

# Leonurine attenuates OVA-induced asthma via p38 MAPK/NF- $\kappa$ B signaling pathway

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## ABSTRACT

Leonurine (Leo) is a natural alkaloid extracted from *Herba leonuri*, which has many biological activities. However, whether leonurine has a protective effect on asthma remains unknown. The purpose of this study was to investigate the protective effect of leonurine on asthma. We evaluated its therapeutic effect and related signal transduction in LPS-induced RAW264.7 cells and OVA-induced asthmatic mice. In addition, we used network pharmacology, molecular docking and molecular dynamics simulation to verify the experimental results. In LPS-induced RAW 264.7 cells, leonurine significantly reduced the production of TNF- $\alpha$  and IL-6, and inhibited the activation of p38 MAPK/NF- $\kappa$ B signaling pathway. In OVA-induced asthmatic mice, leonurine decreased the number of inflammatory cells in the bronchoalveolar lavage fluid (BALF), particularly neutrophils and eosinophils. Leonurine also reduced the contents of IL-4, IL-5, IL-13 in the BALF and OVA-IgE in the serum. Leonurine remarkably improved OVA-induced inflammatory cell infiltration and significantly inhibited mucus overproduction. In addition, leonurine inhibited the activation of p38 MAPK/NF- $\kappa$ B signaling pathway in the lung tissues of asthmatic mice. Network pharmacology suggested that p38 MAPK $\alpha$  was a potential target of leonurine in the treatment of asthma. Molecular docking and molecular dynamics simulations indicated that leonurine could stably bind to p38 MAPK $\alpha$  protein. In summary, leonurine attenuated asthma by regulating p38 MAPK/NF- $\kappa$ B signaling pathway.

## 1. Introduction

Asthma is a recurrent airway inflammatory disease characterized by airway inflammation and airway hyperresponsiveness (AHR), with clinical manifestations of wheezing, cough and shortness of breath [1–4]. Currently, the incidence of asthma is increasing due to the increase of allergens such as pollen, indoor dust, environmental pollutants and toxic particle. Glucocorticosteroids are considered to be the best medicine for asthma, but long-term intake of glucocorticosteroids can cause various adverse effects. Therefore, it is necessary to identify effective therapies to manage asthma.

Th2 cells play a crucial role in asthma. Over-activated Th2 cells produce excess cytokines IL-4, IL-5 and IL-13, exacerbating asthma [5,6]. IL-4 induces B cells to secrete IgE. IL-5 induces the survival, differentiation, growth and recruitment of eosinophils. IL-13 can promote the infiltration of eosinophils into lung tissues, directly leading to airway inflammation and airway hyperresponsiveness [7]. Therefore, inhibition of Th2 cells activation is one of the main therapeutic

strategies for asthma.

Many studies have shown that p38 MAPK is a key signaling molecule that drives the pro-inflammatory response in asthma. p38 MAPK can induce the activation of Th2 cells, thus promoting the excessive secretion of Th2 cytokines [8–10]. There are four isoforms of p38 MAPK (p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$ , p38 $\delta$ ), and p38 $\alpha$  has become a promising target for drug development because of its ability to reduce the secretion of cytokines in chronic inflammatory diseases, including asthma [11]. p38 MAPK acts upstream of the NF- $\kappa$ B signaling pathway in OVA-induced asthma [12,13].

In recent years, herbal monomers have received more and more attention because of their good pharmacological effects. Many herbal monomers such as salidroside, luteolin, baicalin and astragalus membranaceus are of great value in the treatment of asthma [14]. Leonurine (Fig. 1) is a natural alkaloid extracted from *Herba leonuri*, which has many biological activities, such as anti-inflammation, angiogenesis promotion, anti-apoptosis and cardioprotection [15]. Studies have shown that leonurine has a good therapeutic effect on a variety of

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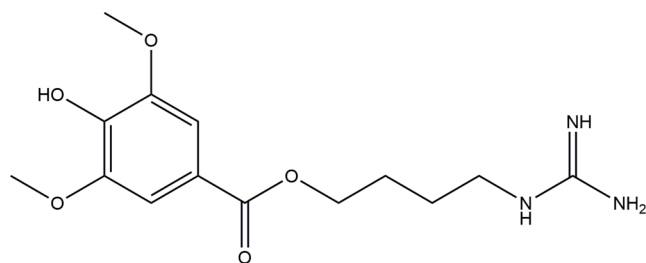


Fig. 1. The structure of leonurine.

inflammatory diseases. Leonurine could treat gouty arthritis through inhibiting COX-2, 5-LOX and mPGES-1 [16]. However, whether leonurine has a protective effect on asthma remains unknown. The purpose of this study was to investigate the protective effect of leonurine on asthma.

## 2. Materials and methods

### 2.1. Drugs and reagents

Leonurine (Leo, purity  $\geq 98\%$ ) was obtained from Nanjing Spring & Autumn Biological Co., Ltd. (Nanjing, China). Aluminum hydroxide [Al(OH)<sub>3</sub>] was obtained from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA) and ovalbumin (OVA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (Dex) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). NO assay kits and BCA protein detection kits were purchased from Beyotime Biotechnology (Shanghai, China). Mouse TNF- $\alpha$ , IL-6, IL-4, IL-5, IL-13 and OVA-IgE enzyme-linked immunosorbent assay (ELISA) kits were obtained from Shanghai FANKEW Industrial Co., Ltd. (Shanghai, China). Anti-bodies against p-NF- $\kappa$ B p65, p-p38 MAPK, NF- $\kappa$ B p65, p38 MAPK and GAPDH were obtained from Cell Signaling Technology (CST; Beverly, MA, USA).

### 2.2. Cell culture and treatment

RAW264.7 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO<sub>2</sub>. RAW264.7 cells ( $2 \times 10^4$  cells/well) were seeded in 96-well plates for 12 h. Then, RAW264.7 cells were treated with 1  $\mu$ g/mL lipopolysaccharide (LPS) and different concentrations of leonurine for 24 h [17–20].

### 2.3. MTT assay

After treating RAW264.7 cells with different concentrations (10, 25, 50, 100, 200, 400, 600  $\mu$ mol/L) of leonurine for 24 h, the effect of leonurine on cell viability was measured by MTT assay. Add 20  $\mu$ L MTT solution (5 mg/mL in PBS buffer) and incubate with RAW264.7 cells for 4 h. Then, the DMEM was removed and 150  $\mu$ L DMSO was added. After 30 min, the absorbance was determined at 490 nm.

