

1 **Pi-Pa-Run-Fei-Tang alleviates lung injury by modulating**
2 **IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF-κB signaling pathway**
3 **and the balance of Th17/Treg cells in murine model of OVA-induced**
4 **asthma**

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Abstract

Ethnopharmacological relevance: Pi-Pa-Run-Fei-Tang (PPRFT) is an empirical prescription of TCM to treat asthma symptoms which contains eight medicinal plants.

However, the underlying molecular mechanisms of PPRFT for the treatment of asthma in asthma are yet to be elucidated. Recent advances have revealed that variations in host metabolism are intimately associated with the progression of asthma. Some natural components could ameliorate asthma contrast injury by affecting host metabolism. We can use untargeted metabolomics to better understand the biological mechanisms underlying asthma development and to identify early biomarkers that can help advance treatment.

Aim of the study: To investigate the metabolic modulating effects, and potential mechanisms underlying the therapeutic effect of PPRFT in asthma.

Materials and methods: A mouse asthma model was built by OVA induction. Inflammatory cell in BALF was counted. The level of IL-6, IL-1 β , and TNF- α in BALF were measured. The levels of IgE in serum and the levels of EPO, NO, SOD, GSH-Px, and MDA in lung tissue were measured. Furthermore, the pathological damage of lung tissues was detected to evaluate the protective effects of PPRFT. The serum metabolomic profiles of PPRFT in asthmatic mice were determined by GC-MS. The regulatory effects on IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF- κ B mechanism pathway of PPRFT in asthmatic mice were explored via immunohistochemical staining and western blotting analysis.

Results: PPRFT showed lung-protective effects through decreasing oxidative stress,

50 airway inflammation, and lung tissue damage in OVA-induced mice, which was
 51 demonstrated by decreasing inflammatory cell levels, IL-6, IL-1 β , and TNF- α levels
 52 in BALF, and IgE levels in serum, decreasing EPO, NO, and MDA levels in lung
 53 tissue, elevating SOD and GSH-Px levels in lung tissue and lung histopathological
 54 changes. Besides, PPRFT could regulate the imbalance of Th17/Treg cell ratios,
 55 suppress the ROR γ t, and increase the expression of IL-10 and Foxp3 in the lung.
 56 Moreover, PPRFT treatment led to the decreased expression of IL-6, p-JAK2/Jak2,
 57 p-STAT3/STAT3, IL-17, NF- κ B, p-AKT/AKT, and p-PI3K/PI3K. Serum
 58 metabolomics analysis revealed that 35 metabolites were significantly different
 59 among different groups. Pathway enrichment analysis indicated that 31 pathways
 60 were involved. Moreover, correlation analysis and metabolic pathway analysis
 61 identified three key metabolic pathways: galactose metabolism, tricarboxylic acid
 62 cycle, and glycine, serine, and threonine metabolism.

63 **Conclusion:** This research indicated that PPRFT treatment not only attenuates the
 64 clinical symptoms of asthma but is also involved in regulating serum metabolism. The
 65 anti-asthmatic activity of PPRFT may be associated with the regulatory effects of
 66 IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF- κ B mechanistic pathways.

67 **Keywords:** PPRFT, asthma, Th17/Treg, Metabolomics

68

Abbreviations:

AKT	protein kinase B
BALF	bronchoalveolar lavage fluid
ELISA	Enzyme-linked immunosorbent assay

EPO	erythropoietin; Foxp3, forkhead box P3
GC-MS	gas chromatography-mass spectrometry
GSH-Px	glutathione peroxidase
HE	Hematoxylin and Eosin
IgE	immunoglobulin E
IL-10	interleukin-10
IL-17	interleukin-17
IL-1 β	interleukin-1 β
IL-6	interleukin-6
JAK2	Janus kinase 2
MDA	malondialdehyde
NF- κ B	nuclear factor Kappa-B
NO	nitric oxide
OPLS-DA	orthogonal partial least squares-discriminant analysis
OVA	Ovalbumin
p-AKT	phosphorylation of protein kinase B
PCA	Principal component analysis
PI3K	Phosphotylinosital 3 kinase
p-JAK2	phosphorylation of Janus kinase 2
PPRFT	Pi-Pa-Run-Fei-Tang
p-PI3K	phosphorylation of signal transducers and activators of transcription 3
ROR λ t	retinoid-related orphan receptor; SOD, Superoxide dismutase
STAT3	started signal transducer and stimulator of transcription 3
TCM	traditional Chinese medicine
Th17	T helper 17
TNF- α	tumor necrosis factor- α
Treg	Regulatory T
WBC	white blood cell
YYQFT	Yang-Yin-Qing-Fei-Tang

1.Introduction

Asthma is a chronic disease that is marked by inflammation, repeated airway blocking, and hyper-responsiveness of the airways(K. et al., 2022). Several countries now face this increasingly prominent public health concern and over 300 million people bear asthma(Liping et al., 2022). It remarkably hampered the quality of people's lives, leading people to experience serious health and economic burden. Therefore, understanding the mechanism of asthma and discovering effective drugs have become important issues in respiratory medicine. However, many types of drugs in treating asthma such as glucocorticoids(Mahesh et al., 2022), β_2 agonists(Lipworth and Jabbal, 2017), aminophylline(Ronghua et al., 2022), leukotriene receptor antagonists(JiSu et al., 2021), and anticholinergics(C, 2019). They could inhibit inflammation for a moment, but not shorten the chronic course of asthma. Furthermore, they also with many side effects, such as double infection, tachycardia, muscle tremor, inhibition of bone metabolism, and adrenal function(Akdis Cezmi, 2012). Unfortunately, there is no single drug that can effectively treat asthma because of the uncertainty and complexity of the pathogenesis of asthma. Therefore, developing safe and effective drugs to relieve asthma has become an increasingly crucial and prolific research field(Beibei et al., 2022; Raquel et al., 2021).

Evolving experimental evidence suggests that the irregular release of cytokines external by the CD4⁺ T cells takes part in the pathogenesis mechanism and progression of asthma(K and Peter, 2006). T helper 17 (Th17) cells is a hypotype of CD4⁺ T cells that act an essential part in the process of asthma by secreting

interleukin-17 (IL-17). They strikingly increase lung tissue inflammation, exacerbating airway remodeling, and pulmonary airway hypertension of asthmatic patients in contrast with healthy people(Elin and Anders, 2012; Julie et al., 2014). Regulatory T (Treg) cells, the other one subtypes of CD4⁺ T cells, are reflecting a kind transcriptions factor forkhead box P3 (Foxp3)(Tiantian et al., 2018). Moreover, it protects the body and avoids damage by inhibiting immunoreaction by restricting T cells to an appropriate level(Yuling et al., 2017). Therefore, the equilibrium of Th17 and Treg is vital for the onset and progression of asthma. The retinoid-related orphan receptor (ROR λ t), an indispensable transcription factors in Th17 differentiation, and started signal transducer and stimulator of transcription 3 (STAT3) are positively relation to the regulation of ROR λ t as well as IL-17 expression(N et al., 2007). Then, some researchers indicated that the prominently increased of Th17 cells and the markedly reduced of Treg cells in bronchoalveolar lavage fluid (BALF) of a OVA-induced asthma model, and the variation were markedly inhibited by stimulate JAK2/STAT3 signaling pathway(Ruping et al., 2022; Xu et al., 2017). Also, interleukin-6 (IL-6) accelerate Th17 development and restrain Treg growth(Aqel et al., 2019). Furthermore, Janus kinase 2 (JAK2) activates the expression of the signaling pathways PI3K/AKT/NF- κ B via activating p-PI3K(Sadat et al., 2021). Regulation of IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF- κ B signaling pathways would affect Th17/Treg homeostasis(Caihong et al., 2021; Jina et al., 2022). However, whether it is involved in the development of PPRFT protects asthma has not been studied.

Metabolomics or Metabonomics biochemically analyzes organisms, cells, tissue,

biofluid, and cell membrane(Zsuzsanna et al., 2012). As a systemic-overall method, metabolomics adopted “top-down” tactics to respond to the feature of bionts from end-stage symptom of the metabolic network and it elaborate the system-wide metabolic alters meddles in an integrated environment, meet the requirement of integrated environment and syndrome differentiation of traditional Chinese medicine (TCM)(Tao et al., 2017). Simultaneously, it is a versatile tool for evaluating various TCM prescriptions’ therapeutic and toxic effects(Yong-Min et al., 2009). For example, asthma is an intricate disease with variety pathogenesis and is well suited to study through a metabolomics approach to understand its pathophysiology(Chao et al., 2021).

TCM has a long and rich history in China, which is receiving wide attention and can function as a safe and effective immunomodulator for treating and preventing viral infections(YuBo et al., 2022; ZHANG and XU, 2009). YYQFT is a classical prescription record in Chonglou Yuyao by Zheng Honggang in the Qing Dynasty, is an important approach in the treatment of lung jury(Li et al., 2014). PPRFT was add some medicinal flavors to enhance the effect in asthma on the basis of YYQFT and it has a long history be used for asthma in clinic. This prescription consists of eight species of medicinal herbs, including Loquat Leaf, *Radix Scrophularia*, *Radix Ophiopogonis*, *Radix Platycodonis*, *Terminalia Chebula Retz.*, *Rhizoma Phragmites*, *Mentha Haplocalyx*, *Radix Glycyrrhizae*. PPRFT with the feature of multicomponent, multi-target, as well as multipath and has a long history in the treat of asthma. While, the related mechanisms of PPRFT treatment asthma are still elusive.

Therefore, this present adopted an OVA-induced asthmatic model to explain the functional and mechanisms of PPRFT. In addition, the anti-asthma mechanism correlated with the IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF- κ B mechanistic pathways, along with the serum metabolome, was elucidated.

2. Material and methods

2.1. Reagents

Ovalbumin (OVA) was bought from Shengong Bioengineering Co., Ltd. (Shanghai, China). Dexamethasone was bought from Guangzhou Nanguo Pharmaceutical Co., Ltd. (Guangzhou, China), and aluminum hydroxide was acquired from Aladdin Reagent Co., Ltd. (Shanghai, China). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), Hematoxylin and Eosin (HE), PAS, and Masson assay kits were obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits of mouse IL-6, immunoglobulin E (IgE), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), erythropoietin (EPO), and nitric oxide (NO) were obtained from Shanghai Enzyme Linked Biotechnology Co. (Shanghai, China). ROR λ t, phosphorylation of Janus kinase 2 (p-JAK2), phosphorylation of signal transducers and activators of transcription 3 (p-STAT3), JAK2, and phosphorylation of Phosphotylinositol 3 kinase (p-PI3K) were obtained from Affinity Biosciences Co., Ltd. (Jiangsu, China). IL-6, IL-1 β , TNF- α , nuclear factor Kappa-B (NF- κ B), Phosphotylinositol 3 kinase (PI3K), protein kinase B (AKT), STAT3, IgG(H+L), and phosphorylation of protein kinase B (p-AKT) were obtained from Proteintech, Co.,

Ltd. (Hubei, China). FOXP3 was obtained from Abcam (Hubei, UA). interleukin-10 (IL-10), and IL-17 were obtained from Boster Biology (Hubei, China).

