitative RT-PCR, Western blot, and other molecular biological means. The results revealed that FSL and its active components could improve the expression of PI3K/Akt-NLRP3-related proteins and mRNA to varying extents. Notably, Ribavirin and Phillyrin demonstrated effectiveness in reducing the expression of Nrf2 mRNA and protein in the lung tissue of RSV-infected mice. This signifies their ability to effectively regulate the inflammatory reaction mediated by the NLRP3 inflammasome during RSV infection. Clinical manifestations of RSV infection encompass inflammatory responses, including pneumonia and capillary inflammation, with severe cases leading to acute lung injury (ALI). These inflammatory responses are often accompanied by the release of inflammatory factors such as IL-1β and IL-18, products of NLRP3 inflammasome activation.

Studies have shown that numerous viruses, including RSV, can activate the NLRP3 inflammasome, and its activation mediates cell death, when it is dysfunctional, it will further lead to lung injury [31] RSV infection will activate a variety of virus recognition receptors in the host airway epithelial cells, stimulate the body to produce innate immune response [32], thus activating NF-κB signaling pathway and up-regulating the expression of pro-IL-18, pro-IL-1β and protein NLRP3. In this process, if the host cell state changes, it

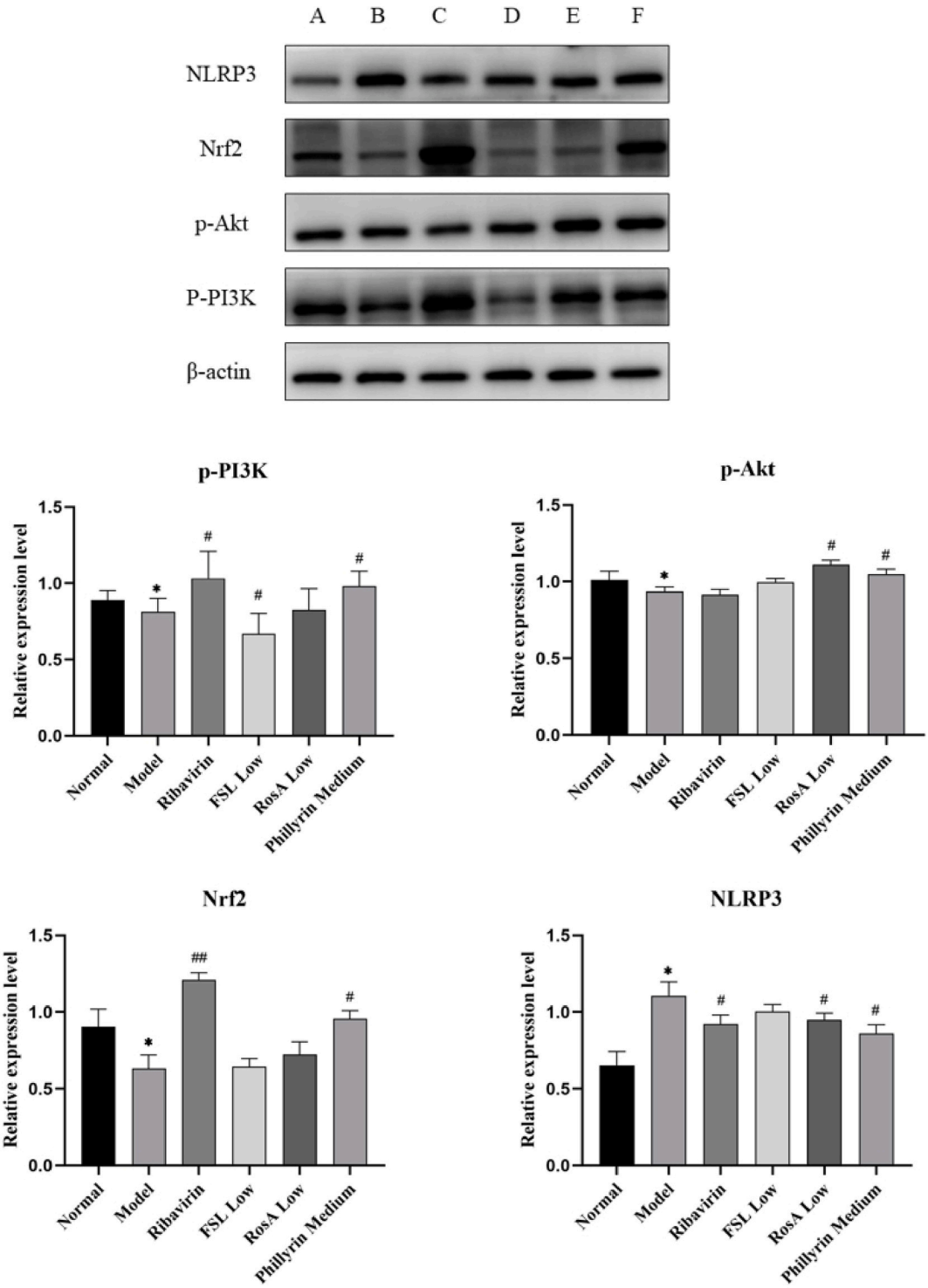

Fig. 11. Effects of FSL and its active components on the expression of p-PI3K, p-AKT, Nrf2, and NLRP3 proteins (A, Supplementary information, Figs. S1–S9. In Figs. S1–S5, the seventh sample is added to control the number of samples in the gel, so it does not participate in data statistics.) in lung tissue of RSV infected mice ($\bar{x} \pm s$, $n = 6$). A. Normal; B. Model; C. Ribavirin; D. FSL Low; E. RosA Low; F. Phillyrin Medium. Data were subjected to one-way ANOVA to determine if changes in data between groups were significant. Compared with the normal group, $^{*}P < 0.05$; Compared with the model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

will accelerate the assembly of NLRP3 inflammatory corpuscles. For example, RSV-SH hydrophobin can promote the activation of NLRP3 inflammatory bodies by affecting the permeability of cell membrane [33]. In addition, ROS produced in infected cells can also promote the formation of NLRP3 inflammatory bodies [34], but the mechanism of ROS production still needs further study. Inflammatory corpuscles can activate active substances such as caspase-1 and promote the secretion of IL-1β and IL-18 after assembly. IL-1β will recruit neutrophils to the inflammatory site to eliminate the invading virus. Then, they will jointly induce cell death related to inflammation, that is, cell scorch. Therefore, determining the involvement of inflammatory corpuscle-related protein NLRP3 in RSV pneumonia and regulating its over-activation can effectively ameliorate the excessive inflammatory response induced by viral infection, consequently improving lung injury.