### 2.4. Detection of NO content

NO content in cell supernatants was determined by Griess reagent. RAW264.7 cells were treated with LPS and different concentrations (10, 25, 50, 100  $\mu$ mol/L) of leonurine for 24 h. Take 50  $\mu$ L supernatant from each well, mix with 50  $\mu$ L Griess Reagent I and 50  $\mu$ L Griess Reagent II, and incubate at room temperature for 10 min. Finally, the absorbance was measured at 540 nm.

### 2.5. Detection of TNF- $\alpha$ and IL-6

RAW264.7 cells were incubated with LPS and different concentrations (10, 25, 50, 100  $\mu$ mol/L) of leonurine for 24 h. TNF- $\alpha$  and IL-6 contents in cell supernatants were measured by ELISA kits, as described in the manufacturer's instructions.

### 2.6. Animals

Female BALB/c mice (6–8 weeks) were purchased from Jinan Pengyue Laboratory Animal Co., Ltd. (Jinan, China). All mice were kept in cages and given adequate food and water at 25 °C. Before the experiment, the mice were feed adaptively in the experimental environment for 7 days. All procedures were approved by the Animal Experiments Ethics Committee of Ocean University of China.

## 3. Construction of the asthma model

35 mice were randomly assigned into five groups ( $n = 7$ ): the control group, OVA group, OVA + Leo 15 group, OVA + Leo 30 group and OVA + Dex group. Dex was a positive drug. The asthma model was induced as previously described [21–24]. Except for the control group, mice in other groups were sensitized by intraperitoneal injection of 0.2 mL sensitized solution (20  $\mu$ g OVA and 2 mg aluminum hydroxide) on days 0, 7 and 14. Then, OVA-sensitized mice were challenged with 5% OVA by ultrasonic atomization for 30 min on days 21, 22 and 23. From day 17 to 23, mice were treated with 15 mg/kg leonurine, 30 mg/kg leonurine, 1 mg/kg Dex or equal amounts of physiological saline by intraperitoneal injection for seven consecutive days. 24 h after the last challenge, all mice were sacrificed for further experiment.

## 4. Bronchoalveolar lavage fluid (BALF) collection and leukocyte counts

24 h after the last OVA challenge, endotracheal intubation was performed and BALF was collected by lavage with 0.7 mL cold PBS twice. After centrifugation (1500 rpm, 4 °C, 10 min), the supernatant of BALF was collected to analyze cytokine content. The cell precipitates were suspended by 100  $\mu$ L cold PBS, and the number of total inflammatory cells, neutrophils, lymphocytes, monocytes and eosinophils in the BALF were measured using ProCyt Dx<sup>®</sup> Hematology Analyzer (IDEXX Laboratories Inc., Westbrook, Maine, USA).

### 4.1. Determination of Th2 cytokine levels in the BALF and OVA-IgE levels in the serum

The contents of IL-4, IL-5, IL-13 in the BALF and OVA-IgE in the serum were detected by ELISA kits, as described in the manufacturer's instructions.

### 4.2. Hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) staining

The lungs were immobilized in 4% paraformaldehyde. Lung tissues were dehydrated, paraffin-embedded and sectioned. The sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Finally, observing the histopathologic changes and mucus secretion of the lung tissues under a light microscopy.

### 4.3. Western blotting

RAW264.7 cells or lung tissues were lysed in cold Radio Immunoprecipitation Assay (RIPA) lysis buffer containing phosphatase inhibitors and protease inhibitors for 20 min. The protein concentration was detected using BCA protein detection kits. 30  $\mu$ g protein was separated by 10% SDS-PAGE gel and then transferred to PVDF membranes

for western blotting. The membranes were sealed with 5 % skim milk for 90 min at room temperature and then incubated with primary antibodies at 4 °C overnight. After washing three times with TBST, the membranes were incubated with a suitable secondary antibody for 1 h at room temperature. Finally, the enhanced chemiluminescence (ECL) kit (Pierce biotechnology, Rockford, IL, USA) was used to visualize the bands.

#### 4.4. Target prediction

We searched the Genecards Gene Database (<https://www.genecards.org/>) for genes associated with the search term “inflammation” and “asthma”. Potential targets of leonurine were obtained from the ETCM (<http://www.tcmip.cn/ETCM/>), HERB (<http://herb.ac.cn/>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov/>).

#### 4.5. GO and KEGG analysis

DAVID online database (<https://david.ncifcrf.gov/>) was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to reflect the biological process (BP), molecular function (MF), cellular component (CC) and pathways of leonurine targets.

#### 4.6. Protein-protein interaction (PPI) network construction

Venn diagram was drawn to obtain the common targets of leonurine, asthma and inflammation, and the common targets were imported into STRING (<https://string-db.org/>) for analysis. The STRING database was used to search for interactions between proteins. The system settings are as follows: select the “Homo sapiens” in the organism, select the “Evidence” in the meaning of network edges column, set confidence > 0.9, eliminate free protein, get associated data between targets, and import the obtained data into Cytoscape for the PPI network.

#### 4.7. Molecular docking

All protein structure files of p38 MAPK $\alpha$  were downloaded from RCSB PDB (<https://www.rcsb.org/>). The protein structure was prepared with the Protein Preparation Wizard module in Schrödinger suite (Schrödinger Release 2020–2, Schrödinger, LLC, New York, NY, 2021), and the ligand was optimized with OPLS3e force field. Finally, all the structures were converted to pdbqt format with all hydrogens kept by *rdkit2pdbqt.py* script (<https://github.com/biocheming/watvina>, accessed on 30 November 2021, Ximing Xu, Qingdao, China). The docking box size of each protein is 20 Å × 20 Å × 20 Å. All the protein structures were aligned, and shared the same coordinates of the binding site. Docking studies of 212 p38 MAPK $\alpha$  structures and leonurine were performed using watvina (<https://github.com/biocheming/watvina>, accessed on 30 November 2021, Ximing Xu, Qingdao, China), which was developed by our group. Based on the Autodock vina molecular docking engine, watvina was optimized for both docking scoring and conformation searching. The molecule docking score in watvina was contributed by Van der Waals, hydrogen bond, polar-polar repulsion and weak hydrogen bond. Unlike Autodock vina, watvina considered the contribution of all hydrogen atoms. Conformation searching adopted a simplified genetic algorithm-simulated annealing-BFGS combination strategy, in addition, torsion penalty was introduced for the conjugated rotatable single bonds.

