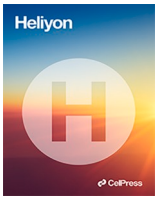
Heliyon 10 (2024) e22971

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Network pharmacology and experimental evidence: MAPK signaling pathway is involved in the anti-asthma roles of *Perilla frutescens* leaf

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ARTICLE INFO

Keywords:
Network pharmacology
Perilla leaves
OVA-Allergic asthma murine model
MAPK pathway

ABSTRACT

Perilla frutescens (PF) leaf is a traditional Chinese medicine and food with beneficial effects on allergic asthma. We sought to elucidate the active compounds, the targets, and underlying mechanisms of PF leaf in the treatment of allergic asthma by using experimental pharmacology and network pharmacology. An OVA-allergic asthma murine model was constructed to evaluate the effect of PF leaf on allergic asthma. And the network pharmacology and western blotting were performed to evaluate its underlying mechanisms in allergic asthma. PF leaf treatment significantly improved the lung function of OVA model mice and mitigated lung injury by significantly reducing of OVA-specific immunoglobulin E in serum, and interleukin 4, interleukin 5 and tumor necrosis factor alpha in the bronchoalveolar lavage fluid. 50 core targets were screened based on 8 compounds (determined by high performance liquid chromatography) through compound-target- disease network. Furthermore, MAPK signaling pathway was identified as the pathway mediated by PF leaf with the most potential against allergic asthma. And the WB results showed that PF leaf could down-regulate the expression of *p*-ERK, *p*-JNK and p-p38, which was highly consistent with the predicted targets and pathway network. In conclusion, this study provides the evidence to support the molecular mechanisms of PF leaf on the treatment of allergic asthma using network pharmacology and *in vivo* experiments.

1. Introduction

Allergic asthma is a chronic allergic inflammatory disease of the airway [1]. It has been reported that more than 300 million people worldwide suffer from asthma, resulting in more than 300,000 hospital admission each year, which is a substantial medical burden [2]. Although the symptoms of most patients with asthma are contingently controlled, repeated attacks cause tremendous psychological burden for patients, and there is no satisfactory treatment. Corticosteroids therapy is still the cornerstone for allergic asthma, but it also

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https://doi.org/10.1016/j.heliyon.2023.e22971
Received 4 March 2023; Received in revised form 22 November 2023; Accepted 22 November 2023
Available online 6 December 2023

has limitations, with severe adverse effects such as generalized immunosuppression [3,4]. Unlike corticosteroids, increasing herbal medicine studies published in recent decades, have shown efficacy in treating patients with asthma without immunosuppressive effects, such as anti-asthma herb medicine intervention (ASHMI) formula and its active compounds isoliquiritigenin and 7,4′- dihydroxyflavone (from Glycyrrhiza uralensis), ganoderic acid B (from Ganoderma lucidum), etc, found in our laboratory [4–7]. Clinical and experimental studies have showed that ASHMI and its active compounds could improve asthma symptom scores, suppress the production of immunoglobulin E (IgE), Th2 cytokines and tumor necrosis factor alpha (TNF-α), and significantly reduce recurring asthma attacks without adverse effects [8–10].

PF is approved for use as medicine and food. Recent studies have shown that PF is highly safe, contains a variety of nutrients, and has anti-inflammatory and anti-allergic effects [11]. Compounds in PF leaf, such as rosmarinic acid [12] and luteolin [13], have been reported to have anti-allergic asthma effects. PF is also often included in clinical prescriptions for the treatment of asthma, such as Chai Pu Decoction [14] and Shen Su Yin [15]. However, the mechanisms underlying the anti-allergic asthma activities of PF have not clearly elucidated.

With strong data statistics and management capabilities, network pharmacology has been widely used to predict the mechanism of action of drugs in diseases [16]. Therefore, this study is based on network pharmacology and bioinformatics technology and comprehensively analyzes the mechanism by which PF acts in allergic asthma. Combinations of experiments conducted to further verify this mechanism provide a definite theoretical reference for the treatment of allergic asthma. This study aims to promote research on the material basis and molecular mechanism of the efficacy of PF leaf. Fig. 1 shows the flow chart of this research.

2. Materials and methods

2.1. Materials and reagents

PF leaf was obtained from Zhang Zhongjing pharmacy (Zhengzhou, Henan, China). Ovalbumin (V and II grade) and $Al(OH)_3$ gels were obtained from Sigma Aldrich (Shanghai, China). ELISA kits for detection of OVA-*s*-IgE, IL-4, IL-5, TNF-α and IFN-γ were purchased from Biosciences (Pharmingen, BD Biosciences, USA).

2.2. Animals

Female Balb/c mice, weighing 18–20 g, were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. (Jinan,

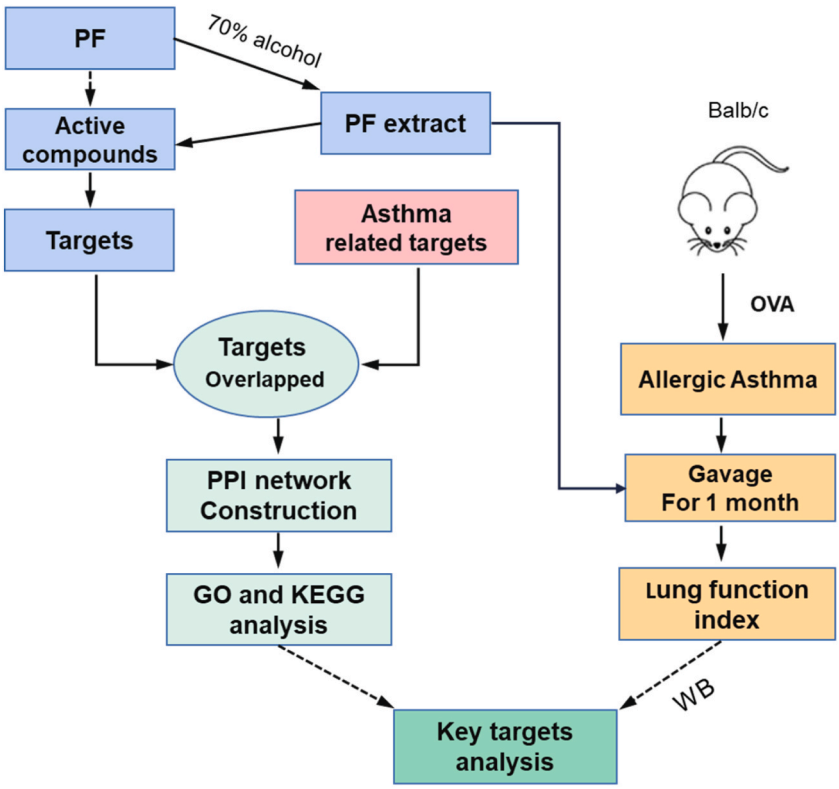

Fig. 1. The workflow of the study on the mechanism of action of PF in treating allergic asthma.

