



Integrated systems pharmacology and transcriptomics to dissect the mechanisms of Loki Zupa decoction in the treatment of murine allergic asthma

Cong Xie^a, Aman Gul^{a,b}, Hang Yu^{a,b}, Xi Huang^{a,b}, Lingling Deng^{a,b}, Yue Pan^{b,c}, Shuangshuang Ni^d, Mammatt Nurahmat^e, Muhammadjan Abduwaki^e, Qingli Luo^{a,b,**}, Jingcheng Dong^{a,b,*}

^a Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China

^b Institute of Integrative Medicine, Fudan University, Shanghai, China

^c School of Pharmacy, Fudan University, Shanghai, China

^d Sinopharm Xinjiang Pharmaceutical Co., Ltd., Urumqi, Xinjiang, China

^e College of Xinjiang Uyghur Medicine, Hotan, Xinjiang, China

ARTICLE INFO

Keywords:

Loki zupa

Asthma

Inflammation

Pharmacology

Transcriptomics

ABSTRACT

Ethnopharmacological relevance: Loki zupa (LKZP) decoction, a traditional Uyghur medicine prescription, has been commonly used to treat numerous respiratory ailments in the Xinjiang region of western China, especially chronic airway inflammatory diseases such as allergic asthma. Due to its complex chemical composition, however, the mechanism of action of LKZP has yet to be fully elucidated.

Aim of the study: Based on the balanced regulation theory of pro-inflammation and anti-inflammation, we tried to investigate the effectiveness of LKZP on asthma and its related protective mechanisms.

Materials and methods: In this study, an experimental model of asthma was established using ovalbumin (OVA) in BALB/c mice to assess the effects of LKZP. The potential mechanism of LKZP anti allergic asthma were researched by the combination of *in silico* systems pharmacology and *in vivo* transcriptomics.

Results: Our data revealed that LKZP exerted a therapeutic effect against OVA-induced asthma by reducing airway hyperresponsiveness (AHR), peribronchial inflammation, and mucus hypersecretion. Meanwhile, LKZP downregulated the expression of OVA-induced IgE, interleukin (IL)-4, IL-5, IL-13, and tumor necrosis factor (TNF)- α and concurrently promoted the expression of interferon (IFN)- γ in serum and bronchoalveolar lavage fluid (BALF). Systems pharmacology analysis identified 10 core bioactive ingredients and 26 hub targets of LKZP against asthma. Transcriptomic analysis confirmed 246 differentially expressed genes (DEGs) after LKZP treatment. These were mainly expressed in cytokine-cytokine receptor interactions and immune and inflammatory response-related signaling pathways. Additionally, the real-time quantitative PCR (qPCR) results for the nine selected DEGs matched those of the RNA-seq analysis. Nuclear factor (NF)- κ B and hypoxia-inducible factor (HIF)-1 signaling pathways were identified as candidate targets involved in the action of LKZP on allergic asthma, which was highly consistent with the findings *in silico*. By qPCR, Western blot, and immunohistochemical analysis, it was verified that LKZP treatment dramatically inhibited the activation of NF- κ B p65 and HIF-1 α stimulated by OVA in asthmatic mice.

Conclusions: Taken together, our experimental data revealed that LKZP could be a candidate for the treatment of allergic asthma via NF- κ B and HIF-1 signaling pathways.

* Corresponding author. Department of Integrative Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, China.

** Corresponding author. Department of Integrative Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, China.

E-mail addresses: xiecong21@163.com (C. Xie), aytegin@outlook.com (A. Gul), banjiao374740075@outlook.com (H. Yu), 20211220091@fudan.edu.cn (X. Huang), lldeng1996@126.com (L. Deng), panyue1107@qq.com (Y. Pan), 409884975@qq.com (S. Ni), 1830468427@qq.com (M. Nurahmat), 1070871718@qq.com (M. Abduwaki), qingqingluo2010@163.com (Q. Luo), jcdong2004@126.com (J. Dong).

<https://doi.org/10.1016/j.jep.2022.115351>

Received 2 December 2021; Received in revised form 17 April 2022; Accepted 3 May 2022

Available online 6 May 2022

0378-8741/© 2022 Elsevier B.V. All rights reserved.