#### 4.8. Molecular dynamics simulation

The molecular dynamics (MD) simulation was to detect the ligand conformational stability of ligand-binding sites and was performed by the Desmond module of Schrödinger suite 2020. The complex in the explicit solvent system with the OPLS3e force field was studied using the

Desmond module in Schrödinger. The molecular system was solvated with crystallographic water (TIP3P) molecules under orthorhombic periodic boundary conditions for a 9 Å buffer region. The overlapping water molecules are deleted, and the system was neutralized by adding Na<sup>+</sup> as counter ions. A 100 ps Brownian motion simulation is performed and a harmonic position constraint is imposed on the backbone (force constant 10 kcal·mol<sup>-1</sup>·Å<sup>-2</sup>). 5 ns canonical ensemble (NVT) simulations were carried out at 300 K, with harmonic position constraints on the backbone (force constant 10 kcal·mol<sup>-1</sup>·Å<sup>-2</sup>) and side chains (force constant 5 kcal·mol<sup>-1</sup>·Å<sup>-2</sup>). An ensemble (NPT) was performed for 5 ns at 300 K temperature and 1 bar pressure with 5 kcal·mol<sup>-1</sup>·Å<sup>-2</sup> force constant restraint for backbone. Nose-Hoover Chain thermostat and Martyna-Tobias-Klein barostat were utilized to maintain the temperature and pressure, respectively. Then 100 ns equilibrium MD simulation was performed with NPT ensemble, which adopts a hybrid energy minimization algorithm with 1000 step steepest descent and conjugate gradient algorithm. A time step of 2 fs was set, and the energies and structures were recorded at every 20 ps.

#### 4.9. Statistical analysis

GraphPad Prism 9.0 software was used for statistical analysis and one-way ANOVA was used followed by multiple comparison tests. Statistical significance was accepted at  $p < 0.05$ . All data were expressed as mean ± SEM.

### 5. Results

#### 5.1. Effect of leonurine on inflammatory cytokines in LPS-induced RAW264.7 cells

After treatment with different concentrations of leonurine, it was found that leonurine had no obvious toxic effect within 600 μmol/L (Fig. 2a). Compared with the LPS group, NO content did not change after treatment with different concentrations of leonurine, while TNF- $\alpha$  and IL-6 contents decreased significantly in a dose-dependent manner (Fig. 2b-d). These results indicated that leonurine had anti-inflammatory activity in LPS-induced RAW264.7 cells.

#### 5.2. Effect of leonurine on p38 MAPK/NF- $\kappa$ B signaling pathway in LPS-induced RAW264.7 cells

To further investigate the therapeutic mechanism of leonurine, we examined the expression of p-p38 MAPK and p-NF- $\kappa$ B p65 in LPS-induced RAW264.7 cells (Fig. 3). The expression of p-p38 MAPK, p-NF- $\kappa$ B p65 in the LPS group were significantly increased, while leonurine inhibited the overactivation of these proteins. These results suggested that leonurine could exert anti-inflammatory effect by regulating the p38 MAPK/NF- $\kappa$ B signaling pathway.

#### 5.3. Effect of leonurine on inflammatory cells in the BALF

We examined the effect of leonurine on the number of inflammatory cells in the BALF. Compared with the control group, the number of total inflammatory cells, neutrophils, lymphocytes, monocytes and eosinophils in the BALF were significantly increased in the OVA group (Fig. 4). After leonurine treatment, the number of inflammatory cells in the BALF decreased significantly, particularly neutrophils and eosinophils, while the number of lymphocytes and monocytes did not decrease significantly. These results indicated that leonurine could reduce inflammatory cell infiltration in the lung of asthmatic mice.