Shandong, China). Quality certificate number and license number are 370726201100464524 and SCXK(Lu) 2019-0003, respectively. Mice were housed in polypropylene cages in a room with a temperature of 23∼25 °C, a relative humidity of 40 %–60 % and a 12-h light/dark cycle. The mice were given free access to food and tap water. All the animal studies were approved by the Animal Care and Use Committee of Henan University of Chinese Medicine and were performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (Approval Num. DWLL 202009103).

2.3. Preparation of PF leaf extract

The crude extracts of PF leaf were obtained according to the method reported in the manuscript with minor modifications [17]. Dried PF was cut into pieces, soaked in a 10-fold volume of 70 % ethanol for 30 min, and extracted twice at 90 °C under reflux. The extracts were concentrated and then freeze-dried. The yield was 25.62 % ± 1.00 % (w/w, dry basis).

The Poroshell-C18 (4.6 × 250 mm, 4 μm, Agilent, American) column was used for gradient elution, and the main compounds in PF were quantitatively analyzed by high performance liquid chromatography (HPLC, waters e2695, Germany) (See supporting information Table S1 for detailed gradient elution protocol). 0.1 % formic acid solution (A) and acetonitrile (B) mixture were used as mobile phases with a flow rate of 1.00 mL/min, and at a wavelength of 330 nm. The quantification was also carried out by integration of the peak using an external standard method.

2.4. Evaluation the pharmacological effect of PF leaf on allergic asthma in vivo

2.4.1. Establishment of OVA-induced allergic asthma mice model

After one week of adaptive feeding, BALB/c female mice were randomly divided into 6 groups (n = 10–12), namely, the naïve group, model group, low-dose PF group (L-PF), medium-dose PF group (M − PF), high-dose PF group (H-PF) and dexamethasone group (Dex). Considering the extraction rate of PF leaf, the dosage used in related studies, and the preliminary experimental results, the dose of PF leaf extract in this study was set as 80 mg/kg, 160 mg/kg, and 320 mg/kg, corresponding to low, medium, and high dose, respectively. And the dose of dexamethasone was set at 0.50 mg/kg according to previous reports [18,19].

The OVA-induced asthma model was established using a previously reported method with minor modifications [6,19]. BALB/c mice were sensitized by intraperitoneal (i.p.) injections of 100 μg OVA (grade V) adsorbed to 2 mg Alum in 200 μL PBS on the 0th, 7th and 14th days. Then, the mice were challenged intratracheally (i.t.) with 100 μg OVA (grade II) in 100 μL PBS on days 21, 28, 35 and 49 (for a total of 4 i.t. challenges). And mice were intragastrically treated with PF or Dex for 4 weeks one day after the first challenge. The naïve group was injected or treated with an equal volume of PBS.

Asthma-symptoms were recorded over 10 min by using a semi-quantitative scoring scale (0–5) as previously described with minor modifications [19]; The criteria were as follows: 0- Asymptomatic; 1- Head and nose disturbance; 2- Flutter, erect hair and decreased activity frequency. 3- Rapid breathing and decreased activity frequency; 4- Mild reaction and nodding breathing; 5- Abdominal breathing.

Mice were fasted on the night of the last i.t. challenge, and the lung function of the mice was measured 24 h after the last i.t. challenge. Then, blood was collected through the orbital venous plexus with a capillary. Half of the mice were subjected to bronchoalveolar lavage with 0.30 mL PBS (washed 3 times) by tracheal intubation, and BALF recovery was approximately 75 %. Finally, the rest mice were anesthetized with urethan, and sacrificed after they lost their response. Lung tissues were collected for follow-up histopathology and mechanism analysis.

2.4.2. Pulmonary function test

Pulmonary function test is an objective method to diagnose and evaluate allergic asthma. Pulmonary function including expiratory time (Te), tidal volume (TV), enhanced pause (Penh) and frequency (F) were detected with a non-invasive pulmonary function tester (EMKA, Guangyuanda, Beijing). Te is a general indicator of lung function, and it represents the time from the beginning of expiration to the end of expiration, reflecting the obstruction of the airway. TV is the lung volume index, which refers to the amount of air inhaled or exhaled during each breath, and it reflects the strength of lung ventilation. Penh is an airway obstruction index that reflects airway resistance. F is a ventilation index, which represents the number of breaths per minute. These four indicators can directly reflect the pathological state of allergic asthma.

2.4.3. Measurement of OVA-s-IgE levels in serum

Blood was collected on the 50th day. OVA-*s*-IgE (Pharmingen, BD Biosciences, American) level was measured by ELISA as previously reported in our laboratory [6].

2.4.4. Measurement of inflammatory cytokines in BALF

BALF was centrifuged at 1200 rpm for 10 min at 4 °C. The supernatant was collected for cytokine analysis. Cytokines of TNF-α (Pharmingen, BD Biosciences, Cat# 555268), IL-4 (Cat# 555232), IL-5 (Cat# 555236) and IFN-γ (Cat# 555138) were determined by ELISA according to the manufacturer's instructions.

2.4.5. Analysis of histopathology

After obtaining BALF, lung tissues were fixed with 4 % paraformaldehyde. Then, the tissues were dehydrated and embedded in paraffin. Embedded tissue samples were sectioned into 4–5 μm thick sections using a Leica RM2235 microtome (Leica, Nussloch,

Germany) and stained with hematoxylin and eosin (H&E).