The PI3K/Akt signaling pathway not only governs cell proliferation and apoptosis but also assumes a pivotal role in regulating oxidative stress. Nrf2, as a key transcription factor implicated in cellular oxidative stress, plays a crucial role in maintaining redox homeostasis. Research indicates that the activation of the PI3K/Akt signaling pathway can upregulate Nrf2 protein expression, thereby mitigating oxidative stress and preserving the equilibrium between oxidation and antioxidation. For instance, 1,2,3,4,6-O-Pentagalloylglucose demonstrates the capability to inhibit oxidative stress by mediating the PI3K/Akt/Nrf2 signaling cascade, effectively combating acute lung injury [35], we speculated that phillyrin played a similar role in the process of fighting RSV pneumonia. The activation of Nrf2 exerts a discernible inhibitory effect on NLRP3, likely through the mediation of reactive oxygen species (ROS) [36]. Upon Nrf2 activation, it modifies the regulatory cysteine residue of its inhibitor Keap1, initiating the expression of a variety of proteins that can effectively "detoxify" ROS. This detoxification process counters NLRP3 inflammatory corpuscles and the inflammatory response they induce, given that the activation of NLRP3 inflammatory corpuscles is contingent upon ROS.

5. Conclusion

To sum up, phillyrin, the active ingredient in FSL, can not only effectively inhibit RSV replication, but also control the inflammatory reaction caused by RSV infection, which is expected to become a potential drug against RSV pneumonia. In addition, in the aspect of controlling inflammatory reaction, it is speculated that phillyrin mainly regulates PI3K/AKT/Nrf2 pathway, inhibits the expression of inflammatory body-related protein NLRP3, and then improves the inflammatory reaction caused by virus infection. However, there are still some limitations in this study. Firstly, In the process of improving RSV pneumonia with phillyrin, it is necessary to further clarify whether phillyrin has the possibility of regulating ROS besides activating PI3K/Akt-Nrf2 pathway. There is also the exact mechanism of Nrf2 regulating NLRP3, which still needs a lot of experiments for in-depth study. Another limitation is that the reverse verification experiments of PI3K inhibitor and Nrf2 inhibitor have not been carried out in this study, which can be verified by supplementary experiments in the future.

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Ethics approval and consent to participate

In this study, all animal experiments were carried out in accordance with the relevant regulations in the management measures for experimental animals of Shandong University of Traditional Chinese Medicine, and the experimental steps were supervised and evaluated by the Experimental Animal Ethics Committee of Shandong University of Traditional Chinese Medicine (batch number: SDUTCM20230509001).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Xiaoxue Wang: Writing – original draft, Visualization, Validation. **Weilian Ren:** Writing – review & editing, Methodology, Investigation. **Ping Wang:** Writing – review & editing, Funding acquisition. **Li Dong:** Writing – review & editing. **Haitao Du:** Writing – review & editing, Supervision, Methodology. **Na Li:** Resources, Investigation. **Guixia Liu:** Data curation. **Ru Zhang:** Formal analysis. **Lin Wang:** Visualization. **Tiefeng Sun:** Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38285.

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