#### 5.4. Effect of leonurine on Th2 cytokines in the BALF and OVA-IgE in the serum

Compared with the control group, the levels of IL-4, IL-5 and IL-13 in

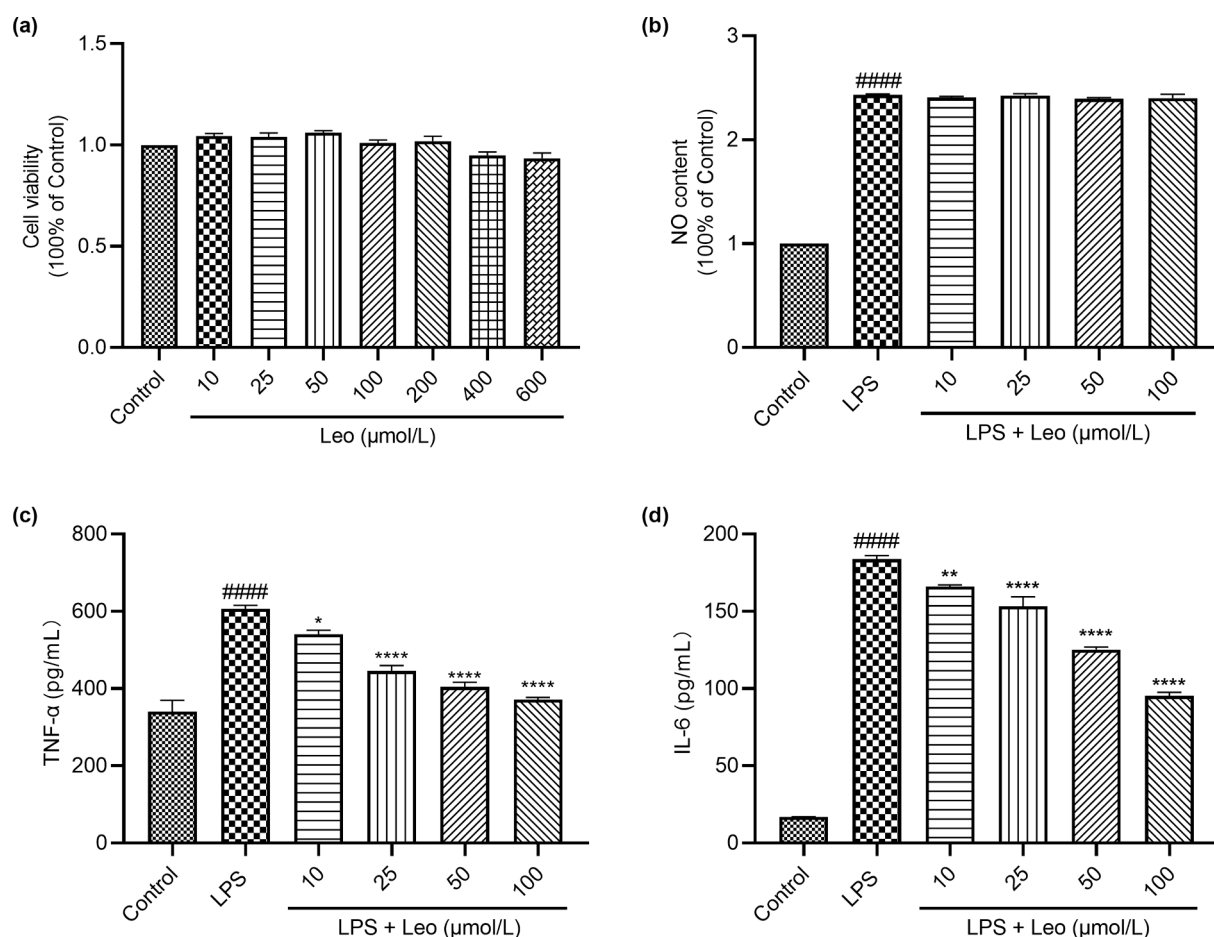


**Fig. 2.** Effect of leonurine on inflammatory cytokines in LPS-induced RAW264.7 cells. The cells were treated with LPS and different concentrations (10, 25, 50, and 100  $\mu\text{mol/L}$ ) of leonurine for 24 h. (a) Cell viability. (b) NO content. (c) TNF- $\alpha$  content. (d) IL-6 content. Values are presented as the mean  $\pm$  SEM ( $n = 4$ ). ####  $p < 0.0001$  vs Control group; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$  vs LPS group.

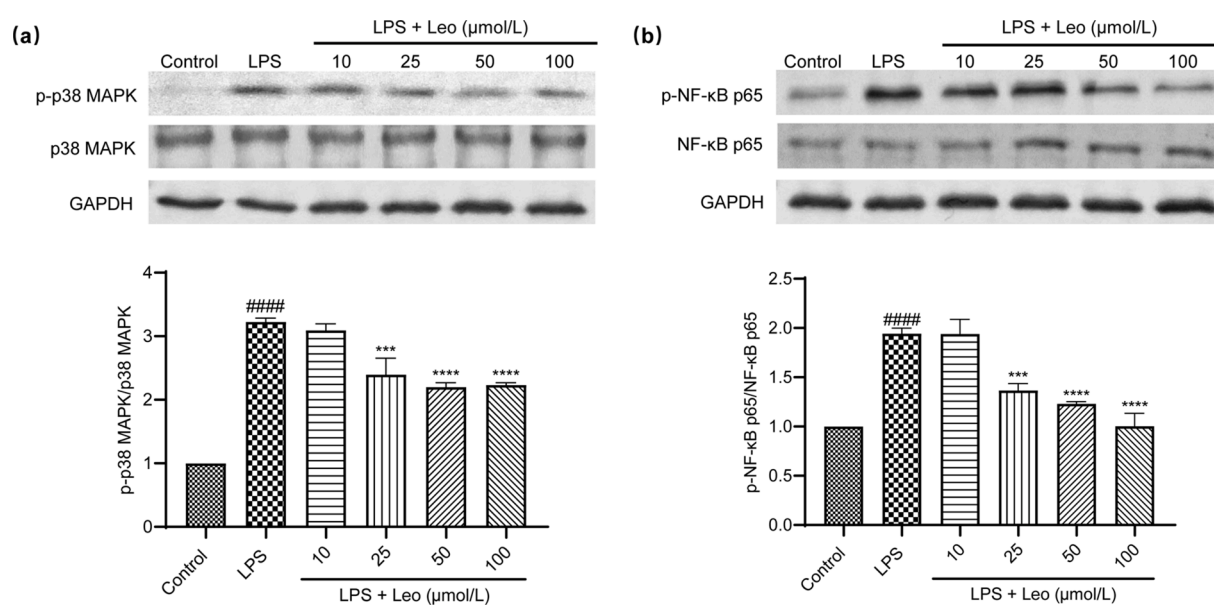


**Fig. 3.** Effect of leonurine on p38 MAPK/NF- $\kappa$ B signaling pathway in LPS-induced RAW264.7 cells. (a) The expression quantity of p-p38 MAPK and p38 MAPK protein. (b) The expression quantity of p-NF- $\kappa$ B p65 and NF- $\kappa$ B p65 protein. Values are presented as the mean  $\pm$  SEM ( $n = 3$ ). ####  $p < 0.0001$  vs Control group; \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  vs LPS group.