2.5. Network pharmacology-based prediction

Firstly, the active compounds of PF leaf were identified with the Traditional Chinese Medicines Integrated Database (http://www.megabionet.org/tcmid/, TCMID) [20] and BATMAN-TCM databases (http://bionet. ncpsb. org/batman-tcm/) [21], and the targets of these compounds were obtained from the Similarity Ensemble Approach (https://sea.bkslab.org/, SEA) and PharmMapper database (http://www.lilab-ecust.cn/pharmmapper/). Then, the Gene Cards (https://www. genecards.org) [22] and Comparative Toxicogenomics Database (http://ctdbase.org/, CTD) [23] was used to obtain allergic asthma-related targets. The String database was used to construct protein-protein interaction (PPI) network and "Compound-Target-Disease" network, which were visualized by Cytoscape3.6.1 software. The core targets were further obtained by the analysis of PPI networks. Finally, the DAVID database was used for annotation and to identify the enrichment pathways of the core targets.

2.5.1. Active compounds and corresponding target collection

Network pharmacology was carried out to identify the interactions between compounds and network target proteins. Firstly, the active compounds of PF leaf were obtained from the TCMID and the BATMAN-TCM database. All compounds are screened according to oral bioavailability (OB) and drug-likeness (DL) through the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (https://tcmsp-e.com/, TCMSD) [24]. Secondly, the SEA and PharmMapper databases were searched to identify the targets of the compounds. The targets were mapped to Gene Cards and CTD, and the targets closely related to allergic asthma were screened. Finally, the obtained targets were further screened by searching UniProt (http://www.uniprot.org/) [25].

2.5.2. Construction of PPI network

In this study, to elucidate the interrelationships among potential targets for investigating the actions of PF on allergic asthma. Targets related to allergic asthma were input into String (https://string-db.org/) [26] to obtain relevant information about protein interactions, and a PPI network was visualized via Cytoscape 3.9.0.

2.5.3. Construction of compound-target-disease network

To visualize and interpret the characterization interactions between compounds and targets, a Compound-Target-Disease network was constructed.

2.5.4. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment

GO and KEGG pathway enrichment analyses were conducted on the core targets using DAVID 6.8. The downloaded results were sorted using P values and count values.

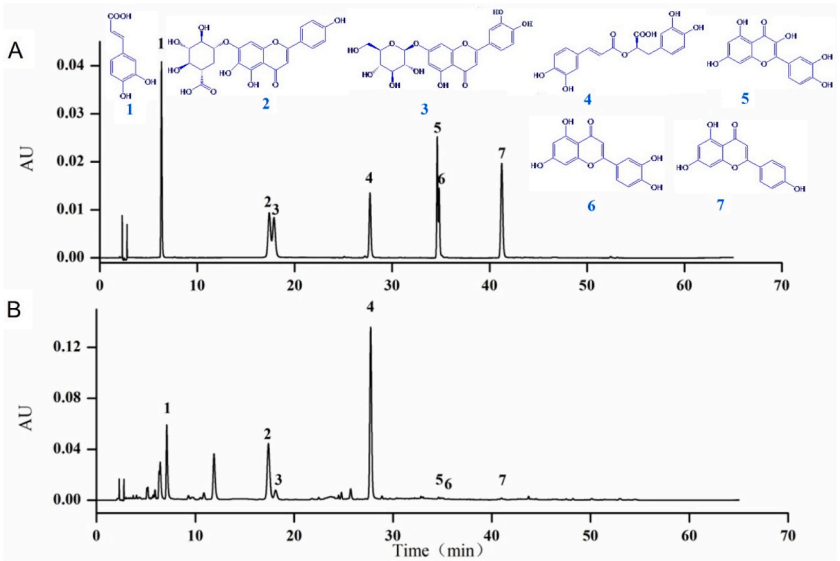

Fig. 2. Chromatograms of mixed standards and PF leaf. (A) is the chromatogram of the mixed standard product, and (B) is the chromatogram of PF leaf extract. 1, 2, 3, 4, 5, 6, 7 represent caffeic acid, scutellarin, luteolin-7-*O*-β-D glucoside, rosmarinic acid, luteolin, quercetin, and apigenin respectively.

2.6. Western blotting analysis

The proteins of lung tissue in each group were harvested, and the protein concentrations were measured with a BCA protein kit (Solar bio biotechnology Co., Ltd. China). The proteins were separated by 10 % SDS-PAGE gel and then transferred to PVDF membranes. The membranes were blocked using 5 % skim milk powder or 2 % BSA blocking solution for 2 h, and then incubated overnight at 4 °C with appropriate primary antibodies. After washing three times with TBST for 15 min each time, the membranes were incubated with secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) for 1 h at room temperature. Immunoreactivity was investigated through ECL method. Images were scanned using an automatic chemiluminescence image analysis system (GE Healthcare Group, American). Quantitative data analysis was performed by Image-Pro Plus 6.0 software (Media Cybernetics, American).

2.7. Statistical analysis

All the data are presented as the mean ± standard deviation (SD). All the statistical analyses were performed using GraphPad Prism 9.1 software (GraphPad Software, Inc., La Jolla, CA). Statistical significance was analyzed by unpaired *t*-test followed by Mann-Whitney test, with p values less than 0.05 considered statistically significant.