2.2. Plant materials

The crude slices of Pipa Runfei decoction (PPRFD) comprised Loquat Leaf (*Eriobotrya japonica* (Thunb.) Lindl., No. 20200910), Radix Scrophularia (*Scrophularia ningpoensis* Hemsl., No. 2200614X), Radix Ophiopogonis (*Ophiopogon japonicus* (L.f) Ker-Gawl., No. 20201005), Radix Platycodonis (*Platycodon grandiflorum* (Jacq.) A.DC., No. 20201012), Terminalia Chebula Retz. (*Canarium album* Raeusch., No. 200303), Rhizoma Phragmitis (*Phragmites communis* Trin., No. 20201114), Mentha Haplocalyx (Mentha Haplocalyx Briq., No. 20070153), and Radix Glycyrrhizae (*Glycyrrhiza glabra* L., No.20070153). They were purchased from Hangzhou Jiuzhou Pharmacy Chain Co. Ltd. The duplicate sample was deposited at School of Pharmacy, Zhejiang University of Technology. These drugs were decocted after being identified by Prof. Yi Tao, a plant taxonomist at the Zhejiang University of Technology. The raw materials were in a ratio of Loquat Leaf: Radix Scrophularia: Radix Ophiopogonis: Radix Platycodonis: Terminalia Chebula Retz: Rhizoma Phragmitis: Mentha Haplocalyx: Radix Glycyrrhizae as 9: 6: 6: 3: 3: 15: 2: 3.

2.3. Preparation of PPRFD

Herbs were refluxed extraction thrice with fifteenfold, twelvefold, and tenfold the volume of water for 3 times for 60 min each time. Then the extracting solution is merged for rotatory evaporation to three concentrations. The intermediate-dose of

PPRFT (PPRFT-M) was 3.0 g/kg/d transform from the patient's (60 kg) daily dose in clinic. The concentration of (PPRFD-H) in the high dosage was twice that of the intermediate-dose and moiety that of the low dosage of PPRFD (PPRFD-L).

2.4. UPLC/Q-TOF-MS/MS conditions

The extract solution was thinning by water, and the mixtures were centrifuged for 15 min with 14000 rpm. An LC-30AD (Shimadzu, Kyoto, Japan) system with a Waters ACQUITY Premier HSS T3 column (2.1 mm × 100 mm, 1.8 μm) and Waters Van Guard FIT cartridge (2.1 mm × 5 mm, 1.8 μm) was applied to separation. The column temperature set as 35 °C. And flow rate set as 0.4 mL/min. The injection volume was 3 μL. The mobile phase was (A): 0.1% formic acid water and (B): 0.1% formic acid acetonitrile, was performed as follows: 0-3 min, 100%-100% A; 3-10 min, 100%-92% A; 10-23 min, 92%-55% A; 23-24 min, 55%-20% A; 24-27 min, 80%-80%; 27-27.1 min, 80%-0.

An AB SCIEX X500R (AB SCIEX, Foster City, CA, USA) system with a Turbo VTM ion source was used to obtain the qualitative mass spectral data with information dependent acquisition (IDA). The ESI ion source was operated in positive and negative ion mode. The voltage of spray was set at 5500 V, and the temperature of the electrospray ion source was 500 °C. Ion source gas 1 (air), ion source gas 2 (air) and curtain gas (N₂) were set at 55, 55 and 35 psi, respectively.

2.5. Experiments animals

54 female Balb/c mice weighting 20 ± 2 g was bought from Zhejiang Weitong Lihua Laboratory Animal Technology Co. Ltd. (No. SCXK (Zhe) 2019-000, Zhejiang,

China). The conditions were: regular 12/12 h light/dark cycle, temperature 22–25 °C, and 60% relative humidity. All experimental were conducted following the Guide for the Care and Use of Laboratory Animals in the Zhejiang University of Technology. The experiment received approval from the ethics committee of Zhejiang University of Technology (Ethical Number: 20210930072).

2.6. Animal grouping, model preparation, and drug treatment

After one week of adaptive feeding, animals were assigning to the following six groups with randomly (n = 9): control group (Control, 0.9%NaCl), model group (Model, 0.9%NaCl), dexamethasone group (DXM, 0.05 mg/kg/d), PPRFD-L group (PPRFD, 1.5 g/kg/d), PPRFD-M group (PPRFD, 3.0 g/kg/d), and PPRFD-H group (PPRFD, 6.0 g/kg/d). As shown in Fig. 1, on days 0, 7, and 14, apart from control group, mice in the rest mice were intraperitoneally injected into 200 µL phosphate-buffered solution (PBS) containing 20 µg OVA emulsion and 2 mg aluminum hydroxide for sensitization. After initial sensitization, the mice continuously accepted an atomization (1%OVA w/v in PBS) challenge for half one hours on days 16, 17, and 18. DXM, PPRFT-L, PPRFT-M and PPRFT-H were oral treatment to mice as gastric gavages from day on 13-18. Normal and control mice use water to replace. On the last time challenge, 24 h later mice were sacrificed by cervical dislocation, and operated was execution(Kim et al., 2020).

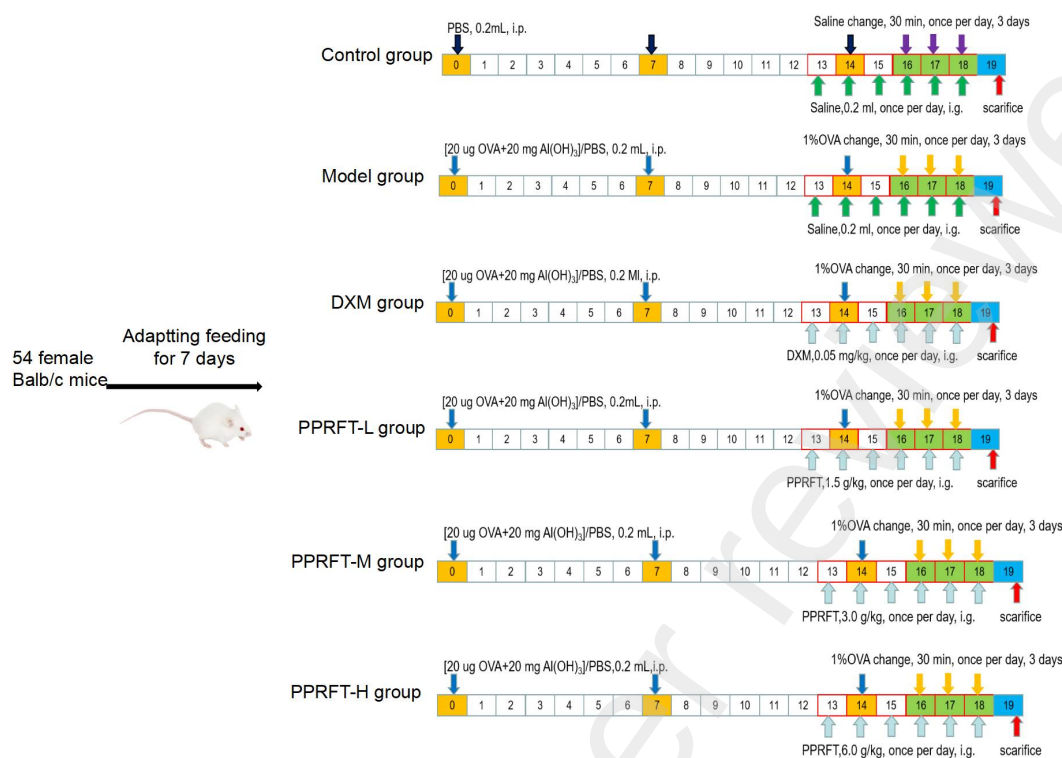


Fig.1. Animal Experimental design of this study.

2.7. Serum and BALF collection

On the 20 days of the experiment, mice were anesthetized. Then, a capillary tube collected blood from the orbital venous plexus. Then the serum was centrifuged with speed at 3500 rpm and temperature was 4 °C for 15 min. Immediately after the animals were sacrificed, the chest cavity was opened, exposed the cervical and a Lanz incision was made over the trachea. A sterile infusion needle was insertion bronchus lobaris inferior dexter. Then the trachea was tied to the right bronchus with catgut. Next, the left lung lobe of mice was securely tied with surgical sutures to guarantee that the left lung was pressure-tight. Using a syringe filled with 0.5 mL saline connected to the ligated gavage needle, saline was flushed into the mice's right lung gently. Then, the saline was retained in lung for an appropriate time, and mild pullout

to acquire BALF. Repetitive operation three times, and roughly collected 1 mL of BALF from every mouse(Kun et al., 2022). BALF was centrifuged, and collected the supernatant, stored at -80 °C. Next, the left lung tissue was soak in 4% paraformaldehyde while the right lung was stored at -80 °C until used.

2.8. Inflammatory cells count in BALF

BALF after centrifugation, collecting precipitation, then the precipitated cells were resuspended and stained with Giemsa stain (Shanghai Bailingwei Chemical Technology Co., LTD, Shanghai, China). Under an optical microscope (Olympus, Japan), the total number of white blood cell (WBC) was counted, which totaled 200 cells, and each slide was classified.

2.9. Histopathological assessment of lung tissue

Left lung tissue cleaned by PBS. Then immobilization in 10% paraformaldehyde (Shanghai Titan Technology Co., Shanghai, China), then stored at 20-25 °C for 72 h. After that, the tissue embedded in the left lung was paraffin. Tissues paraffin slices were stained with Hematoxylin and Eosin, PAS, and Masson. An optical microscope (Olympus, Japan) was adopted to detect the organizational change. Semi-quantitative score was adopted to measure the damage of lung tissue. Grade arranged from 0 (normal) to 4 (very serious) in line with lung injuries(Liyang et al., 2021; Wencheng et al., 2016).

2.10. Measurement of cytokine level in BALF

On basis of the product manual, the BALF standard of IL-6, IL-1 β , TNF- α , EPO, and NO as well as the serum levels of Ig-E were detected by ELISA.

2.11. Measurement of oxidative stress levels in lung tissue

50 mg lung tissue was collected, and saline was added (450 μ L) as a sample. Then, the freezing homogenization was adopted to prepare the lung tissue homogenates, and centrifugation with 4 $^{\circ}$ C for 15 min at 3500 rpm. Lastly, collected the supernatant. Then, standards of SOD, GSH-Px, and MDA in lung tissue were detected with kits in line with the product manual.

2.12. Immunohistochemistry staining

The lung tissues were fixation into paraffin-embedded, then they were divided into 4 μ m sections. After that, dewaxing and ethanol gradient were contributed to tissue rehydration. Then, sections were immersed in a boiling sodium citrate antigen retrieval solution and rinsed three times by PBS. Subsequently, they were incubated in 3% H_2O_2 solution and 5% BSA blocking liquid. Then, hatched by first antibody 12 h at 4 $^{\circ}$ C, next to incubate together by second antibody, and finally stained with DAB. Positive reaction of primary antibody was detection and take a picture with a light microscope.

2.13. Metabolomic study

2.13.1. Sample preparation

With 100 μ L serum was added into 5 μ L Ribitol (CAS:488-81-3) that concluded an isotope labelling standard substance compound. Then, it was added to 200 μ L acetone and vortexed for 60 s. After incubation at ice-water bath ten min and 15 min centrifugation for 4500 rpm at 4 $^{\circ}$ C, 200 μ L supernate was collected. Then, the all supernate was transfer to a GC bottle and blown dry with nitrogen in a room

temperature. Adding Methoxyypyridine solution (75 μ L, 15 mg/ mL) and then imitated 60 min at 70 °C. Next, 75 μ L of MSTFA-derived reagent containing 1% TMCS was joined, mixed, then put at 25 °C for 1 h. After dilution with 150 μ L n-heptane, the supernatant was centrifuged for 15 min with 12000 rpm for 4 °C to gain supernatant for gas chromatography-mass spectrometry (GC-MS) analysis.