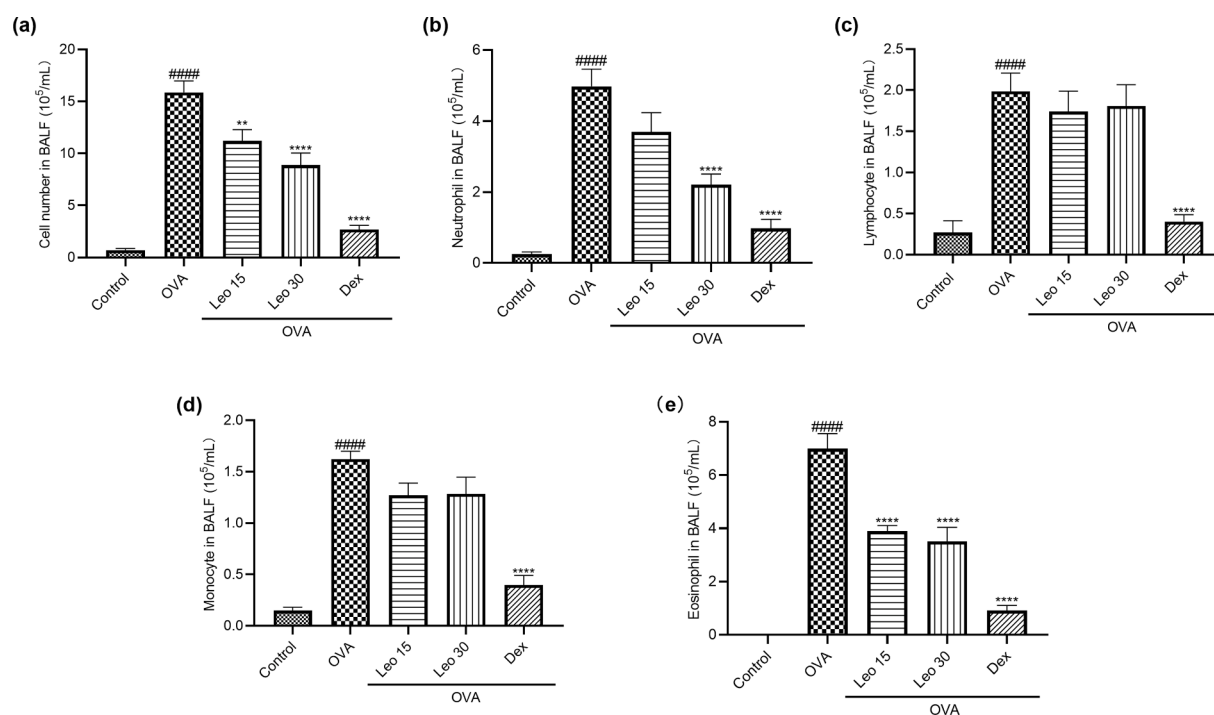


**Fig. 4.** Effect of leonurine on the number of inflammatory cells in the BALF. 24 h after the last challenge, the number of inflammatory cells in the BALF were detected. (a) Total inflammatory cells. (b) Neutrophils. (c) Lymphocytes. (d) Monocytes. (e) Eosinophils. Values are presented as the mean  $\pm$  SEM ( $n = 7$ ). ####  $p < 0.0001$  vs Control group; \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$  vs OVA group.

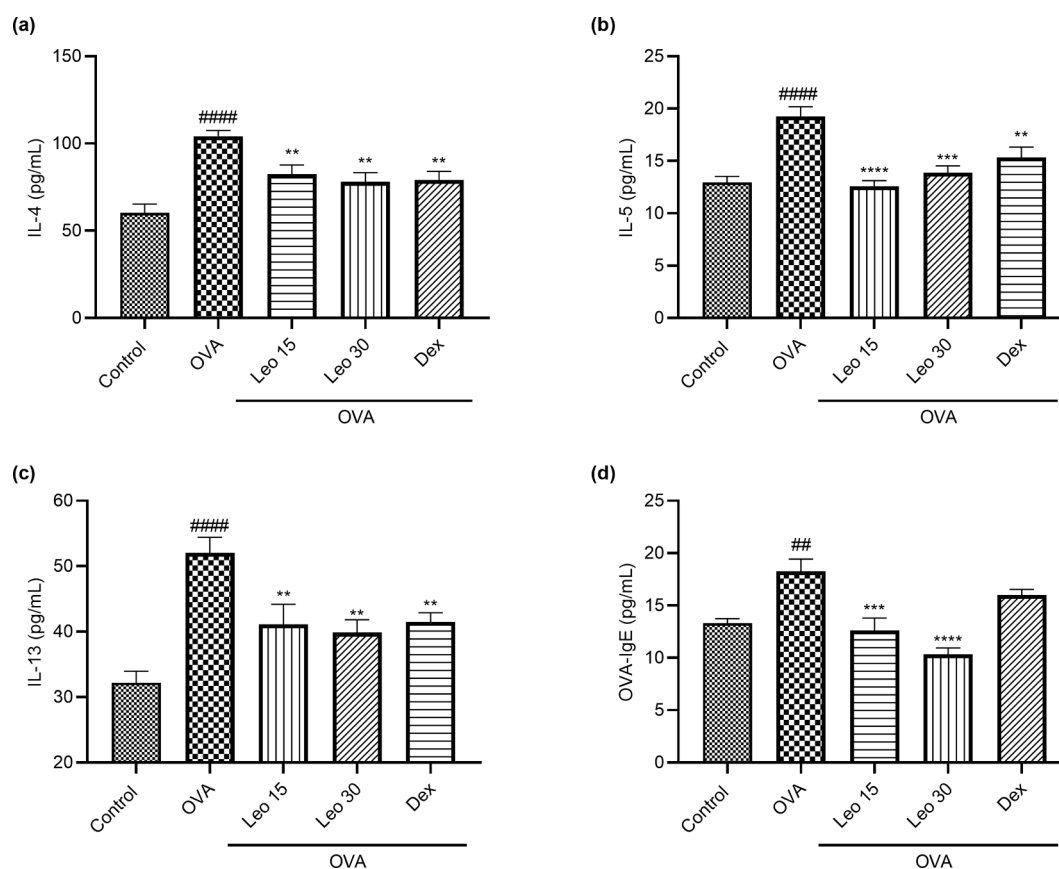


**Fig. 5.** Effect of leonurine on Th2 cytokines in the BALF and OVA-IgE in the serum. (a) IL-4 level. (b) IL-5 level. (c) IL-13 level. (d) OVA-IgE level. Values are presented as the mean  $\pm$  SEM ( $n = 7$ ). ####  $p < 0.0001$  vs Control group; ##  $p < 0.01$  vs Control group; \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  vs OVA group.

the BALF and OVA-IgE in the serum of OVA-induced mice were significantly increased (Fig. 5). However, leonurine significantly inhibited the production of IL-4, IL-5, IL-13 and OVA-IgE, which were superior to dexamethasone.

#### 5.5. Effect of leonurine on lung histopathology in asthmatic mice

Hematoxylin eosin (H&E) and periodic acid-Schiff (PAS) staining were used to test the histological changes of lung tissues. Lung tissues in the OVA group had significant inflammatory infiltration (Fig. 6a). PAS staining showed that OVA-induced mice overproduced mucus (Fig. 6b). Leonurine remarkably improved OVA-induced inflammatory cell infiltration and significantly inhibited mucus overproduction, which were similar to dexamethasone.

#### 5.6. Effect of leonurine on p38 MAPK/NF- $\kappa$ B signaling pathway in asthmatic mice

To investigate the effect of leonurine on p38 MAPK/NF- $\kappa$ B signaling pathway, the expression levels of p-p38 MAPK and p-NF- $\kappa$ B p65 in lung tissues were tested by western blotting (Fig. 7). We observed that the expression levels of p-p38 MAPK and p-NF- $\kappa$ B p65 were significantly increased in OVA-induced mice, while leonurine significantly inhibited the activation of p38 MAPK and NF- $\kappa$ B.

#### 5.7. Network pharmacologic analysis of leonurine in the treatment of asthma

By integrating information from PubChem, ETCM and HERB databases, we obtained 107 potential targets of leonurine. Potential targets of inflammation and asthma were collected from Genecards database, and 10950 targets of inflammation and 4590 targets of asthma were collected. After intersection analysis, 73 common targets of leonurine, asthma and inflammation were acquired (Fig. 8a). Then, 73 common targets were imported into STRING and Cytoscape software to draw the protein-protein interaction network diagram (Fig. 8b). Core targets were screened according to the Degree value, and STAT3, MAPK3, AKT1, MAPK14 and MAPK1 were identified as the top five core targets involved in the pathogenesis of asthma (Fig. 8c). MAPK14, also known as p38 MAPK $\alpha$ , was the fourth core target. GO and KEGG enrichment analysis showed that leonurine could treat asthma by exerting anti-inflammatory activity (Fig. 8d-e).