Abbreviations

ACT	asthma control test
AHR	airway hyperresponsiveness
BALF	bronchoalveolar lavage fluid
COPD	chronic obstructive pulmonary disease
DEGs	differentially expressed genes
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
HIF	hypoxia-inducible factor
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ICS	inhaled glucocorticoids
IHC	immunohistochemistry

IL	interleukin
LKZP	Loki zupa
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEF	peak expiratory flow
PPI	protein-protein interaction
PVDF	polyvinylidene fluoride
RCT	randomized controlled trial
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCM	traditional Chinese medicine
TNF	tumor necrosis factor

1. Introduction

Asthma is a chronic respiratory disease characterized by airway inflammation and airway hyperresponsiveness (AHR) that affects approximately 334 million people worldwide (Aun et al., 2017). Asthma manifests as recurrent wheezing, shortness of breath, chest tightness, or cough, often with aggravation at night and in the early morning. This has seriously depressed the life quality of asthma patients (Papi et al., 2018).

Currently, asthma treatment relies on inhaled glucocorticoids (ICS), β_2 receptor agonists, leukotriene regulators, theophylline, and anticholinergic agents. However, due to the complex etiologies of asthma and its diverse manifestations among different individuals, the effectiveness of conventional therapy remains limited. Additionally, many of these drugs have potentially serious side effects, including metabolic disorders, increased cardiovascular disease risk, and gastrointestinal injury (Car-amori et al., 2021; Derendorf et al., 2006).

Due to these limitations, herbal medicine products have emerged as alternative therapeutic options for asthma. Many traditional Chinese medicine (TCM) formulas and products have been used to treat asthma. Based on an abundant combination of herbs, their sophisticated chemical compositions, and a multi-target therapeutic approach, TCM formulas provide an easier and more flexible approach to asthma therapy.

Loki zupa (LKZP) consists of two herbs, namely, *Hyssopus cuspidatus* Boriss (Shenxiangcao, SXC) and *Iris halophila* Pall (Yuanweigen, YWG). LKZP was originally recorded in the classic “Ehtiyarati bedey” during the 14th century in Xinjiang region of China and was used as a therapy for respiratory inflammatory diseases caused by “excessive mucus”, such as common cold, influenza, bronchitis, asthma, and pneumonia.

Our previous a randomized controlled trial (RCT) demonstrated that adding LKZP to standard ICS produced clinically significant improvements in ACT score, PEF, daytime symptom-free days, and acute exacerbation in patients with chronic asthma (Lv et al., 2018). Animal model studies have shown that LKZP decoction and its extracts significantly reduced AHR, attenuated airway inflammation, promoted Th1, and suppressed Th2 cell functions in an ovalbumin (OVA)-induced asthmatic mouse model (Wei et al., 2016). In subsequent studies, quantitative proteomic profiling of targeted proteins suggested that LKZP’s attenuation of airway inflammation and fibrosis may be associated with extracellular matrix (ECM)-receptor interaction and that RMB 10 and COL6A6 may be the targets (Wuniquimu et al., 2021). In addition, LKZP decoction showed protective effects in the lungs of the a cigarette smoke-induced COPD rat model (Mohammadtursun et al., 2020). However, no studies have reported the anti-asthmatic effects of LKZP decoction at the transcriptional level and its correlative-specific signaling pathways. Therefore, the objective of this study was to determine the effects of LKZP in an asthma mouse model and further explore its underlying mechanisms by means of systems pharmacology

and transcriptome analysis.

2. Materials and Methods**2.1. Preparation and extraction of a LKZP decoction**

All decoction pieces in the LKZP formula were purchased from the Xinjiang Maidisen traditional Uyghur medicine Co. Ltd. (Hotan, Xinjiang, China) and the plant materials were identified morphologically and histologically according to a standard Chinese herbal identification procedure by Professor Feng Ying of the Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences.