2.13.2. Untargeted lipidomics analysis

The GC-MS analysis was acquired to use an Agilent 8890A gas chromatograph (Agilent Technologies) link to Agilent 7000D triple-quadrupole tandem mass-spectrometer. Injecting a 2.0 μ L serum solution as the splitless form into DB-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) using helium as the carrier gas with a flow rate of 1.0 mL/min. Set the injector temperature at 280 °C. The ion-source temperature was switch at 230 °C, and the quadrupole temperature was switch to 150 °C. Electron ionization mass spectra were recorded at 70 eV, 2 scan/s. They range from m/z 35 to 650 and the ratio of scan was quintic per second. The starting temperature was set as 70 °C and maintain five minutes. Following, the temperature was rise to 100 °C with a ratio of 10 °C/min, and kept at 100 °C for 5 min. Then, temperature rise to 250 °C with a ratio of 10 °C/min, and kept at 250 °C for 10 min.

2.13.3. Data analysis

The ChemStation software package (Agilent) was adopted to uniformization the original data on the basis of the values of m/z and retention times. Then input the data into <https://www.bioincloud.tech/> for multi-analysis. Principal component analysis (PCA) was taking on acquire an summarize of changes among groups. The orthogonal

partial least squares-discriminant analysis (OPLS-DA), a monitor model identify method, was adopted to establishment of prediction model to estimate the ovalbumin-induced asthmatic model effect of PPRFT and to distinguish differential metabolites effect for the ovalbumin-induced asthmatic model effects. The parameter setting of models for instance of explanatory power (R^2) Y, and predictive power (Q^2) Y, were calculate to guarantee the quality of the multivariate-models and to avert the danger of overfitting. OPLS-DA models were adopted to obvious noteworthy change metabolites (VIP value ≥ 1.0). A heat map was established to visual the relative-amounts of different metabolites between groups and to analysis their systematic cluster. Furthermore, pathway impact plots were accepted to observe the effect of altered metabolic pathways.

2.14. Western blot

50 mg lung tissue, then adding 0.4 mL RIPA buffer, milling thrice with sixty seconds each time, in rapid sequence cracking at 4 °C for 60 min. The lung tissue was homogenized and centrifuged at 12000 rpm for 15 min, and collected the supernatant. The protein level in lung tissue was measured by BCA method. Initially, the sample was hatched with first antibody for 14-16 h at 4 °C and using TBST to wash the membranes for three times with 10 min each time. Then hatched for 2 h with second antibody at room temperature. In the end, blots were detected by the electrochemiluminescence system G: ChemiScope 3300Pro (Shanghai Qinxiang Scientific Instrument Co., LTD., China), and the Gelpro.32 software was adopted to analysis the densitometric.

2.15. Statistical analysis

The obtained data was showed as mean $\pm$ standard deviation. The statistics analysis was analyzed through the IBM SPSS Statistics 24.0 software. The result was deemed to statistically significance at p -value < 0.05 . #: $p < 0.05$, ##: $p < 0.01$, and ###: $p < 0.001$, compare with the control group; *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$, compare with the model group.

3. Result

3.1. Identified chemical components in PPRFT

The 52 components in PPRFT were characterized under UPLC-QTOF-MS. And shown in Fig.2 and Table 1 based on the mass fragment ions (m/z), retention time (tR), molecular weight.

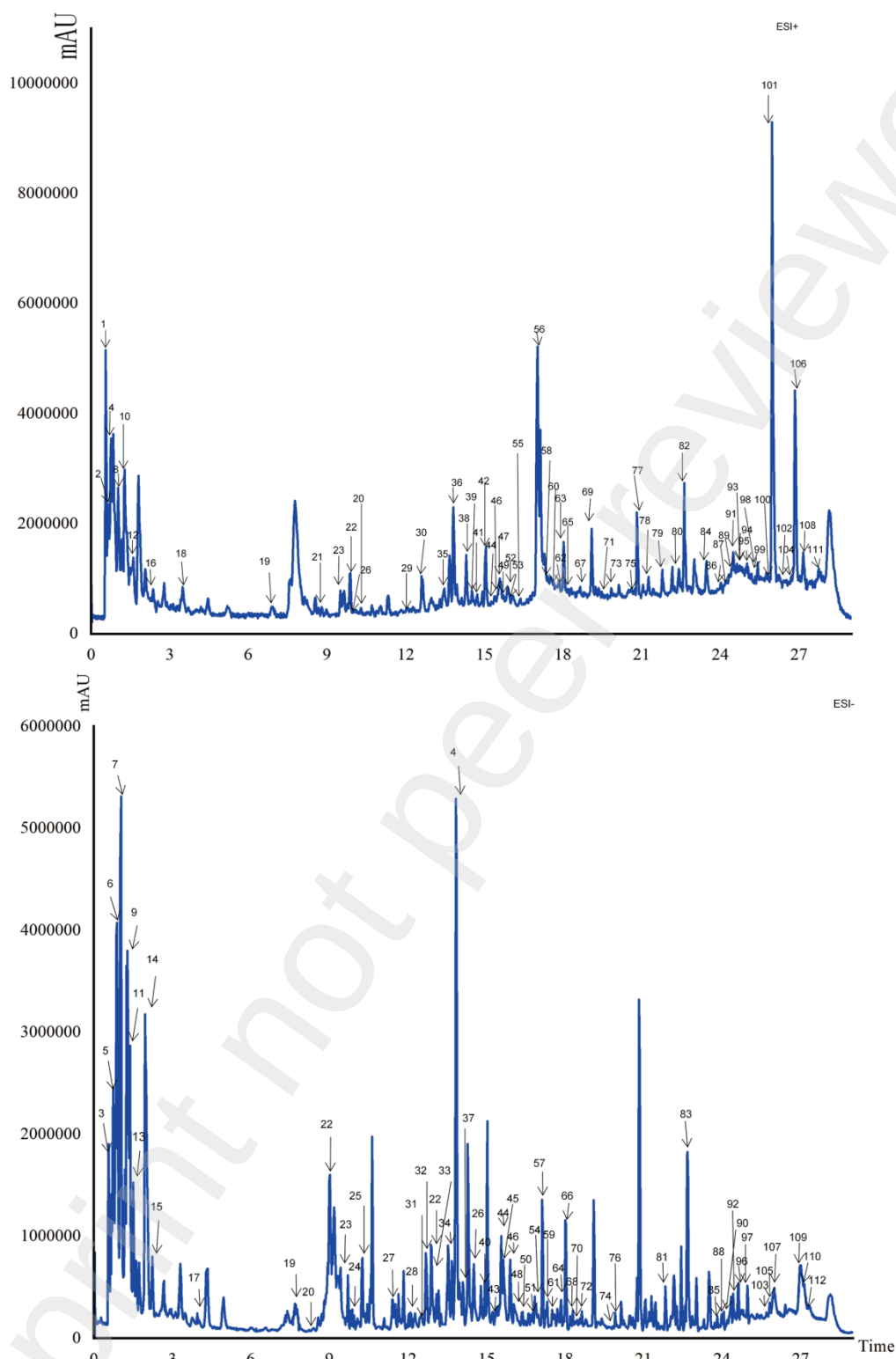


Fig.2. Elementary particle flow graph chromatogram in positive (A) and negative (B) ion mode of PPRFT analyzed with UPLC-QTOF-MS

Table 1. The identification results of compounds in PPRFT

NO.	tR (min)	Identification	Formula	Molecular weight	Mass error (Ppm)	Source
1	0.72	stearic acid	C ₁₈ H ₃₆ O ₂	284.48	2.5	PI
2	0.79	Prunasin	C ₁₄ H ₁₇ NO ₆	295.29	3.6	M
3	0.79	inositol	C ₆ H ₁₂ O ₆	180.16	-0.5	C
4	0.8	Diethyloxalate	C ₆ H ₁₀ O ₄	247.24	-0.5	E
5	0.83	Tartaric acid	C ₄ H ₆ O ₆	150.09	1.7	E
6	1.03	Ascorbic acid	C ₆ H ₈ O ₆	176.12	3.7	PI
7	1.19	Nicotinic acid	C ₆ H ₅ NO ₂	123.11	1.4	PI
8	1.2	salicylic acid	C ₇ H ₆ O ₃	138.12	-1.2	C
9	1.35	licuraside	C ₂₆ H ₃₀ O ₁₃	550.51	6.1	G
10	1.58	Amentoflavone	C ₃₀ H ₁₈ O ₁₀	538.46	-0.2	C
11	1.72	L-Valin	C ₅ H ₁₁ NO ₂	117.15	0.8	PI
12	1.82	1-[5-(5-prop-1-ynylthiophen-2-yl) thiophen-2-yl] ethanone	C ₁₃ H ₁₀ OS ₂	246.35	-1.7	Ph
13	1.95	scoparone	C ₁₁ H ₁₀ O ₄	206.19	-0.3	C
14	2.04	citric acid	C ₆ H ₈ O ₇	192.12	0.3	E
15	2.55	succinic acid	C ₄ H ₆ O ₄	118.09	0.9	S
16	2.79	p-coumaric acid	C ₉ H ₈ O ₃	164.16	-0.5	M
17	4.38	5-hydroxymethylfurfural	C ₆ H ₆ O ₃	126.11	0.2	S
18	4.46	3,4,5-trihydroxybenzoic acid	C ₇ H ₆ O ₅	170.12	-0.7	C
19	7.93	5-Methoxymethyl furfural	C ₇ H ₈ O ₃	140.14	-1.1	O
20	8.5	protocatechuic acid	C ₇ H ₆ O ₄	154.12	-0.4	G
21	8.85	Benzyl alcohol	C ₇ H ₈ O	108.14	0.3	E
22		catapol	C ₂₁ H ₃₂ O ₁₅	524.47	-1.2	G
23	9.77	Vanillic acid	C ₈ H ₈ O ₄	168.15	-0.5	Ph
24	10.07	hydroxytyrosol	C ₈ H ₁₀ O ₃	154.16	1.8	S
25	10.67	6-methoxy-3H-1,3-benzoxazol-2-one	C ₈ H ₇ N	117.15	1.5	E
26	10.69	Progallin A	C ₁₅ H ₂₂ O ₁₀	198.17	0.1	C
27	11.56	3,4-dimethoxyphenol	C ₈ H ₁₀ O	122.16	-0.4	E
28	12.37	5-(2-hydroxyethyl)-2-methoxyphenol	C ₁₉ H ₂₈ O ₁₁	432.42	-0.3	S
29	12.4	1H-Indole-3-carboxylic acid	C ₉ H ₇ NO ₂	161.16	-0.6	G
30	12.64	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	354.31	-0.8	E
31	12.7	Heriguard	C ₁₆ H ₁₈ O ₉	354.31	-0.8	E
32	12.99	(5R)-5-ethyloxolan-2-one	C ₆ H ₁₀ O ₂	114.14	-1	E
33	13.23	(E)-3-(4-Methoxyphenyl) acrylic acid	C ₁₀ H ₁₀ O ₃	178.18	0.4	S
34	13.41	Eriocitrin	C ₂₇ H ₃₂ O ₁₅	596.53	-2	M
35	13.42	Procyanidin	C ₂₇ H ₃₂ O ₁₅	578.52	-1.8	M
36	13.82	Estragole	C ₁₀ H ₁₂ O	148.2	-1.8	E
37	14.05	7-chloroarctinone B	C ₁₃ H ₁₀ OS ₂	246.35	4.2	Ph
38	14.34	vanillin	C ₈ H ₈ O ₃	152.15	-0.8	O
39	14.6	Quercetin-3-O-sophoroside	C ₂₇ H ₃₀ O ₁₇	626.52	-0.2	E