#### 5.8. Molecular docking and molecular dynamics simulation between leonurine and p38 MAPK $\alpha$

Molecular docking was performed to study the interaction between leonurine and p38 MAPK $\alpha$ . The 212 crystal structures of p38 MAPK $\alpha$  were obtained from Protein Data Bank (PDB), and the docking score of these 212 crystal structures and leonurine was calculated by *watvina* respectively. The docking score distribution of leonurine and 212 crystal structures of p38 MAPK $\alpha$  was shown in Fig. 9a. The p38 MAPK $\alpha$  (PDBID: 4dlj) had the best docking score with leonurine, with a score of -9.6, and the docking result were shown in Fig. 9b-c.

The binding stability of leonurine and p38 MAPK $\alpha$  (PDBID: 4dlj) was further simulated by molecular dynamics simulations (MD). The Root Mean Square Deviation (RMSD) of p38 MAPK $\alpha$  backbone in the complex was <3.0 Å, indicating that p38 MAPK $\alpha$  had a stable conformation during the simulation (Fig. 9d). The Root Mean Square Fluctuation (RMSF) values of atoms 15 and 17 fluctuated greatly relative to the protein, while the other atoms showed relatively stable atomic fluctuations, indicating a relatively firm binding to the protein pocket (Fig. 9e). As shown in Fig. 9f-g, Trp197, Ser251, Asp294, Lys249 and Ser252 were key residues. The hydrogen bond value corresponding to Ser251 and the hydrophobic interaction value of Trp197 both exceeded 1.0, indicating that the interaction between two residues and leonurine maintained specific interactions throughout the simulation time. Hydrogen bonding played an important role in ligand binding, because it had a great influence on specific metabolism and adsorption of drugs. Therefore, the hydrogen bonding between Ser251 and leonurine played a key role in the stability of complex.

## 6. Discussion

Asthma is a complex disease characterized by chronic inflammation of the respiratory system [25]. In recent years, the prevalence of asthma has been consistently increasing and is considered as a major public health problem. Many components with good anti-inflammatory activity have been isolated from traditional Chinese medicine and have been widely used in the treatment of various inflammatory diseases [26–28]. The application of leonurine in respiratory diseases has not been reported. In this study, for the first time, we confirmed that leonurine could attenuate asthma through p38 MAPK/NF- $\kappa$ B signaling pathway.

A large number of studies have shown that inflammation plays a crucial role in the development of asthma. anti-inflammatory treatment is considered to be the basic treatment for asthma [29]. Macrophages also play a vital role in allergic airway inflammation by expressing inflammatory factors such as NO, TNF- $\alpha$  and IL-6. TNF- $\alpha$  and IL-6 are pro-

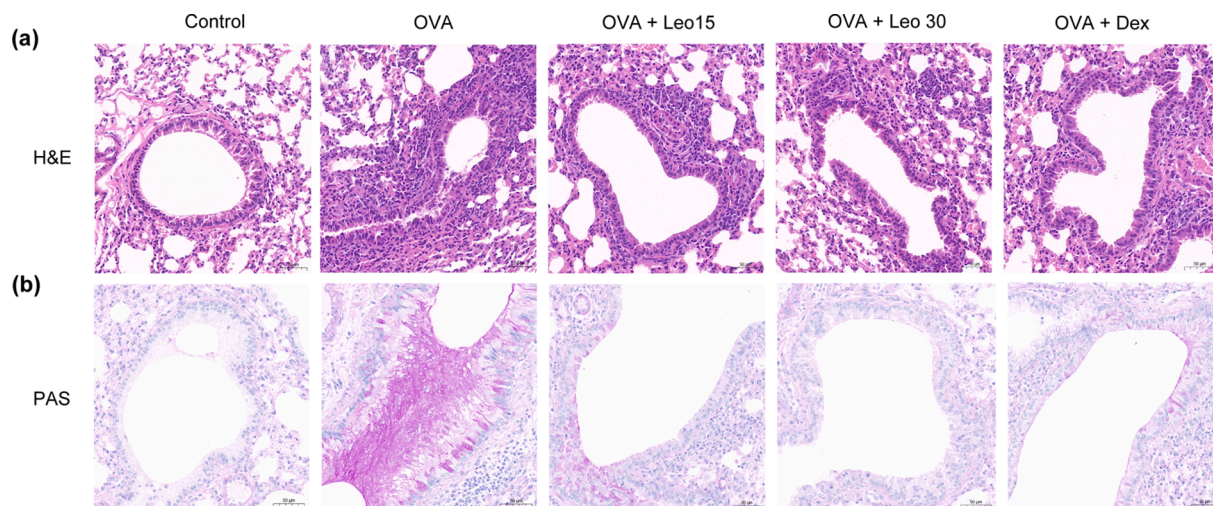


Fig. 6. Effect of leonurine on lung histopathology in asthmatic mice. (a) H&E staining. (b) PAS staining. 200 × magnification; scale bar: 50  $\mu$ m.