LKZP decoction was performed using a water extraction and alcohol precipitation method. The herb *Hyssopus cuspidatus* Boriss. (60 g) and *Iris halophila* Pall. roots (60 g) were extracted at a 1:1 ratio twice, each for 2 h. Twelve (12) units of water was added and soaked for 2 h. Another 10 times water was added. The two decoctions were then blended and filtered. The extract was concentrated to a relative density of 1.05 (60 °C), and ethanol was added to 60% of the alcohol content. The extract was left at room temperature for 12 h and then centrifuged. Ethanol was recovered from the supernatant by decompression and concentration to a relative density of 1.05 (60 °C). The finished decoctions were prepared in Sinopharm Xinjiang Pharmaceutical Co., Ltd. (Urumqi, Xinjiang, China). The mass spectra of LKZP extract was determined for quality control by high performance liquid chromatography (HPLC, Supplementary Material 1).

2.2. Animals and experimental design

The asthma model was established and treated as shown in Fig. 1A. Sixty specific pathogen-free (SPF) female BALB/c mice (aged 6–8 weeks) weighing 18.6 g–21.2 g were procured from Shanghai JieSiJie Laboratory Animal Co., Ltd. (Shanghai, China). The mice were supplied with standard maintenance mouse chow and sterile water ad libitum. All procedures were conducted in accordance with the “Laboratory Animal Guideline for ethical review of animal welfare (GB/T 35892–2018, China)” and were approved by the Animal Welfare and Ethics Committee of the Department of Laboratory Animal Science, Fudan University. The Institutional Animal Care and Use Committee (IACUC) number was 2020-HSYY-JS-675 (Supplementary Material 2).

After adaptive feeding for 1 week at the School of Pharmacy, Fudan University Efficacy Evaluation Experiment Animal Center (Shanghai, China), sixty (60) mice were arbitrarily divided into six groups (10 mice/group). Ten mice were in the control group, and the rest of the mice were multi-sensitized with 100 μ g of OVA (Grade V; Sigma-Aldrich; St. Louis, MO, USA) mixed with 1 mg aluminum hydroxide adjuvant (Thermo Fisher Scientific, Waltham, MA, USA) in 0.2 ml of saline solution by intraperitoneal injection (i.p.) on days 0, 7, and 14.