40	14.7	4-Methoxybenzaldehyde	C ₈ H ₈ O	120.15	0	E
41	14.71	schaftoside	C ₂₆ H ₂₈ O ₁₄	564.49	-0.5	G
42	15.16	Kaempferol-3-O-sophoroside	C ₂₇ H ₃₀ O ₁₆	610.52	7.1	E
43	15.18	naringenin	C ₂₇ H ₃₀ O ₁₆	610.52	-1.6	M
44	15.45	violanthin	C ₂₇ H ₃₀ O ₁₄	578.52	-0.4	G
45	15.51	naringin	C ₂₇ H ₃₂ O ₁₄	580.53	-2.2	C
46	15.62	liquiritin	C ₂₁ H ₂₂ O ₉	418.39	-1.5	G
47	15.76	Hyperoside	C ₂₁ H ₂₀ O ₁₂	464.38	1	E
48	15.9	acteoside	C ₂₉ H ₃₆ O ₁₅	624.59	-1.5	S
49	16.16	hexyl acetate	C ₈ H ₁₆ O	128.21	1.4	M
50	16.27	Robinin	C ₃₃ H ₄₀ O ₁₉	740.66	-2.1	PI
51	16.46	4-Hydroxyacetophenone	C ₂₈ H ₃₂ O ₁₆	136.15	0.9	G
52	16.47	Narcissoside	C ₂₈ H ₃₂ O ₁₆	624.54	-1.1	G
53	16.67	4-Methoxycinnamic acid	C ₁₀ H ₁₀ O ₃	178.18	-1.1	S
54	16.7	Vincetoxicoside B	C ₂₁ H ₂₀ O ₁₁	448.38	-1	E
55	16.72	quercetin	C ₁₅ H ₁₀ O ₇	302.24	-0.3	G
56	16.93	Diosmetin	C ₁₆ H ₁₂ O ₆	300.26	0.4	M
57	16.96	6-Methylhept-5-en-2-one	C ₈ H ₁₄ O	126.2	0.2	E
58	17.15	(E)-Ferulic acid	C ₁₀ H ₁₀ O ₄	194.18	-1.5	O
59	17.23	rosmarinic acid	C ₁₈ H ₁₆ O ₈	360.31	-1.4	M
60	17.52	glycyroside	C ₂₇ H ₃₀ O ₁₃	562.52	-0.1	G
61	17.52	(Z)-caryophyllene	C ₂₇ H ₃₀ O ₁₃	170.25	-3.9	G
62	17.62	Luteolin	C ₁₅ H ₁₀ O ₆	286.24	0.2	PI
63	17.87	o-Thymol	C ₁₀ H ₁₄ O	150.22	-0.4	M
64	17.94	Cinchonain	C ₂₄ H ₂₀ O ₉	452.41	-2.2	E
65	18.06	Isoviolanthin	C ₁₅ H ₁₀ O ₄	254.24	-1.5	G
66	18.19	cistanoside D	C ₃₁ H ₄₀ O ₁₅	652.64	-2	S
67	18.2	(Z)-Hex-3-enyl acetate	C ₈ H ₁₄ O ₂	142.2	-0.4	M
68	18.59	Isoliquiritigenin	C ₁₅ H ₁₂ O ₄	256.25	-0.9	G
69	18.65	Fortunellin	C ₂₈ H ₃₂ O ₁₄	592.54	-0.6	M
70	18.82	Diosmetin	C ₁₆ H ₁₂ O ₆	300.26	-0.5	M
71	19.1	Coumaran	C ₈ H ₈ O	120.15	0.7	E
72	19.1	cis-Zimtsaeure	C ₉ H ₈ O ₂	148.16	0.5	S
73	19.48	Elemicin	C ₁₂ H ₁₆ O ₃	208.25	0	E
74	20.28	naringenin	C ₁₅ H ₁₂ O ₅	272.25	-1.8	M
75	20.33	Tricin	C ₁₇ H ₁₄ O ₇	330.29	1	Ph
76	20.41	Apigenin	C ₁₅ H ₁₀ O ₅	270.24	-1.9	M
77	20.75	rosmarinic acid	C ₁₈ H ₁₆ O ₈	360.31	0	M
78	21.32	Neric acid	C ₁₀ H ₁₆ O ₂	168.23	0.5	E
79	21.87	2-Butyl-4-hydroxyanisole	C ₁₁ H ₁₆ O ₂	180.24	-0.6	E
80	22.16	glabrolide	C ₃₀ H ₄₄ O ₄	468.67	-0.8	G
81	22.21	Vestitol	C ₁₆ H ₁₆ O ₄	272.3	-1.6	G
82	22.67	Damasione	C ₁₃ H ₂₀ O	192.3	-0.4	E
83	23.19	Vomifolol	C ₁₃ H ₂₀ O ₃	224.3	-1.7	E

84	23.29	3-(2,4-dihydroxyphenyl)-5-hydroxy-7-methoxy-6-(3-methylbut-2-enyl)-2,3-dihydrochromen-4-one	C ₂₁ H ₂₂ O ₆	370.39	-2.1	G
85	24.1	apioglycyrrhizin	C ₄₁ H ₆₂ O ₁₄	778.92	-5.3	G
86	24.18	Nevadensin	C ₁₈ H ₁₆ O ₇	344.31	0	O
87	24.37	Sigmoidin-B	C ₂₀ H ₂₀ O ₆	356.37	-0.2	G
88	24.37	Glycycoumarin	C ₂₁ H ₂₀ O ₆	368.38	-2	G
89	24.49	Lobetyol	C ₁₄ H ₁₈ O ₃	234.29	-3.3	Pl
90	24.5	Jaranol	C ₁₇ H ₁₄ O ₆	314.29	-1.6	G
91	24.63	5,7-dihydroxy-3-(4-methoxyphenyl)-6-(3-methylbut-2-enyl)-4H-chromen-4-one	C ₂₀ H ₁₈ O ₆	354.35	0.1	G
92	24.71	Tormentic acid	C ₃₀ H ₄₈ O ₅	488.7	-1.4	E
93	24.72	1-alpha-Terpinyl acetate	C ₁₂ H ₂₀ O ₂	196.29	0	M
94	24.81	diosgenin glucoside	C ₃₃ H ₅₂ O ₈	576.76	-2.4	O
95	25.02	Pentanoic acid	C ₅ H ₁₀ O ₂	102.13	-5.6	E
96	25.05	oleic acid	C ₁₈ H ₃₄ O ₂	282.46	0.9	S
97	25.13	Glabrone	C ₂₀ H ₁₆ O ₅	336.34	-0.8	G
98	25.15	glyasperins D	C ₂₂ H ₂₆ O ₅	370.44	0.5	G
99	25.41	Methyl oleate	C ₁₉ H ₃₆ O ₂	296.49	0.5	C
100	25.6	Chokegard	C ₁₆ H ₃₀ O	238.41	-0.7	C
101	25.82	Pomolic acid	C ₃₀ H ₄₈ O ₄	472.7	-1.4	E
102	26.03	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	278.34	0	G
103	26.03	Decyl acetate	C ₁₂ H ₂₄ O ₂	200.32	-3.3	M
104	26.23	methyl tridecanoate	C ₁₅ H ₃₀ O ₂	242.4	0.2	C
105	26.31	Linoleic Acid	C ₁₈ H ₃₂ O ₂	280.44	-5.4	M
106	26.42	aucubigenin	C ₂₀ H ₂₈ O ₂	184.19	-1.8	S
107	26.43	sugiol	C ₂₀ H ₂₈ O ₂	300.43	-1.5	S
108	27.08	Ethyl linoleate	C ₂₀ H ₃₆ O ₂	308.5	-4.2	C
109	27.14	stearic acid	C ₁₈ H ₃₆ O ₂	284.48	0.4	Pl
110	27.39	12-methyltetradecanoate	C ₁₆ H ₃₂ O ₂	256.42	-0.1	G
111	27.43	lutidine	C ₇ H ₉ N	107.15	-1.6	E
112	27.63	myristic acid	C ₁₄ H ₂₈ O ₂	228.37	1.5	E

337 Note:"E": Eriobotrya japonica, "S": Scrophularia ningpoensis, "O": Ophiopogon japonicus, "Pl":

338 Platycodon grandiflorum, "C": Canarium album, "Ph": Phragmites communis, "M": Mentha

339 Haplocalyx, and "G": Glycyrrhiza glabra

340 3.2. PPRFT reduce the inflammatory cells in BALF of asthmatic mice

341 To verify the influence of PPRFT on relief asthma, researchers counted and

342 converted the number of WBCs, neutrophil, and eosinophil percentages in BALF.

Results indicate that (Fig. 3A), the number of WBCs markedly increased in model group in BALF contrast to control group ($p < 0.001$). In contrast to model group, WBCs reduced significantly in the DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups ($p < 0.05$). Results shown that (Fig. 3B, C), in contrast to control group, increased significantly the percentage of neutrophils and eosinophils in model group ($p < 0.001$). The percentage of neutrophils and eosinophils decreased markedly in the DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups contrast with the model group ($p < 0.05$).

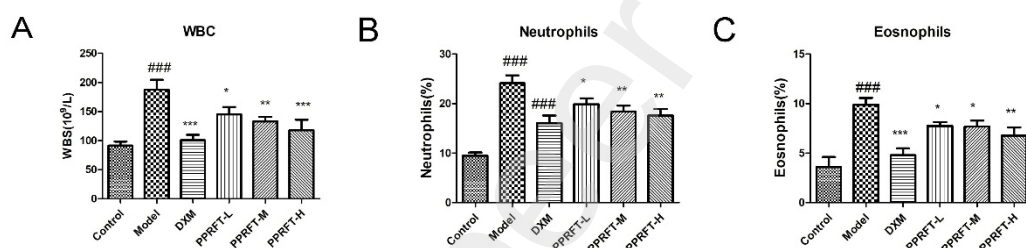


Fig.3. Inflammatory cell in BALF on mice with asthma were measured (A-C).

3.3. Pathological assessment of the lung tissues

Histological change in lung tissues was used to be observed by H&E staining. Compared with control group, the anomalous arrangement, branchial wall thickening, and inflammatory cell permeation were noticed in model group. The treatments DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups relieve the pathological symptoms in lung (Fig. 4A). Similarly, the lung damage grades were improved markedly in model group contrast to control group ($p < 0.001$). However, the lung damage grades were reduced significantly in the DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups contrast with it in model group (Fig. 4B) ($p < 0.05$).

Results were also observed in airway collagen sediment and mucus secretion in

the lung by Masson and PAS staining (Fig. 4A). It shows collagen deposition and mucus production in lung of model group. Less collagen deposition as well as mucus production in lung of DXM and PPRFT groups in comparison with model group. Correspondingly, the semiquantitative result indicated that the Masson score (Fig. 4C) and PAS score (Fig. 4D) were higher remarkable in model group contrast to control group, while DXM and PPRFT groups could decrease significantly the score contrast with the model group ($p < 0.05$).