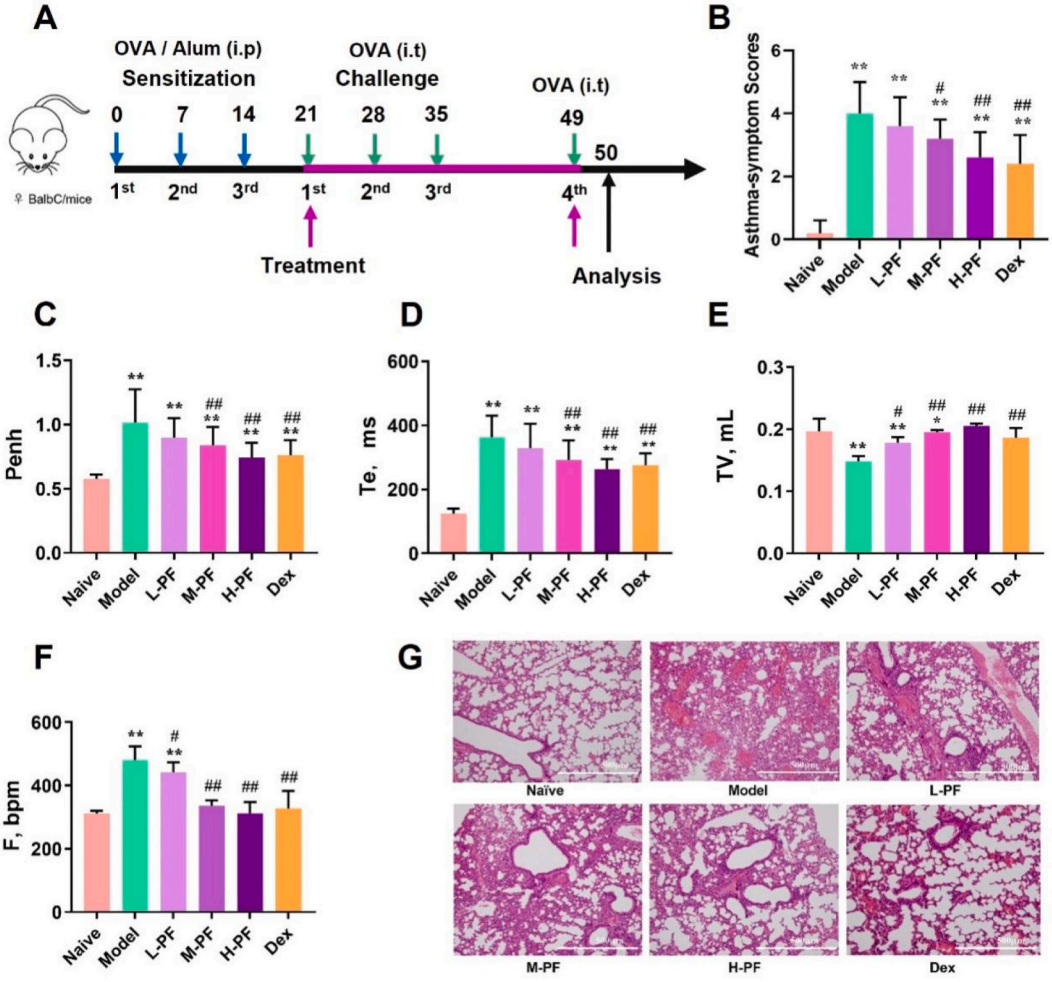

Fig. 3. PF leaf treatment can significantly reduce the asthma symptom score and improve the lung function of OVA-induced asthma model mice. (A) Timeline schematic for the development of OVA-induced asthma mouse model. Mice were sensitized three times intraperitoneally (i.p.) on days 0, 7 and 14, and challenged intratracheally (i.t.) on days 21, 28, 35 and 49 with 100 μg OVA in 100 μL PBS. And mice were intragastrically treated with PBS (Naïve group and Model group), PF leaf or Dex (i.g. once daily) from 21st to 49th days. (B), Asthma symptom scores. The results of pulmonary function test in the six groups (n = 6–8 for per group); (C), Penh; (D), Te; (E) TV and (F) F. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the Naïve group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus the Model group. (G) Lungs were sectioned and stained with hematoxylin-eosin (H&E, 100 × magnification). (Naive) naïve group, (Model) OVA-induced asthma model group, (L-PF) low-dose PF group, (M − PF) medium-dose PF group, (H-PF) high-dose PF group, (Dex) Dexamethasone group.

3. Results

3.1. Evaluation of anti-allergic asthma effects of PF leaf in vivo

3.1.1. HPLC identification of main compounds in PF leaf

Seven major compounds, caffeic acid, scutellarin, rosmarinic acid, luteolin-7-*O*-β-D glucose, rosmarinic acid (RA), luteolin, quercetin, and apigenin, were identified by comparing their retention times with those of standard compounds (Fig. 2 A and B). It can be seen from Fig. 2 that caffeic acid, scutellarin, and rosmarinic acid are the main compounds in PF extract. The average contents of caffeic acid, scutellarin and rosmarinic acid (Figs. 2B, 1 and 2 and 4) were 3.21 ± 0.15 mg/g (equivalent to the content in the raw *perilla frutescens* leaf), 16.79 ± 0.45 mg/g and 33.42 ± 1.10 mg/g, respectively.

3.1.2. Effect of PF leaf extract on pulmonary function

Fig. 3A shows the timeline of an OVA-induced asthma mouse model. OVA sensitization/challenge could cause the typical symptoms of asthma in mice. All the model mice showed significant decreased activity and responses of anaphylaxis such as increased scratching behavior, hypothermia and difficulty breathing. PF leaf treatments significantly reduced the asthma symptom score in a dose-dependent manner (Fig. 3B). Compared with naïve group, the levels of Te, Penh, and F in the model group were significantly increased, while TV level was sharply decreased. PF extract could significantly improve the pulmonary function in mice with allergic asthma in a dose-dependent manner. After treatment with high dose PF extract, TV level was significantly increased by 38.69 % ($p < 0.01$, Fig. 3E), meanwhile the levels of Te, Penh and F were significantly reduced by 27.39 %, 26.89 % and 35.10 % ($p < 0.01$, as shown in Fig. 3C, D, F), and the improvement effect was comparable to dexamethasone (Dex).

The H&E results of lung tissue were consistent with those of asthma symptom score and pulmonary function test. As shown in Fig. 3G, the alveolar structure of mice in the model group almost disappeared, the alveolar interval was significantly thickened, the alveolar wall was severely congested, and the airway lumen was significantly narrowed. And a large amount of inflammatory cell infiltration was observed around the trachea. PF leaf extract could significantly alleviate the pulmonary inflammation (such as above-mentioned bronchial congestion, inflammatory cell infiltration, etc.) of mice in a dose-dependent manner.