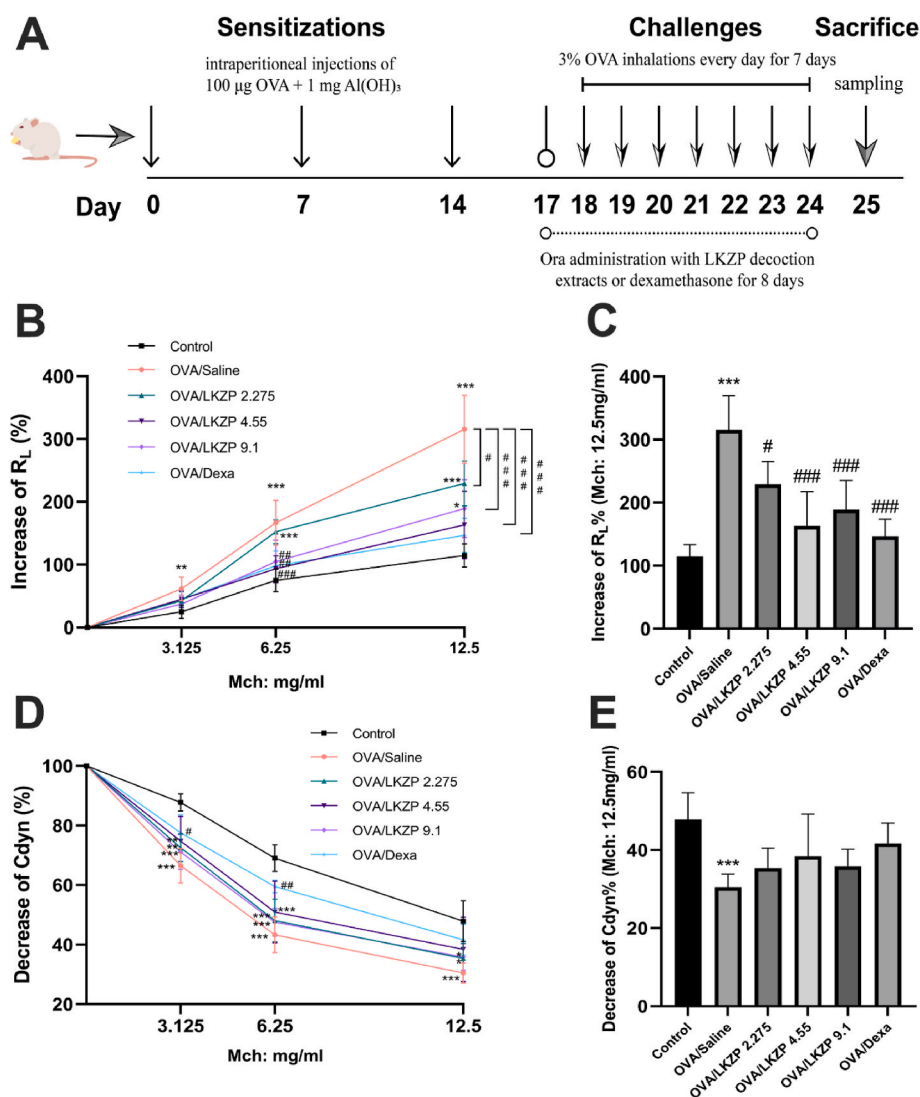


Fig. 1. LKZP decoction reduced AHR in OVA-induced murine allergic asthma model. (A) Protocol of the asthma model establishment and treatment as described in **Materials and Methods**. (B) Increase of R_L %. (C) Changes in R_L to Mch dose of 12.5 mg/ml. (D) Decrease of C_{dyn} %. (E) Changes in C_{dyn} to Mch dose of 12.5 mg/ml. The data are presented as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs OVA/Saline group. $N = 6$ mice/group.

These mice were then exposed to 3% OVA (Grade II, w/v) solution using an ultrasonic nebulizer (402AI, Yuwell, Zhenjiang, Jiangsu, China) for 30 min from day 18 to day 24. Mice in the control group were sensitized and challenged with saline. The remaining 50 OVA-treated mice were randomly assigned to the following five groups: the OVA-induced asthma model group (OVA/saline group), low-dose LKZP decoction (2.275 g/kg/d)-treated group, middle-dose LKZP decoction (4.55 g/kg/d)-treated group, high-dose LKZP decoction (9.1 g/kg/d)-treated group, and dexamethasone treated group ($n = 10$ for each group).

Drug treatment was initiated on the day before the first ultrasound nebulizer inhalation of OVA antigen fluid. According to the drug dose conversion formula for humans and mice, the gastric perfusion doses were 2.275, 4.55, and 9.1 g/kg in the low-dose, middle-dose and high-dose LKZP decoction group, respectively. The final concentrations used for oral administration were equivalent to 15, 30, and 60 g of raw herbal medicine per kg body weight. For dexamethasone treatment, mice were intragastrically administered 1 mg/kg dexamethasone (Winherb, Shanghai, China) for 8 consecutive days from day 17 to day 24. In addition, the mice in control group and the asthma model group were treated with equal volume of saline only.

2.3. Measurement of AHR

On day 25, 24 h after the last OVA aerosol exposure, a bronchial

provocation test was performed to determine AHR in mice. Here, we adopted 2% pentobarbital sodium (50 mg/kg body weight) as the anesthetic drug. Changes in airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were recorded by a whole-body plethysmograph (DSI Buxco FinePointe RC System, Troy, NY, USA) as described previously (Jin et al., 2013). Briefly, the mice were exposed to methacholine (Mch) at sequentially increasing concentrations (0, 3.125, 6.25 and 12.5 mg/ml) under anesthesia to evaluate lung function. The results were expressed as a percentage change relative to the baseline phosphate buffered saline (PBS) value (Glaab et al., 2001).

2.4. Collection and detection of blood and bronchoalveolar lavage fluid (BALF)

Blood samples were left at room temperature for 2 h, and then centrifuged at 2500 rpm at 4 °C for 15 min (Centrifuge 5804R, Eppendorf, Leipzig, Germany). The collected serum was repackaged and stored in -80 °C for further analysis of inflammatory factors. BALF was collected by intratracheal perfusion with 300 µl aliquots of cold PBS three times (total volume 0.9 ml), centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were separated and preserved at -80 °C for cytokine testing. Cold PBS (60 µl) was used to resuspend the bottom pellet from the BALF for cell count and classification analysis using a fully automatic hematology analyzer (BC-5000 Vet, Mindray, Shenzhen,

Guangdong, China).