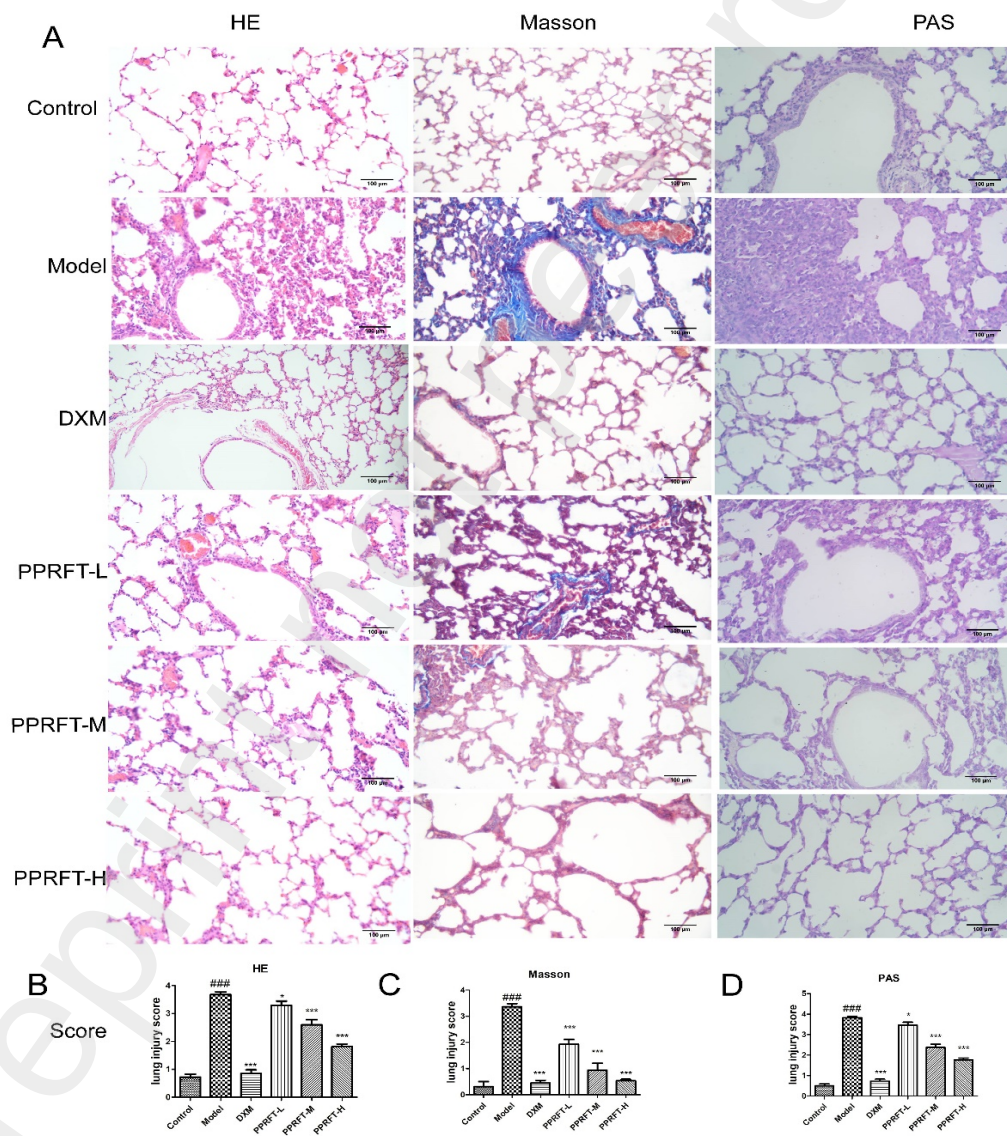


Fig.4. (A) H&E staining, Masson staining, and PAS staining. (B) H&E scores. (C) Masson scores.

(D) PAS scores (magnification: 400×)

3.4. Effect of PPRFT on the inflammatory cytokine in mice with asthma

Higher content of Ig-E and disturbance in Th17/Treg cytokines steady state are key elements of allergic lung inflammation. The result showed an obvious increase of Ig-E activities in serum in model group mice ($p < 0.001$). Conversely, Ig-E activities decreased markedly in the DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups contrast with model group (Fig. 5G) ($p < 0.05$). Next, the analysis about Th17 and Treg cytokines revealed a remarkable aggrandize in expression of IL-1 β , and TNF- α in asthmatic mice ($p < 0.001$). Inversely, in contrast those with model group IL-6, IL-1 β , and TNF- α decline in DXM group, PPRFT-L group, PPRFT-M group, and PPRFT-H group (Fig. 5D-F) ($p < 0.05$). PPRFT treatments lessen the expression of those cytokines in a dose-dependent relationship, far more the therapeutic effect was enhanced with the increase in concentration. Additionally, the expression of IL-1 β (Fig. 5A, B), and TNF- α (Fig. 5A, C) in different groups was detected by IHC. In brief, compared with the model group, total protein expression of IL-1 β , and TNF- α decreased markedly in DXM group, PPRFT-L group, PPRFT-M group, and PPRFT-H group ($p < 0.05$). While, the expressions of IL-1 β , and TNF- α noticeable rise in model group contrast to normal group ($p < 0.001$).

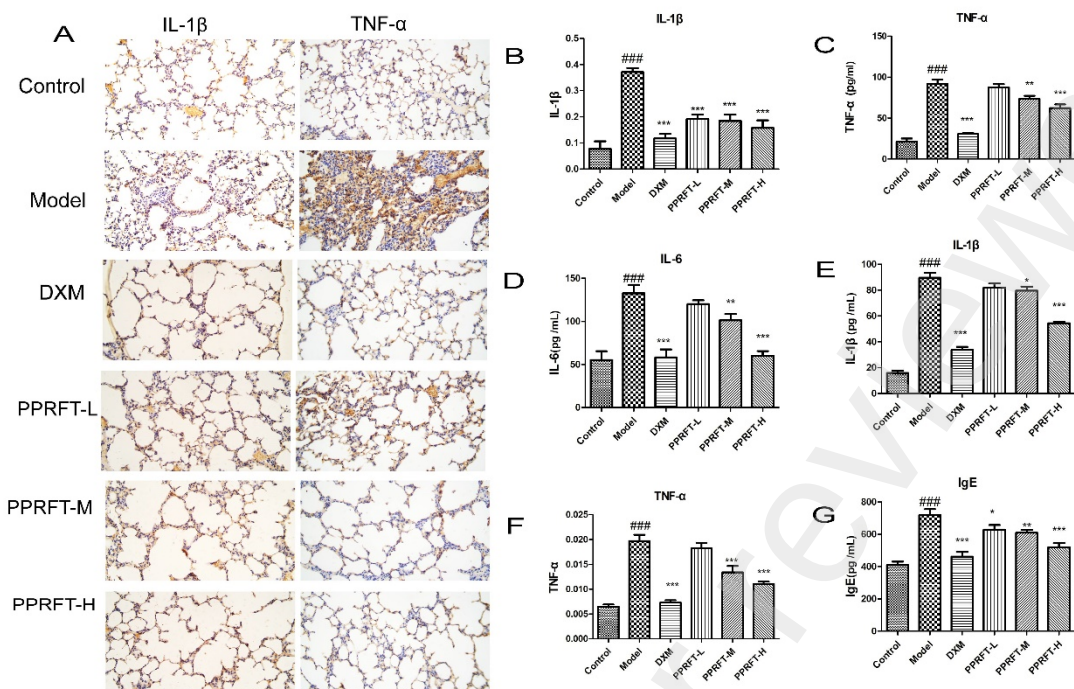


Fig.5. (A) The protein level of IL-1 β , and TNF- α in lung were observed of immunohistochemistry staining (magnification: 400 $\times$). (B-C) Expression score of IL-1 β , and TNF- α by immunohistochemistry staining. (D-F) Levels of IL-6, IL-1 β , and TNF- α in BALF. (G) Levels of Ig-E in serum.

3.5. Effect of PPRFT on oxidative stress in lung tissue in mice with asthma

EPO, NO, SOD, GSH-Px, and MDA activities in lung-homogenate was measured to evaluate PPRFT on oxidative stress, too. (Fig. 6A-E). The result showed that NO, EPO, SOD, as well as GSH-Px levels decrease markedly while MDA augment obvious in model group contrast to control group ($p < 0.05$). Inversely, contrast to those in model group NO, EPO, SOD, as well as GSH-Px activities rise significantly, and MDA activities reduced markedly in DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups ($p < 0.05$). Strikingly, there is a concentration depending on the NO, EPO, SOD, GSH-Px, and MDA activities varying in PPRFT-L, PPRFT-M, and

PPRFT-H groups.

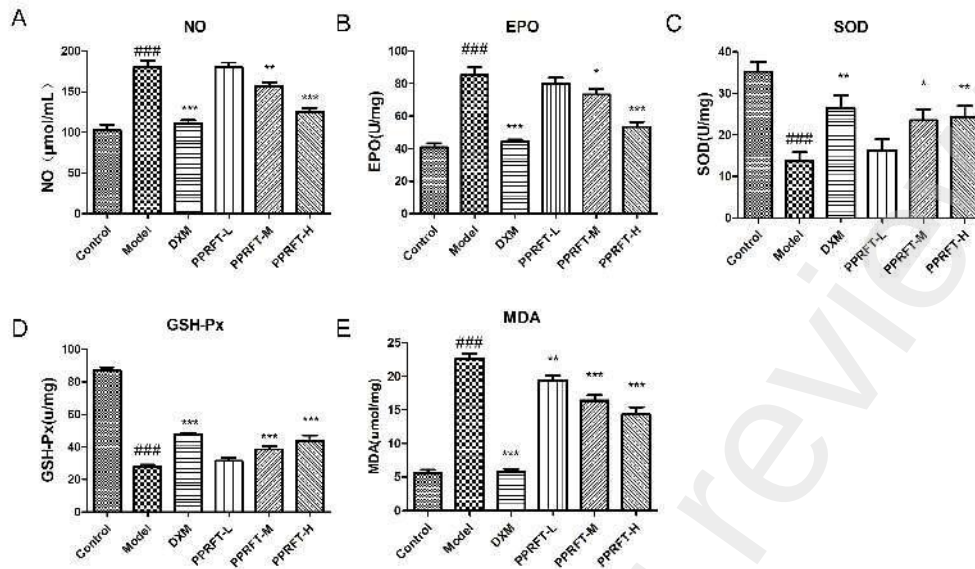


Fig.6. Effect of PPRFT on levels of NO, EPO, SOD, GSH-Px, and MDA (A-E) in lung of asthmatic mice.

3.6. PPRFT treatment suppresses IL-6/JAK2/STAT3/IL-17 signaling pathway

The role of IL-6/JAK2/STAT3/IL-17 signaling is widespread in induction to lung tissue in mice with asthma. To validate the OVA challenge asthma mice in the histopathological analysis, expression of IL-6, Jak2, STAT3, Foxp3, RORγt, IL-17, IL-10, p-JAK2, and p-STAT3 were detected in lung tissue. The influence of PPRFT on asthmatic mice was researched via the expression of IL-6 (Fig. 7A, B), p-JAK2 (Fig. 7A, C), Jak2 (Fig. 7A, D), p-STAT3 (Fig. 7A, E), and STAT3 (Fig. 7A, F) in the lung through immunohistochemistry staining. IHC analysis revealed no obvious difference about total protein activities of JAK2 and STAT3 between each group. However, the positive unit of IL-6, p-JAK2 and p-STAT3 in lung of model group mice was obviously higher than control group. While DXM, PPRFT-L, PPRFT-M, and PPRFT-H groups decreased IL-6, p-JAK2, and p-STAT3 contrast to model group

420 ($p < 0.05$).