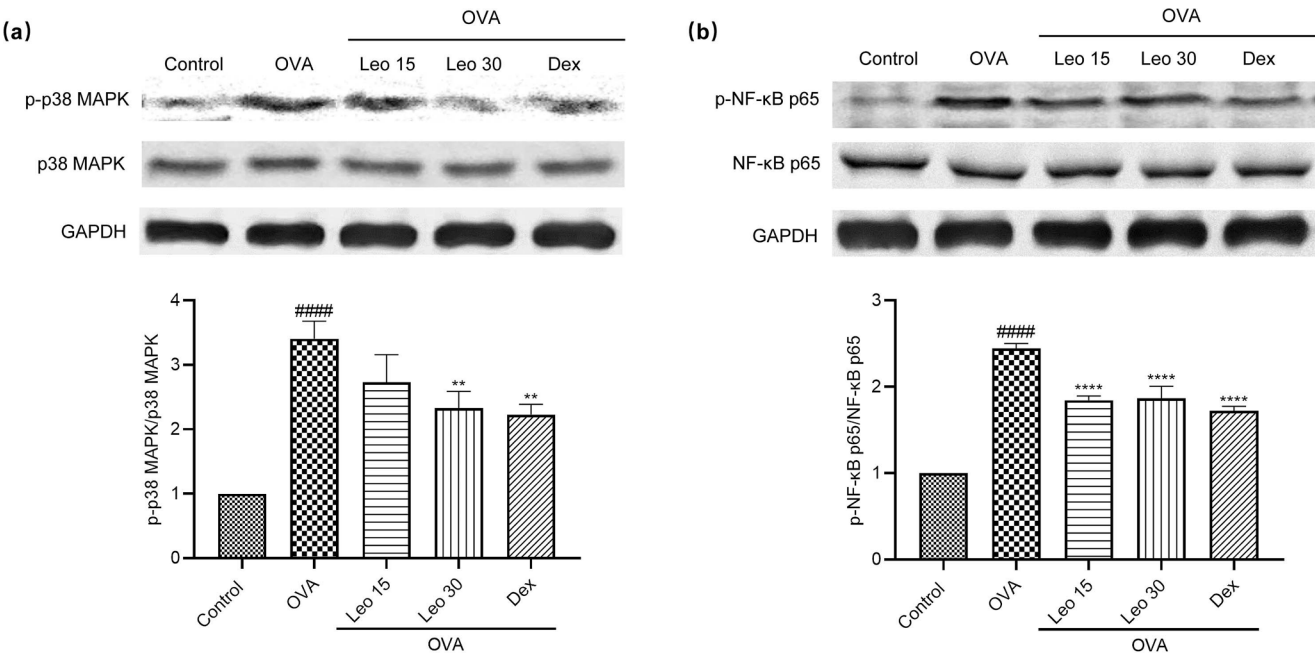


**Fig. 7.** Effect of leonurine on p38 MAPK/NF-κB signaling pathway in the lung tissues of asthmatic mice. (a) The expression quantity of p-p38 MAPK and p38 MAPK protein. (b) The expression quantity of p-NF-κB p65 and NF-κB p65 protein. Values are presented as the mean ± SEM (n = 3). ####  $p < 0.0001$  vs Control group; \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$  vs LPS group.

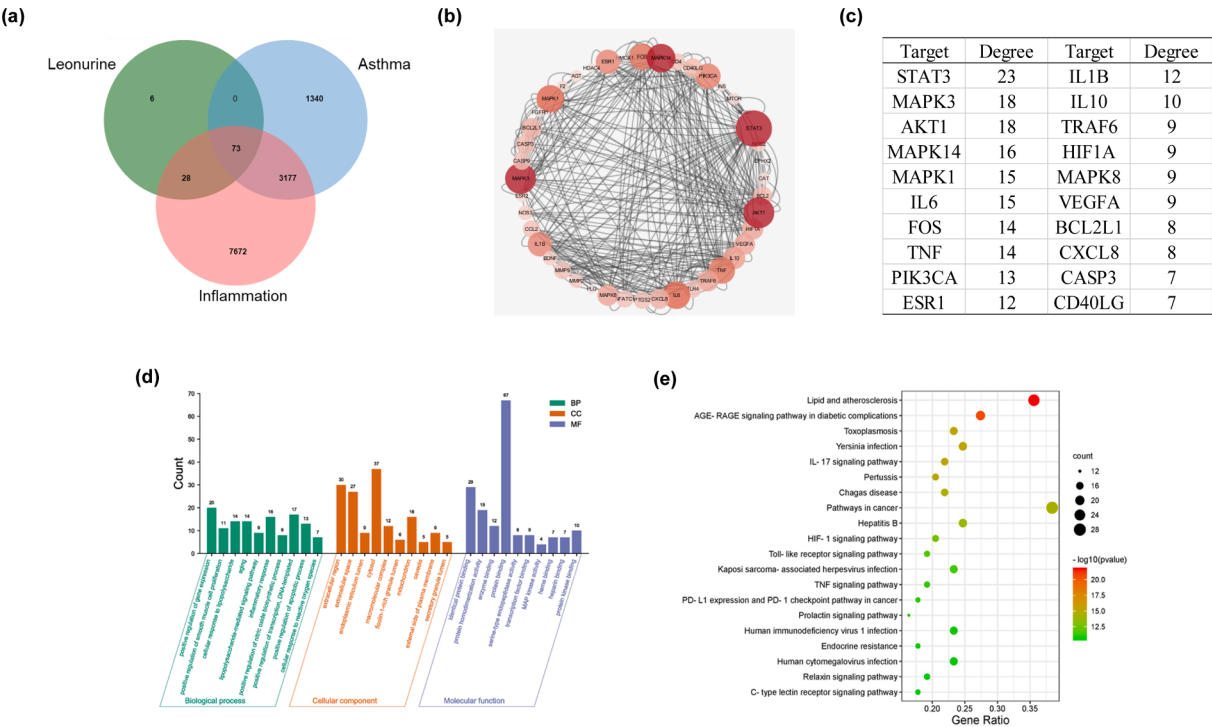


**Fig. 8.** Network pharmacologic analysis of leonurine in the treatment of asthma. (a) Venn diagram of common targets of leonurine, asthma and inflammation. (b) Protein-protein interaction network diagram. (c) Top 20 core targets. (d) GO analysis of the common targets. (e) KEGG analysis of the common targets.

inflammatory cytokines that induce macrophage activation, lung inflammation and mucus oversecretion [19,30–32]. In this study, we demonstrated that leonurine significantly inhibited TNF-α and IL-6 release in LPS-induced RAW264.7 cells.

As previously mentioned, p38 MAPK is one of three major MAPKs that are thought to play a key role in allergic asthma. p38 MAPK inhibitors can regulate the expression of a variety of inflammation-related

genes and are used for the treatment of inflammatory diseases such as rheumatoid arthritis and asthma [33]. NF-κB is a classical transcription factor that is widely believed to play a regulatory role in inflammatory responses. Studies have shown that inhibition of NF-κB activation prevents OVA-induced asthma, and NF-κB may be a promising molecular target for the treatment of asthma [34,35]. In our study, leonurine inhibited the phosphorylation of p38 MAPK and NF-κB in LPS-induced