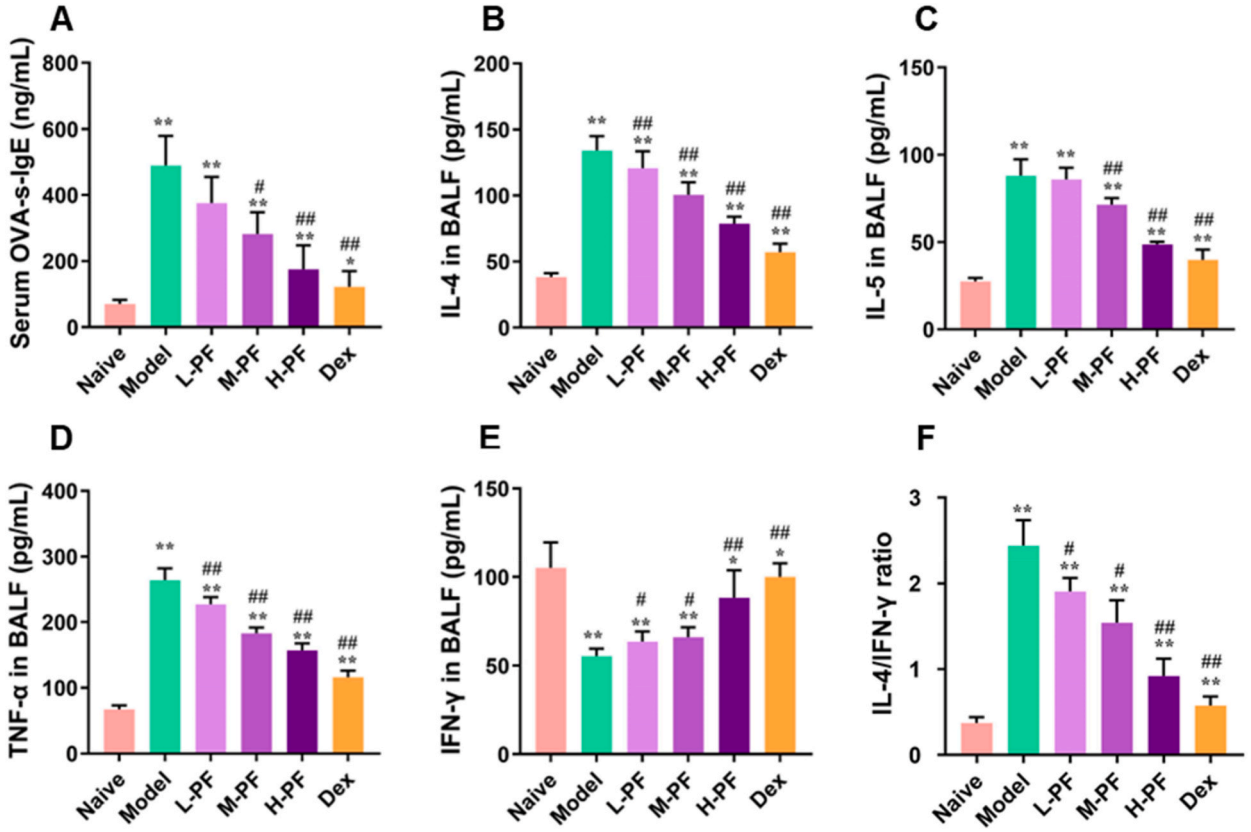

Fig. 4. PF leaf extract significantly suppressed the production of OVA-*s*-IgE and Th2 cytokines. (A) Levels of OVA-*s*-IgE in serum (n = 10), and levels of the cytokines of IL-4 (B), IL-5 (C), TNF-α (D) and IFN-γ (E) in BALF in the six groups (n = 5–6). (F) Fold changes of the IL-4/IFN-γ ratio in the six groups. Data represent the mean ± SD, and statistical analysis was performed by unpaired *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the naïve group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus the Model group.

3.1.3. Effects of PF leaf extract on OVA-s-IgE in serum and inflammatory factors in BALF

The level of OVA-*s*-IgE in serum and inflammatory factor levels of TNF-α, IL-4, IL-5, and IFN-γ in the BALF were estimated by ELISA. As shown in Fig. 4A, compared with naïve group, serum OVA-*s*-IgE level in the model group was sharply increased ($p < 0.01$), indicating that the allergic asthma model was successfully established. In comparison with model group, L-PF, M − PF, H-PF significantly decreased OVA-*s*-IgE levels by 23.23 %, 42.37 % ($p < 0.05$), and 64.18 % ($p < 0.01$), respectively.

In the model group, the levels of IL-4 (Fig. 4B), IL-5 (Fig. 4C) and TNF-α (Fig. 4D) cytokines in the BALF were significantly increased ($p < 0.01$) and the level of IFN-γ (Fig. 4E) significantly decreased ($p < 0.01$). PF leaf treatment significantly suppressed the Th2 immune response (Fig. 4F) in mice with allergic asthma. Compared with the model group, H-PF, M − PF, and L-PF reduced the level of IL-4 inflammatory cytokine in BALF by 41.32 %, 25.08 % and 9.97 %, respectively. TNF-α cytokine was decreased by 40.54 %, 30.68 % and 14.00 %, respectively. IL-5 cytokine was decreased by 44.72 %, 18.87 % and 2.50 % respectively. While IFN-λ level was increased by 59.66 %, 19.28 %, and 14.95 % ($p < 0.05$, $p < 0.01$), respectively.

3.2. Network pharmacology-based analysis

3.2.1. Active compounds in PF leaf

Based on the TCMID, BATMAN-TCM database and together with the seven detected compounds, 36 active compounds in PF were initially identified. The chemical components of PF were further screened from TCMSP, and OB ≥ 30 %, DL ≥ 0.18 were selected by according to the previous report [24]. In addition, caffeic acid, scutellarin, rosmarinic acid, apigenin and astragalin were also chosen, which didn't meet the conditions but had significant pharmacological activities. Basic information of the active compounds is shown in Table S2.

3.2.2. PPI network analysis

A total of 558 PF-related targets were obtained via the SEA and Pharm Mapper databases. In addition, 2326 targets in allergic asthma were identified through Gene Cards and CTD databases. After overlapping the above targets, 254 targets were obtained (Fig. 5A). Then these 254 targets were imported into the STRING 11.0 database to obtain a PPI network file. The higher the degree value of the target node, the more important it is considered to be in the network, thus top 50 targets nodes were chosen as core targets for further analysis, and its PPI network was visualized by the Cytoscape 3.9.0 (Fig. 5B).