421 Moreover, no obvious distinction was detected for protein activities of JAK2 and
422 STAT3 among different groups (Fig. 7G). An obvious distinction was discovered in
423 ratio of p-JAK2/JAK2 (Fig. 7G, M) and p-STAT3/STAT3 (Fig. 7G, N) in lung
424 homogenate of OVA-induced mice was augment remarkable, contrast to control
425 group ($p < 0.05$). Similarly, the expression of IL-6 (Fig. 7G, H), ROR γ t (Fig. 7G, L),
426 and IL-17 (Fig. 7G, J) increased significantly, whereas the expression levels of
427 FOXP3 (Fig. 7G, K) and IL-10 (Fig. 7G, I) decreased markedly in model group,
428 contrast to control group ($p < 0.01$). We can conclude that PPRFT may play a
429 protective role in lung tissue by IL-6/JAK2/STAT3/IL-17 signaling axes.

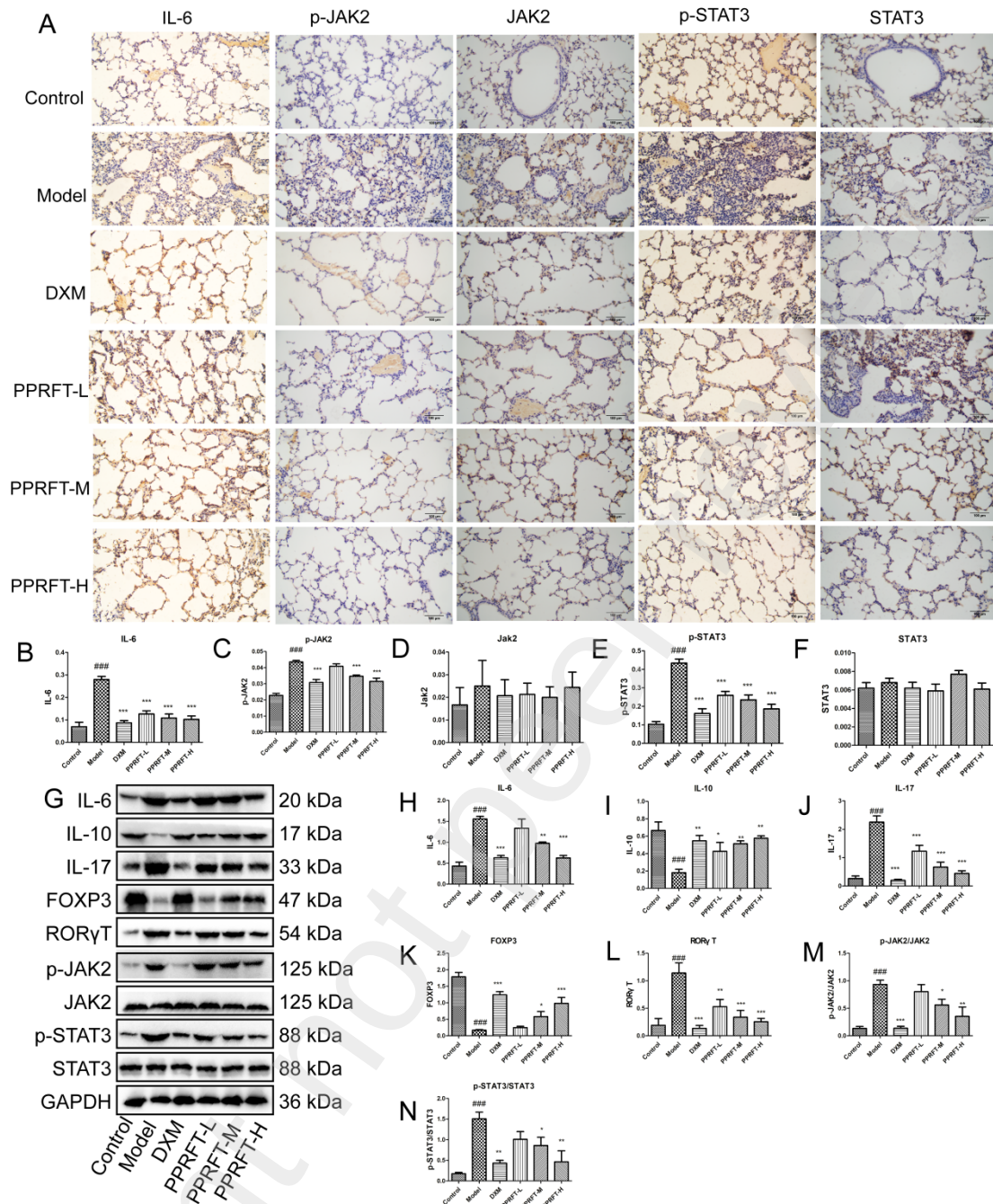


Fig.7. (A) The protein levels of IL-6, p-JAK2, JAK2, p-STAT3, and STAT3 in lung tissue were observed by immunohistochemistry staining (magnification: 400×). (B-F) The expression score by immunohistochemistry staining. (G) Western blot bands of target molecular. (H-N) The expression score by WB.

3.7. PPRFT treatment suppresses PI3K/AKT/NF-κB signaling pathway

IHC showed the effect of PPRFT on the expression of p-PI3K (Fig. 8A, B), PI3K (Fig. 8A, C), p-AKT (Fig. 8A, D), AKT (Fig. 8A, E), and NF- κ B (Fig. 8A, F) in lung tissue. The result indicated that the activities of PI3K and AKT no remarkable among groups, whereas NF- κ B, p-PI3K, and p-AKT were noticeable rise in lung tissue of OVA challenge mice in model group contrast with control group, while DXM, PPRFT-L, PPRFT-M, and PPRFT-H group reduce the level of NF- κ B, p-PI3K, and p-AKT ($p < 0.05$). Besides, in WB results, the protein expression of NF- κ B (Fig. 8G, J), p-PI3K/PI3K (Fig. 8G, I), and p-AKT/AKT (Fig. 8G, H) were rise in model group opposites to control group ($p < 0.05$). DXM, PPRFT-L, PPRFT-M, and PPRFT-H group treatment down-regulated the protein activities of NF- κ B, p-PI3K/PI3K, and p-AKT/AKT in OVA challenge model mice ($p < 0.05$). In brief, we speculate that PPRFT may play an active act in lung tissue by PI3K/AKT/NF- κ B signaling axes.

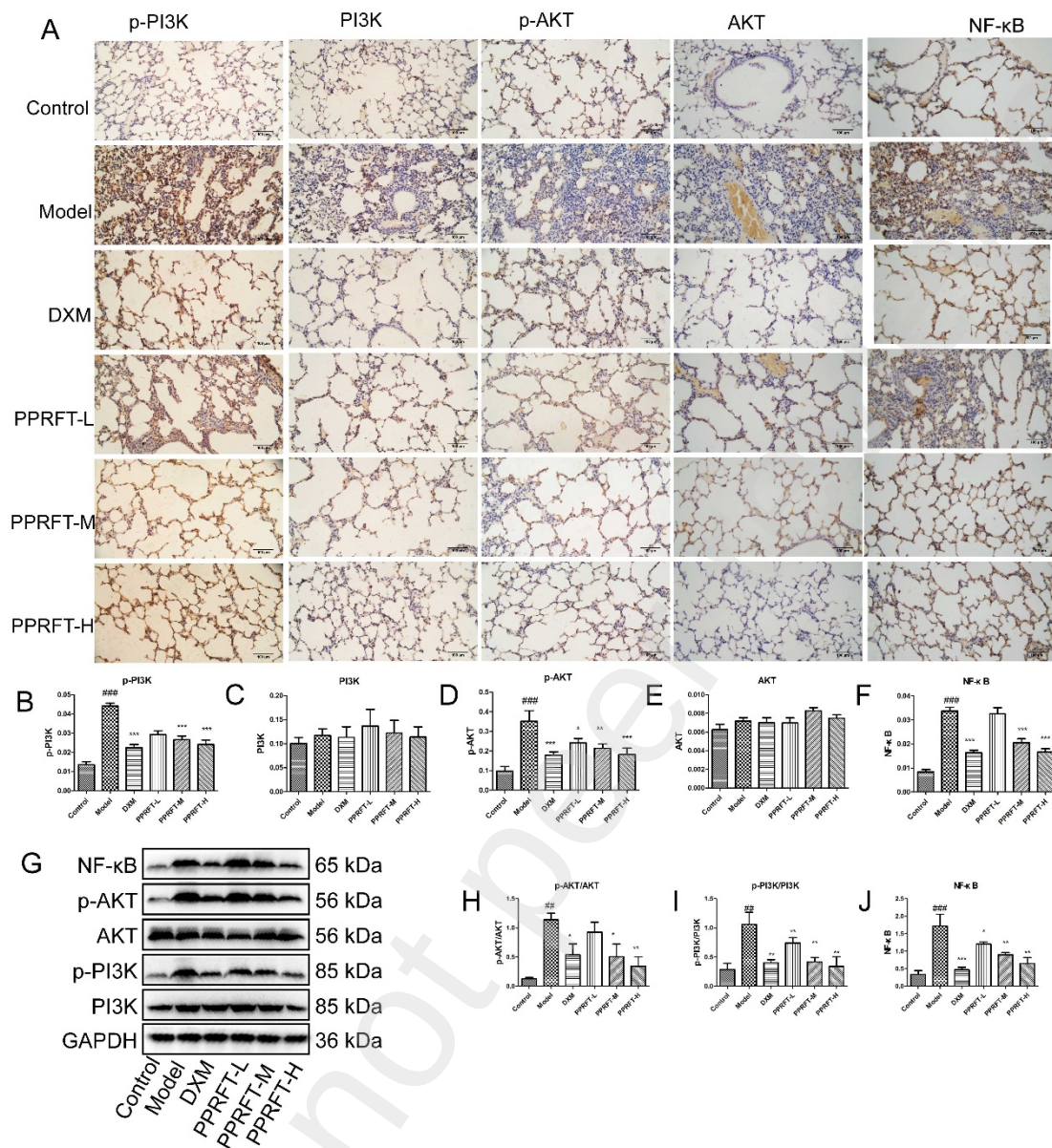


Fig.8. (A) The protein levels of p-PI3K, PI3K, p-AKT, AKT and NF-κB in lung tissue were observed by immunohistochemistry staining (magnification: 400×). (B-F) The expression score by immunohistochemistry staining. (G) Western blot bands of target molecular. (H-J) The expression score by WB.