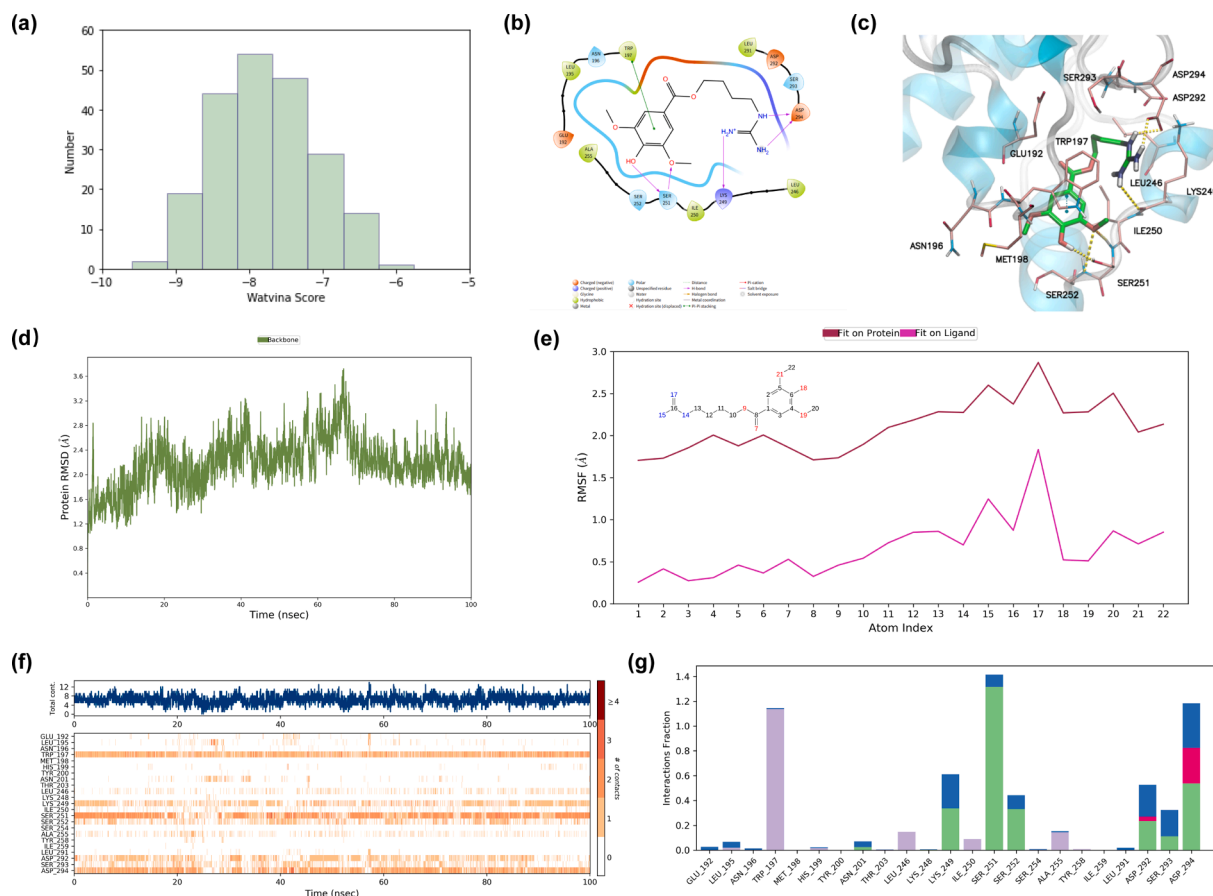


**Fig. 9.** Molecular docking and molecular dynamics simulation between leonurine and p38 MAPKα. (a) The docking score distribution diagram of leonurine and 212 crystal structures of p38 MAPKα. (b) The 2D interaction structures between leonurine and p38 MAPKα. (c) The 3D interaction structures between leonurine and p38 MAPKα. (d) The Root Mean Square Deviation (RMSD) of p38 MAPKα backbone. (e) The Ligand Root Mean Square Fluctuation (L-RMSF) of the ligand in the complex. (f) The interactions between residues and ligands during the whole simulation process. (g) The main interactions between leonurine and p38 MAPKα. Hydrogen bonding in green color, hydrophobic interaction in purple color, water bridge in blue, while the ion interaction in red.

RAW264.7 cells.

It has been shown that estrogen can promote the onset of asthma [36–40]. Therefore, we used female mice to construct asthma model. In OVA-induced mice, our results showed that leonurine greatly prevented inflammatory infiltration in the lung of asthmatic mice, as evidenced by reducing the number of inflammatory cells in the BALF, particularly neutrophils and eosinophils. In our study, it was found that OVA could induce the overproduction of Th2 cytokines and OVA-IgE. Leonurine treatment significantly reduced Th2 cytokines (IL-4, IL-5 and IL-13) in the BALF and OVA-IgE in the serum of asthmatic mice. The H&E and PAS results showed that leonurine treatment also significantly improved inflammatory infiltration and mucus overproduction in the lungs of asthmatic mice. In addition, leonurine could significantly inhibit the phosphorylation of p38 MAPK and NF-κB in the lung tissues of asthmatic mice. These results indicated that leonurine could alleviate asthma through p38 MAPK/NF-κB signaling pathway.

Network pharmacology is a novel approach that combines system network analysis with pharmacology to elucidate the synergies and potential mechanisms of compound - compound, compound - target, and target - disease networks at the molecular level [41]. This helps to clarify the interconnections among compounds, genes, proteins and diseases. In this study, network pharmacology was used to verify the experimental results. Through the publicly available databases, we obtained 73 potential common targets among leonurine, inflammation and asthma. Then, the protein-protein interaction network of the common targets was constructed, and the core targets were obtained. STAT3, MAPK3, AKT1, MAPK14 and MAPK1 were identified as the top five core targets

involved in the pathogenesis of asthma. MAPK14, also known as p38 MAPKα, was the fourth core target. GO and KEGG enrichment analysis showed that leonurine could treat asthma by exerting anti-inflammatory activity. Moreover, molecular docking results showed that leonurine had good binding activity to p38 MAPKα. Molecular dynamics simulation results showed that leonurine and p38 MAPKα could bind stably under simulated physiological conditions.

In conclusion, leonurine could effectively alleviate asthma by regulating p38 MAPK/NF-κB signaling pathway. Leonurine may be a promising drug for the treatment of asthma.

#### Ethics statement

The animal study was reviewed and approved by the Animal Experiments Ethics Committee of Ocean University of China (OUC-SMP-2021-02-02).

#### Author contribution

Donghui Bai conducted the research and wrote the paper; Yujie Sun contributed new reagents or analytic tools; Qiong Li, Haihua Li and Yuerun Liang performed the experiments and analyzed the data; Ximing Xu and Jiejie Hao designed and directed the project. All authors have read and approved the published version of the manuscript.

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## 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

The data that has been used is confidential.

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