3.2.3. Construction of compound-target-disease network

To fully understand the molecular mechanisms of PF leaf in allergic asthma, a compound-target-disease network (Fig. 6) were constructed. The network uncovers the underlying interactions between PF and allergic asthma, where different shaped nodes represent the compounds, targets, disease, and herb, respectively, and the interactions between them were signified by edges. The importance of a node was marked by its size, which was defined as the number of edges associated with the node. The compounds with a degree value ≥ 10 were successively scutellarin (degree = 37), luteolin (degree = 35), apigenin (degree = 34), caffeic acid (degree = 33), rosmarinic acid (degree = 33), quercetin (degree = 33), luteolin-7-*O*-β-D glucoside (degree = 30), and beta-sitosterol (degree = 21). Most of the targets in the network have been reported to play important roles in the pathogenesis of allergic asthma. The higher the degree of targets in the network, the more important they are considered to be, and they may play the most vital roles in regulating

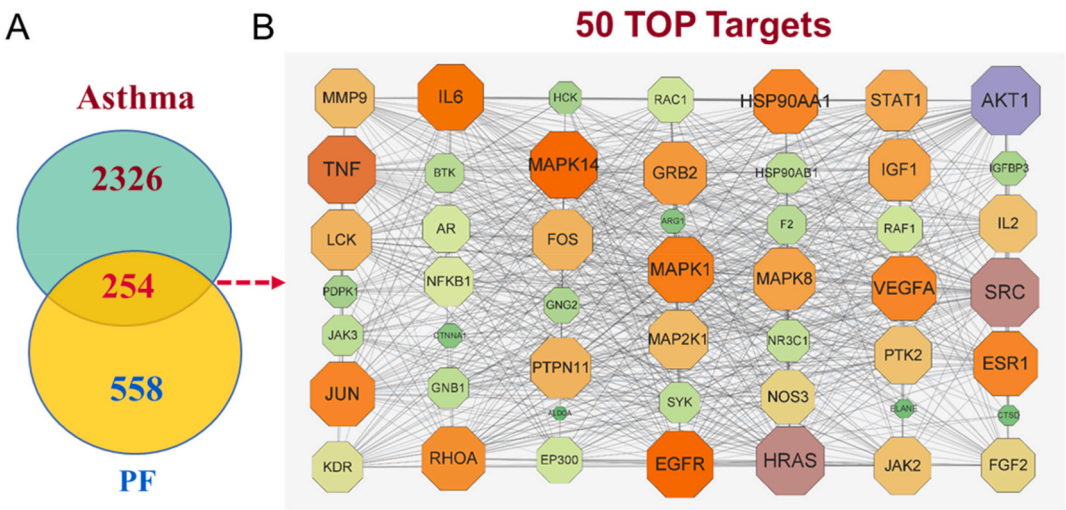

Fig. 5. Identification of the therapeutic targets. (A) Venn diagram showing the number of targets predicted. There are 254 targets for PF and Asthma. (B)The PPI network of 50 targets. Bright orange (or violet) represents key genes, the size of the octagonal circle is related to its – Log(*p*) of genes. Having a more multilateral target indicates that there are more targets connected to it, thus suggesting the target has a more central role within the network.

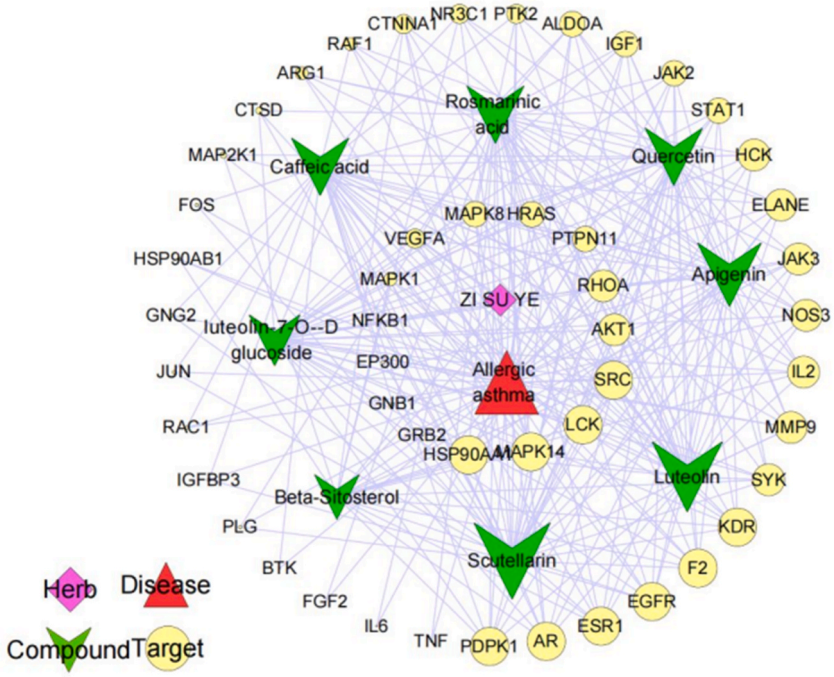

Fig. 6. Compound-Target-Disease network for PF leaf extract on asthma treatment. The size of a node is proportional to its degree value. Bright yellow represents key targets, the size of a circle is related to its – Log(p) of targets. Having a more multilateral target indicates that there are more compounds and targets connected with it, thus suggesting the target has a more central role within the network.

allergic asthma. Among them, MAPK14, AR, ESR1, F2, KDR, LCK, SRC, EGFR, HSP90AA1 and PDPK1 were the most critical targets hit by all compounds.

3.2.4. GO pathway enrichment analysis

To reveal its underlying pharmacological mechanisms of PF leaf in allergic asthma, the above 50 core targets were entered into the DAVID 6.8 database for GO enrichment analysis. The top 20 GOs with a *p*-values less than 0.01 were analyzed, as shown in Fig. 7. Most targets are found to be highly enriched to several biological processes, such as the positive regulation of transcription from RNA polymerase II promoter, signal transduction, positive regulation of GTPase activity, inflammatory response, and innate immune response. Studies have found that the transcription of RNA polymerase II is associated with the inflammatory response mediated by macrophages [27]. Particularly, the positive regulation of transcription from RNA polymerase II promoter with core targets, such as IL-2, IL-6, TNF and NFκB1, is an important regulatory in immunity and inflammation. The results indicate that the mechanisms of PF in allergic asthma were associated with the immune/inflammatory cascade.