3.8. Effect of PPRFT on metabolite levels in serum on asthmatic mice

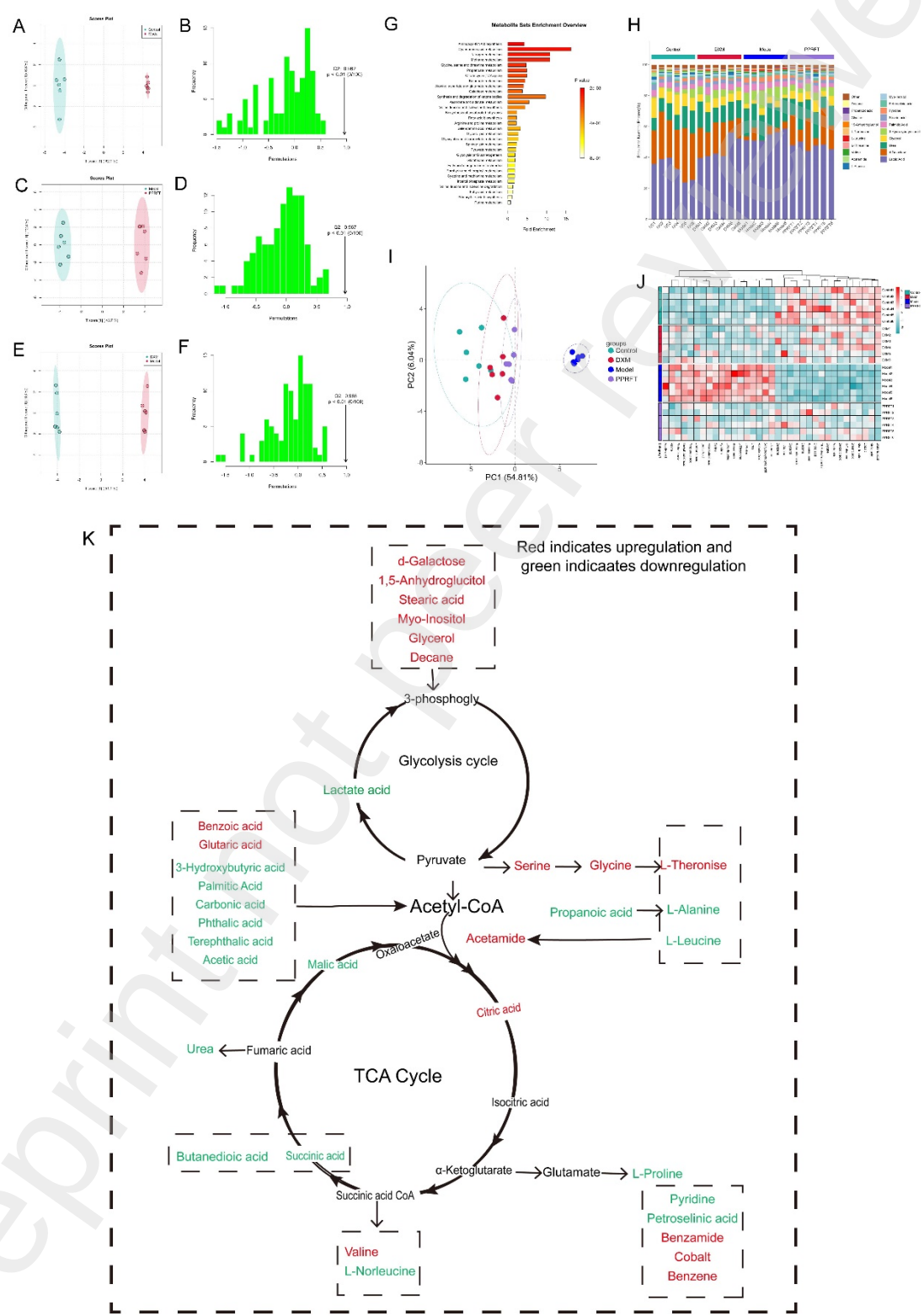
The influence of PPRFT in serum metabolites in OVA-induced mice was studied using the untargeted metabolomics method, which revealed that groups were well

differentiated in PCA. PCA analysis indicated that the length from model to control groups is farther than the length from model to treat groups (Fig. 9I). Subsequently, OPLS-DA was adopted to analysis among control, model, DXM, and PPRFT groups to acquire VIP of each metabolite. The model group had a $R^2 Y = 0.998$ and a $Q^2 Y = 0.962$ contrast with the control group (Fig. 9A, B), while the PPRFT group had an (R^2) $Y = 0.994$ and a (Q^2) $Y = 0.967$ contrast with the model group (Fig. 9C, D), DXM group had an (R^2) $Y = 0.999$ and a (Q^2) $Y = 0.985$ contrast with the model group (Fig. 9E, F). The OPLS-DA results implied the model was stable without over-fitting, implying that the model was suitable for differential metabolite identification.

3.9. Differential metabolites among groups and their comparisons

A percentage accumulation chart (Fig. 9H) and heat map (Fig. 9J) were applied to visual the relative-amounts of differential metabolites between groups and analyze their hierarchical cluster. Results showed that, 35 differential metabolites (a: $p < 0.05$, b: $VIP > 1$, and c: fold change (FC) > 1.2 or $FC < 0.8$ (among each other group compare with control group)) were detected in PPRFT group, as contrast to the model group (Table1)(Xuehua et al., 2021). In contrast to those in control group, standards of Glycine, L-Alanine, Glycerol, Succinic acid, Butanedioic acid, Stearic acid, Myo-Inositol, 1,5-Anhydroglucitol, d-Galactose, Decane, Acetamide, Benzoic acid, Cobalt, Malic acid, and Glutaric acid were downregulated in model group whereas upregulated after treating with PPRFT. Contrast with control group, Lactic Acid, 3-Hydroxybutyric acid, Urea, Valine, Pyridine, L-Threonine, Benzamide, Propanoic acid, Serine, Citric acid, Petroselinic acid, Palmitic Acid, Carbonic acid, Phthalic acid,

478 L-Leucine, Acetic acid, L-Norleucine, Benzene, L-Proline, and Terephthalic acid in
 479 the model group were upregulated. These were downregulated in the PPRFT group.



480
 481 Fig.9. (A, C, E) Scores plots of OPLS-DA between control and model group (A), between model

and PPRFT group (C), and between model and DXM group (E). (B, D, F) Permutation tests of OPLS-DA models between control and model group (B), between model and PPRFT group (D), and between model and DXM group (F). (G) Pathway analysis of differential metabolites in lung. (H) Percentage accumulation chart of the 35 differential endogenous metabolites. (I) Score plot of PCA among control, model, PPRFT and DXM group. (J) Heat map of the 35 differential endogenous metabolites. (K) Metabolic network of the significantly changed endogenous metabolites.

3.10. Pathway analysis of differential metabolites

A map of potential pathways was investigated according to the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes). A total of 31 pathways were discovered based on the relative-amounts of differential metabolites portray above (Fig. 9G), and three metabolic pathways were integrated (Fig. 9K).

Table 2 Differential metabolites of OVA-induced mice after the treat of PPRFT

NO.	Formula	RT [min]	Metabolites	VIP	FC		Trend	
					M vs. C	T vs. M	M vs. C	T vs. M
1	C ₈ H ₂₁ NO ₂ Si ₂	9.835	Glycine	1.229257566	4.4915	6.317	↓###	↑**
2	C ₉ H ₂₂ O ₃ Si ₂	8.273	Lactic Acid	1.095879837	0.75158	0.50404	↑###	↓**
3	C ₁₃ H ₃₃ NO ₃ Si ₃	9.3575	L-Threonine	1.072247296	2.9125	3.5253	↓#	↑*
4	C ₁₀ H ₂₄ O ₃ Si ₂	11.2808	3-Hydroxybutyric acid	1.14350011	0.50418	0.27755	↑###	↓**
5	C ₇ H ₂₀ N ₂ O ₂ Si ₂	14.4685	Urea	1.133107574	0.7108	0.6003	↑###	↓**
6	C ₁₃ H ₃₀ O ₅ Si ₃	13.6972	Malic acid	1.010750451	0.48089	0.46545	↑##	↓**
7	C ₁₂ H ₃₂ O ₃ Si ₃	15.7931	Glycerol	1.098654219	1.4982	2.2807	↓###	↑**
8	C ₁₂ H ₃₁ NO ₃ Si ₃	16.8382	Serine	1.251145152	4.6567	6.9488	↓##	↑*
9	C ₁₇ H ₉ ClF ₃ NO	26.2366	Pyridine	1.009638568	0.3954	0.2812	↑###	↓***
10	C ₉ H ₂₃ NO ₂ Si ₂	18.2414	L-Alanine	1.288366035	0.4845	0.34	↑###	↓***
11	C ₁₂ H ₁₅ NO	16.5538	Benzamide	1.021189497	2.2213	4.2339	↓###	↑*

12	C21H44O2Si	27.9239	Stearic acid	1.06923282	1.5542	2.1102	↓###	↑**
13	C24H60O6Si6	26.879	Myo-Inositol	1.061274474	1.2943	1.5725	↓###	↑**
14	C10H22O4Si2	15.8023	Butanedioic acid	1.111090113	0.3207	0.28314	↑##	↓**
15	C7H15NO3Si	8.0079	Propanoic acid	1.289175891	0.55268	0.35314	↑###	↓**
16	C18H12Br2F4O4	17.7299	Succinic acid	1.193498319	0.51718	0.5007	↑##	↓**
17	C18H40O7Si4	24.0829	Citric acid	1.102535973	1.9332	2.3736	↑##	↓*
18	C18H44O5Si4	24.4341	1,5-Anhydroglucitol	1.123275399	1.7329	1.9525	↓##	↑*
19	C21H42O2Si	27.7063	Petroselinic acid	1.049425753	0.7886	0.68471	↑###	↓*
20	C19H40O2Si	26.1292	Palmitic Acid	1.097663329	0.6604	0.69554	↑###	↓***
21	C22H55NO6Si5	25.2032	d-Galactose	1.027749219	3.4778	6.0092	↓##	↑**
22	C25H47NO2	26.2561	Carbonic acid	1.113953966	0.48496	0.10485	↑###	↓**
23	C25H24O4	33.9733	Phthalic acid	1.107011309	0.35675	0.096792	↑##	↓*
24	C20H38ClNO4	9.9205	L-Leucine	1.179904911	0.62646	0.031725	↑###	↓**
25	C5H10O4	6.1137	Acetic acid	1.007839842	0.55919	0.32779	↑##	↓*
26	C12H29NO2Si2	15.5907	L-Norleucine	1.119450831	0.24328	0.27558	↑###	↓***
27	C21H26OS	14.4677	Benzene	1.035114617	3.1255	7.3431	↑##	↓
28	C12H26	8.0331	Decane	1.259040261	2.8001	3.4308	↓##	↑*
29	C15H21NO3S	23.8002	L-Proline	1.097130089	0.29145	0.13734	↑###	↓***
30	C12H14N2O2	16.1464	Acetamide	1.188207703	2.4246	4.8724	↓###	↑*
31	C15H23NO2	15.7975	Benzoic acid	1.30969759	2.1215	3.4757	↓###	↑*
32	C20H28CoN2	33.9318	Cobalt	1.191321079	4.4592	8.4529	↓###	↑*
33	C11H27NO2Si2	19.9072	Valine	1.05665834	2.5493	4.364	↓###	↑*
34	C14H18O6	23.4493	Terephthalic acid	1.076843607	0.64326	0.39494	↑###	↓*
35	C17H24F8O4	17.3753	Glutaric acid	1.323023655	1.7128	11.925	↓###	↑

4. Discussion

Asthma is a chronic respiratory disease with airflow limitation, trachea inflammation, and reactivity bronchial hyperresponsiveness (G. and H., 2022; Reddel et al., 2021). Crude drugs recently acquired significant widespread in the word by the reason of that their effectiveness, safety, and acceptability (E. et al., 2022; Shizuo et al., 2021). From an ethnomedical perspective, the focus has shifted to respiratory diseases.

Although many medications are used for the treatment of asthma, their efficacies are generally limited, and side effects are common. PPRFT is an in-hospital TCM prescription with a mild and long-lasting effect with few side effects in both short and long-term treatment. This study aims at discovery of its activity of PPRFT on asthmatic mice by systematic pharmacology and metabolomics.

Oxidative stress is a crucial pathological response in asthma, in which long-term trachea inflammation can result to the overproduction of reactive oxygen species (ROS) in lung tissue (Mishra et al., 2018). In addition, surpassing ROS contribute to the damage of oxidative DNA and the lipid overoxidation in lung cells, even damaging airway smooth muscle (Kun et al., 2022). The present study, results showed that PPRFT increased SOD as well as GSH-Px activities and decreased EPO, NO, and MDA levels in lung. It indicated that modulation of antioxidant defense parameters mediated by PPRFT confirmed its protective effect in asthma.