KEGG pathway enrichment analysis of the core targets was performed with the DAVID 6.8 database and a total of 104 pathways

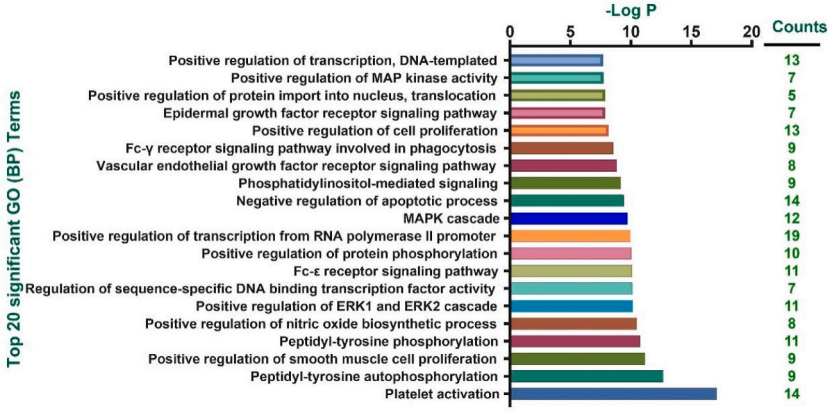

Fig. 7. GO biological process analysis of the core targets. Y-axis: top 20 biological processes relevant to the enriched targets; X-axis: gene counts.

were obtained. The top 20 most significant pathways and core targets in the pathway are listed in Fig. S1. Most pathways are strongly associated with the immune, inflammatory, airway remodeling, endocrine and metabolism. Among them, MAPK signaling pathway, which is an important transmitter of signals from the surface of the cell to the inside of the nucleus [28], might play an important role in the treatment of asthma with PF. Further analysis found that the MAPK pathway has a cross relationship with the other 19 pathways, and the three main proteins, MAPK1 (ERK), MAPK8 (JNK) and MAPK14 (p38 MAPK) are core proteins, so the MAPK pathway was selected as the research pathway.

3.3. Effects of PF leaf on the core targets

Considering the pathogenesis of allergic asthma, the biological activities of potential compounds in PF leaf and the results of GO and KEGG pathways enrichment, we speculate that the MAPK pathway is a key pathway due to its cross-correlation with 19 other pathways. Coincidentally, the three major proteins acting on this pathway were also the core targets of the previous analysis, namely ERK, JNK and p38 MAPK. Therefore, the above three proteins were selected for western blotting verification.

Compared with naïve group, ERK, JNK and p38 protein expression in the lung tissues in other groups (Model, PF or Dex) had no significant difference ($p > 0.05$) (Fig. 8A). However, the protein expression of pERK, pJNK and p-p38 in lung tissue of model group were significantly increased ($p < 0.05$, $p < 0.01$ and $p < 0.01$) (Fig. 8A). While both M − PF and H-PF treatment could significantly decrease the phosphorylation of the above-mentioned proteins ($p < 0.05$, or $p < 0.01$) in a dose-dependent manner, and their quantification results are shown in Fig. 8B, C and D.

4. Discussion

PF is a popularly used traditional Chinese medicine which is clinically used to treat cough, colds, allergies, vomiting, depression, tuberculosis, chronic gastritis and other diseases [29,30]. Studies have reported that PF could suppress the Th2 responses and augment T-bet activity to inhibit systemic allergic reactions [31,32]. Similarly, daily oral PF supplements have preventive and therapeutic effects on asthma [33]. Interestingly, the leaf, stem, and seed of PF herb have been equally commonly used since Song dynasty. However, the active compounds of PF leaf and underlying molecular mechanisms are poorly understood. In this study, we employed

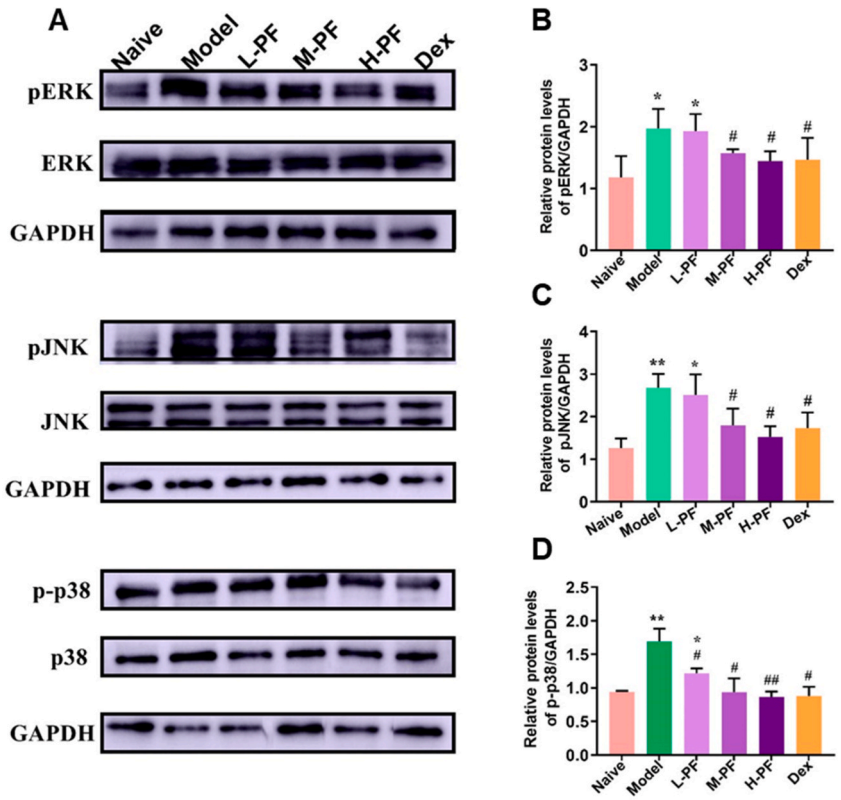

Fig. 8. PF leaf extract inhibited the protein expression of pERK, pJNK and p-p38. (A) The expression levels of ERK, JNK and p38 MAPK proteins in lung tissue in each group; (B) Phosphorylated ERK quantitative analysis; (C) Phosphorylated JNK quantitative analysis; (D) Phosphorylated p38MAPK quantitative analysis. The values are expressed as the mean ± SD (n = 5 for per group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the naïve group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, versus the Model group).

network pharmacology and, for the first time, elucidated the possible mechanism by which PF leaf acts on asthma and the potential compounds involved.