The main features associated with OVA-induced asthma inflammatory cell secretion, increased mucus secretion, tracheal thickening, and collagen deposition (Vijay et al., 2021). OVA-sensitized mice emerged analogous pathological and mitigated by PPRFT administration. This was confirmed for a reduce in IgE standard in serum of PPRFT-treated mice contrast with OVA-induced mice. The decreased IgE excreta is an important target of suppression about inflammatory reaction and effect cells (Hailing et al., 2022). Allergens are known as attract increased levels of IgE, which stimulates immune cell to liberate various inflammatory cytokines (Hee et al., 2022).

Dendritic cells (DCs) assume an effect of "gatekeeper" in congenital and acquired immunity, serving the most efficient antigen presentation in the lung tissue(R et al., 2021). DCs have become a promising tool for modulating antigen-specific immune responses(Schülke, 2018). Th17/Treg immune imbalance has become a focus and hot topic in mediating neutrophil involvement in asthma airway inflammation(Yu-heng et al., 2011). Th17 cells act a vital link in pathogenesis of asthma(Yang et al., 2019). Th17 cell transdifferentiation could induced through exciting antigen-activated T cells with IL-6, IL-1 β , and TNF- α that accelerate phosphorylated JAK2(Akihiro and Tadamitsu, 2010; Liang et al., 2008) and phosphorylated STAT3(Zhou et al., 2016). The phosphorylated STAT3, which is the precondition of the downstream promoter element ROR λ t expression(Shigeru et al., 2014), IL-17 generated via Th17 cells activated inflammation gather in infection location(R and R, 2012). A study reported that STAT3 may combine with IL-17 promoter immediately, it indicated that STAT3 as well as ROR λ t is cooperating management of the transcription of Th17 cells(Chen et al., 2010). Our data turn out that the reaction of IL-6, IL-1 β , and TNF- α in BALF were significantly increased in the OVA groups. The reaction of IL-6, p-JAK2/JAK2, p-STAT3/STAT3, ROR λ t and IL-17 in lung tissue were remarkably increased. Therefore, it is logical to speculate that with PPRFT adjusted after OVA stimulation, inhibiting the phosphorylated JAK2 and STAT3 can into the cell nucleus and restrain its downstream transcriptional regulator ROR λ t, which can remit the differentiation of initial Th17 cells and relieve the attack of asthma. Foxp3, a transcription factor that regulates the Treg phenotype,

appears to act a critical effect in Treg cell regulation(Xu et al., 2017). The Epidemiological investigations indicate that Treg cells, traditionally called "suppressor cells", which has been linked to IL-10 secreted in humans' asthma pathogenesis(Tzu-Hsuan et al., 2016). In this study, PPRFT come to light induce the Treg cell differentiation Foxp3 and increase IL-10 secretion, which is according to the preceding study. PPRFT may alleviate asthma symptoms by inducing the IL-10 secretion and Foxp3 differentiation, then promoting the differentiation of initial T cells to Treg cells. Thus, through the IL-6/JAK2/STAT3/IL-17 mechanistic pathway, the Th17/Treg immune balance recovery might imply a new therapeutic strategy in asthma.

Therefore, inhibition of inflammatory cytokines, and the increase percentage of IL-10 to IL-17 is vital for reducing asthma symptoms(Dhakal et al., 2019; ElSaber et al., 2022). To further validate this, we assessed the effectiveness and the mechanism of PPRFT in attenuating OVA-induced asthma. The results showed that PPRFT could also regulate the PI3K/Akt/NF- κ B signaling pathway and relieve asthma symptoms.

As a result of OVA-challenged, lungs can give rise to inflammation and oxidative stress. In present study, metabolomic analysis of the serum indited that, 35 metabolites have been point as possible target of PPRFT for the therapy of asthma, which are engaged with modulation of trachea inflammation, trachea remodeling, and trachea hyperresponsiveness. Therefore, Galactose metabolism, the TCA cycle, and the metabolism of glycine, serine, and threonine were affected by OVA-induced lung injury.

Carbohydrate substances provide energy through the production of lactate by glycolysis under anaerobic conditions and the aerobic oxidation of sugars (including the citric acid cycle) under aerobic conditions. Lactic acid(Eugene et al., 2013) is the intermediate product of the citric acid cycle. The experimental results showed that the serum levels of lactate acid, citric acid, malic acid, and succinic acid increased in OVA asthmatic mice and were adjusted back after administration with PPRFT in the treatment group(Yang et al., 2017). On the other hand, most carbohydrate substances such as glycerol, stearic acid, Myo-inositol, 1,5-anhydroglucitol, d-galactose, and decane were decreased in the serum of OVA asthmatic mice, indicating that abnormal energy metabolism in OVA asthmatic mice(Mingjie et al., 2015). As it reported, the altered energy metabolites may be produced by abnormal respiratory movements in asthma, especially in hypoxic or inflammatory states(Zhuo et al., 2016). Lactate(Zi-Teng et al., 2016) is an intermediate product of carbohydrate and non-essential amino acid metabolism under anaerobic conditions. Although it is not a major energy production pathway, it has important pathological and physiological implications.

Glucose can be broken down into pyruvate under anaerobic conditions or damaged mitochondria, while pyruvate can be converted into lactate by catalyzing lactate dehydrogenase(Zengjie et al., 2019). The liver and kidneys reintroduce the lactic acid produced by the body's tissues to participate in metabolism(Wang et al., 2019). Stress or exercise elicits high glycolytic rates, lactate reuptake decreases, and lactate accumulation(C et al., 1995; Gelabert-Rebato et al., 2019). Abebayehu D

found increased levels of lactic acid in sera of human asthmatics(Abebayehu et al., 2019). Furthermore, our present display a better positive dependency among lactate, inflammatory cells, and the immunoreaction, accord to the experimental results of Papamichael(Michelle et al., 2021). Malic acid, citric acid, and succinic acid in mitochondrial energy metabolism serve as intermediates in the TCA cycle(Matteo et al., 2022). Our present indicated that citric acid, succinic acid, and malic acid were increased in asthmatic mice serum. Most notably, increased citric acid levels in plasma have been found in asthmatics during exacerbation(Ran et al., 2019). According to previous study that patients with moderate asthma and children with asthma will have a higher level of succinic acid in their serum(Ran et al., 2019). This indicate that the conform to the injury of the TCA cycle in these respiratory diseases.

Glycine, the simplest unnecessary amino-acid, take part in synthesizing many important nitrogenous substances, including purine base, heme, porphyrin ring of cytochrome, creatine, glutathione, and glycocholic acid(Dongqing et al., 2017). Glycine reduces the oxidative stress occurrence through inhibiting the production of ROS(Ilkun et al., 2015). Gutsch et al found that it could reduce mitochondrial swelling, ROS, and lipid peroxidation by reversing the phenotype by replenishing glycine(Annelie et al., 2020). In this study, glycine content in serum of mice in model group was lower than control group, but it increased after PPRFT treatment. Glycine, l-threonine, and serine have antioxidant and anti-inflammatory effects during asthma. Besides, l-alanine and l-threonine have been reported that they participation both immune response and oxidative damage(Chan et al., 2017). In present study, PPRFT

significantly interfered with glycine, serine, and threonine metabolism, suggesting that PPRFT may alleviate asthma symptoms by affecting the metabolism of serine, glycine, and threonine.

The PPRFT treatment reversed aberrant levels of metabolites in OVA mice, it is concerned to Galactose metabolism, the TCA cycle, and the metabolism of glycine, serine, and threonine. These changes of endogenous metabolites indicated that the protected action of PPRFT contrast asthma was systematical.

5. Conclusion

This paper indicated that PPRFT treatment can relieve asthma symptoms through suppressive inflammatory responses, oxidative stress, airway remodeling, and regulating Th17/Treg balance. The mechanism of PPRFT treating of asthma associated with IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF- κ B pathways. Furthermore, the metabolic mechanism of PPRFT for asthma may also be related to Galactose metabolism, TCA cycle, and glycine, serine, and threonine metabolism. Simultaneity, pharmacology, biomechanics, and metabolomics analysis verified the conservation effect of PPRFT contrast asthma and which provided scientific connotation and basis for explaining the objective effect of famous medicine prescription.

Ethics statement

The experiment received approval from the ethics committee of Zhejiang University of Technology (Ethical Number: 20210930072).

CRedit authorship contributions statement

Ji-ping Lan and Ping Wang: Investigation, Validation, Xiao-lu Jie, Zi-rui Luo: Writing – original draft, Formal analysis. Jin Yu, Zhe-ren Tong, and Ji-ping Lan: Methodology, Writing-review & edit-ing, Conceptualization, Supervision, Funding acquisition. Zi-rui Luo: Methodology, Data analysis, Software. Qiao-qiao Li and Jia-hui Wu: Data curation, Software. Pei-shi Feng and Yi Tao: Data curation, Visualization. All authors read and approved the final manuscript.

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.

Data availability

Data will be made available on request.

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655

656 **Figure captions**

657 Fig.1. Animal Experimental design of this study.

658 Fig.2. Elementary particle flow graph chromatogram in positive (A) and negative (B)
659 ion mode of PPRFT analyzed with UPLC-QTOF-MS.

660 Fig.3. Inflammatory cell in BALF on mice with asthma were measured (A-C).

661 Fig.4. (A) H&E staining, Masson staining, and PAS staining. (B) H&E scores. (C)
662 Masson scores. (D) PAS scores (magnification: 400×).

663 Fig.5. (A) The protein level of IL-1 β , and TNF- α in lung were observed of
664 immunohistochemistry staining (magnification: 400×). (B-C) Expression score of
665 IL-1 β , and TNF- α by immunohistochemistry staining. (D-F) Levels of IL-6, IL-1 β ,
666 and TNF- α in BALF. (G) Levels of Ig-E in serum.

667 Fig.6. Effect of PPRFT on levels of NO, EPO, SOD, GSH-Px, and MDA (A-E) in
668 lung of asthmatic mice.

669 Fig.7. (A) The protein levels of IL-6, p-JAK2, JAK2, p-STAT3, and STAT3 in lung
670 tissue were observed by immunohistochemistry staining (magnification: 400×). (B-F)
671 The expression score by immunohistochemistry staining. (G) Western blot bands of
672 target molecular. (H-N) The expression score by WB.

673 Fig.8. (A) The protein levels of p-PI3K, PI3K, p-AKT, AKT and NF- κ B in lung
674 tissue were observed by immunohistochemistry staining (magnification: 400×). (B-F)
675 The expression score by immunohistochemistry staining. (G) Western blot bands of
676 target molecular. (H-J) The expression score by WB.

Fig.9. (A, C, E) Scores plots of OPLS-DA between control and model group (A), between model and PPRFT group (C), and between model and DXM group (E). (B, D, F) Permutation tests of OPLS-DA models between control and model group (B), between model and PPRFT group (D), and between model and DXM group (F). (G) Pathway analysis of differential metabolites in lung. (H) Percentage accumulation chart of the 35 differential endogenous metabolites. (I) Score plot of PCA among control, model, PPRFT and DXM group. (J) Heat map of the 35 differential endogenous metabolites. (K) Metabolic network of the significantly changed endogenous metabolites.

Table captions

Table 1 The identification results of compounds in PPRFT.

Table 2 Differential metabolites of OVA-induced mice after the treat of PPRFT.

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