Among the 8 compounds involved in the material basis of PF for the treatment of allergic asthma, most have been reported having anti-allergic asthma effect. Quercetin can inhibit allergic asthma by promoting Th1/Th2 balance [34]. Luteolin can significantly inhibit arachidonic acid-induced ear edema and oxazolone-induced allergic edema, and has good anti-inflammatory and anti-allergic effects [35]. Rosmarinic acid can inhibit eosinophilic airway inflammation in mice caused by mite allergens, and reduce the expression of local eosinophilic chemokines and the production of allergen-specific IgE [36]. Previous studies have found that apigenin treatment significantly alleviates airway hyper-responsiveness in an OVA-sensitized mice, and reduced the percentage of eosinophils and neutrophil infiltration in the BALF and lung tissue [37,38]. Scutellarin has favorable antioxidant, anti-inflammatory and anti-fibrotic effects [39]. Caffeic acid is a polyphenol organic acid compound that can reverse LPS-induced anxiety behavior via regulating LPS-TLR signaling, reducing oxidative stress and directly inhibiting the inflammatory cascade [29,40]. β-sitosterol significantly increased the tidal volume (TV), decreased the respiration rate, and decreased eosinophilic and neutrophil counts in blood and BALF in OVA-sensitized guinea pigs [31,41]. The above results suggest that the pharmacological effects of the 8 compounds are highly overlapping. Indeed, the results of network pharmacology also show that the core targets interact with multi-compounds, indicating that targets with highest degrees may be the central signaling molecules for PF action on OVA-induced allergic asthma. MAPK14, MAPK8 and MAPK1 are the most important members of the mitogen-activated protein kinase (MAPK) family. MAPK14 (p 38), which could aggravate airway obstruction by inducing epithelial cells to secrete mucus and goblet cells proliferation [42], was targeted by all compounds. MAPK8 (JNK, degree = 6) has been reported to involved in epithelial-mesenchymal transition and airway remodeling [43]. While MAPK1 (ERK, degree = 4) regulates the biosynthesis of pro-inflammatory and anti-inflammatory cytokines to participate in airway inflammation [44]. KEGG analysis provided more details of pathways, the top 20 pathways could be broadly divided into five categories based on their main biological functions. They are airway inflammation-related pathways (P1, P3, P5, P12 and P19), immune-related pathways (P7, P16, P13 and P17), airway remodeling related pathways (P2, P4, P14 and P15), endocrine and metabolism-related pathways (P6, P9, P11 and P18), and other pathways (P8, P10 and P20). Interestingly, almost all of these pathways are related to the MAPK pathway.

To validate the results of network pharmacology, we established a mouse model of OVA-induced allergic asthma, and we performed a pleiotropic and systemic evaluation of PF on the above aspects by poly-pharmacology, including protection of lung tissue , modulation of immunity, and reduction of inflammation.

Mice in each group were placed in a non-invasive lung monitor to test their lung function. Although the error bar in some parameters were a little large, they can still reflect the actual lung function status of the mice in each group to a certain extent (Fig. 3B–E). Compared with the model group, PF leaf can significantly improve the lung function of allergic mice, and the H&E results of pathological section also confirmed the therapeutic effect of PF leaf on allergic asthma mice. As shown in Fig. 3F, the integrity of alveolar structure of PF- treated mice significantly improved, the congestion of alveolar walls and the infiltration of inflammatory cells around the trachea was significantly reduced, and the above changes were dose-dependent. To further evaluate the protective effect of PF leaf on allergic mice, we evaluated levels of OVA-specific IgE in serum and Th2 type cytokines in BALF. The inhibitory effects of PF leaf on IL-4, IL-5, TNF-α in BALF and OVA-sIgE in serum, as well as on the elevation of IFN-γ in BALF, suggest that PF leaf has the role of reducing the inflammatory response and modulating immunity. Network pharmacology analysis showed that PF can alleviate allergic asthma by regulating multiple pathways including inflammation, Th2 immune response, airway remodeling, endocrine and metabolism. Since the MAPK pathway is a terminal pathway and has a cross-correlation effect with other pathways, three main proteins in this pathway, namely ERK, JNK and p38 MAPK, were finally selected for verification. Western-blot analysis showed that the expression of *p*-ERK, *p*-JNK and p-p38 could be significantly down-regulated by medium and high doses of PF leaf. A limitation in the study is the absence of adding inhibitors of ERK, JNK, and p38 to further validate the effect of PF leaf on them. Nevertheless, these results suggest that PF leaf plays an important role in inhibiting inflammation and regulating Th2/Th1 immune balance in allergic mice, validating the biological function of predicted MAPK signaling, and further confirming the feasibility of network pharmacology in predicting the mechanisms by which drugs act on disease targets.

Mounting evidence indicates that these compounds in PF leaf have the pharmacological activities of regulating immunity, inhibiting the production of inflammatory cytokines, antioxidation and anti-fibrosis, all of which contribute to the treatment of allergic asthma. In this study, we identified the seven compounds from the PF extract, with rosmarinic acid, scutellarin, and caffeic acid being the three compounds with the highest content in sequence. A recently published paper reported that rosmarinic acid was screened from *Perilla frutescens* Britton as the bioactive compound targeting β2-adrenergic receptor with a binding constant $2.95 \times 10^4\ M^{-1}$ [45]. And one similar study confirmed that roseoside, vicenin-2 and rosmarinic acid compounds which were identified from *Perilla* leaves by using Syk-conjugated beads alleviate allergic airway inflammation by synergistically targeting on syk pathway [18]. Based on these studies, we can assume that rosmarinic acid possibly is one of the key components of PF leaf in the treatment of allergic asthma, and the effect of PF leaf on alleviating allergic airway inflammation is a consequence of the collective action of these compounds. Another limitation lies in this study that it does not clearly elucidate how these compounds exert synergistic effects.

5. Conclusions

In summary, PF leaf has a definite therapeutic effect on allergic asthma. PF leaf extract can significantly improve the lung function and alleviate lung inflammation and allergic symptoms in a dose dependent manner in mice with allergic asthma. From the results of experimental pharmacology and network pharmacology, it can be concluded that the mechanism of PF leaf extract in the intervention of allergic asthma is through the regulation of airway inflammatory and immune signal pathways, and is closely associated with the

inhibition of ERK, JNK and p38 phosphorylation on MAPK pathway.

Data availability statement

The data supporting the conclusions of this article will be made available by the author without undue reservation.

CRediT authorship contribution statement

Mingzhuo Cao: Writing - review & editing, Project administration, Data curation, Conceptualization. **Mengling Zhan:** Writing - original draft, Methodology, Investigation, Data curation. **Heyun Jing:** Investigation. **Zeqian Wang:** Investigation. **Yuan Wang:** Investigation. **Xiumin Li:** Project administration, Methodology, Conceptualization. **Mingsan Miao:** Supervision, Project administration.

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.

Acknowledgment

This study was supported by the grants including Henan Province Science and Technology Research Project (222102310662), and Henan University of Chinese Medicine Doctor Project (BSJJ2022-13), and Henan Outstanding Foreign Scientist Project (GZS2019006). We thank AJE company for checking our manuscript for language and grammar.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22971.

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