The levels of IgE, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-4, IL-5, and IL-13 were determined using enzyme-linked immunosorbent assay (ELISA) kits (Solarbio, Beijing, China) following the manufacturer's instructions.

2.5. Pulmonary histopathology

Left lung tissue samples were fixed with 4% paraformaldehyde (PFA), embedded in paraffin, and cut into 3 μ m sections. The tissues were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome. The lung pathological sections of ten mice in each group were photographed under an optical microscope (Eclipse Ni-E, Nikon, Japan), and six visual fields were randomly selected in each section for observation and assessment in detail. The pathological scores, which included the degree of inflammatory cell infiltration, mucus secretion, and collagen deposition, were blinded to randomized sections using ImageJ software (NIH, Bethesda, MD, USA). A 5-level scoring system was used to evaluate the severity of inflammatory cell infiltration in the H&E stained samples of lung tissue as previously described (Tachdjian et al., 2010). The level of airway mucus secretion was assessed by PAS staining, and at least six bronchioles were counted per glass slide. The results were quantified as the area of positive airway epithelial mucus staining/the circumference of the bronchiolar basement membrane (Wang et al., 2014). For Masson's trichrome staining, airway collagen deposition in the peribronchial space was scored on a semi-quantitative scale: 0 = no collagen deposition; 1 = a thin layer of collagen deposition; 2 = a cluster of collagen deposits; 3 = a thick layer of collagen deposits (Sulaiman et al., 2018). All pathological results were evaluated by two independent analysts. If the results were different, a third analyst was considered to further analyze the relevant data.

2.6. Data preparation of network pharmacology

2.6.1. Compounds screening

The compounds' information of Shenxiangcao-Yuanweigen were obtained from TCMSP (Ru et al., 2014), TCMID (Huang et al., 2018), CHEM-TCM (<http://chemtcm.com/database.html>), HIT (Ye et al., 2011), BATMAN-TCM (Liu et al., 2016), and the relevant literature. The bioactive components included eight core components of *Hyssopus cuspidatus* Boriss. (methyl caffeate, luteolin 7-O-B-D-glucopyranoside, rosemary acid, etc.) and two core components of *Iris halophila* Pall. roots (irigenin, iristectorigenin A, etc.)

2.6.2. Targets identification

Details on the human gene targets associated with asthma were collected from the DisGeNET (Pinero et al., 2020), CTD (Davis et al., 2021) and GeneCard (Stelzer et al., 2016) databases with "Asthma" and "Allergic Asthma" as the keywords, and only "*Homo sapiens*" genes were acquired. All the asthma gene targets were normalized with R software (version 4.1.1, Alcatel-Lucent Bell Laboratories, Murray Hill, NJ, USA) using the Bioconductor package when redundancy was removed.

2.6.3. Gene Ontology (GO) term and Kyoto Encyclopedia Genes and Genomes (KEGG) pathway enrichment analysis

R and clusterProfiler packages have provided robust power in statistical analysis and visualization, illustrating the molecular function (MF), cellular component (CC), and biological process (BP) implemented by KEGG (Kanehisa et al., 2016) pathway and GO (Ashburner et al., 2000; Gene Ontology, 2021) functional enrichment analysis. In this study, functional annotation and biological relevance of candidate targets were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID, version 6.8, <http://david.ncifcrf.gov/>) (Huang da et al., 2009).

2.7. mRNA-sequencing

2.7.1. RNA isolation and sequencing

In accordance with the manufacturer's order, total RNA was separated and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA amount and fineness of each sample were quantitatively analyzed using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA). RNA completeness was assessed using a Bioanalyzer 2100 (Agilent, CA, USA), with an RIN value > 7.0, and confirmed by agarose gel electrophoresis. Finally, we performed 2 $\times$ 150bp paired-end sequencing (PE150) on an Illumina Novaseq 6000 (LC-Bio Technology Co., Ltd., Hangzhou, China) according to the supplier's recommendations.

2.7.2. Analysis based on sequence for gene differential expression

After generating the final transcriptome, StringTie and ballgown (<http://www.bioconductor.org/packages/release/bioc/html/ballgown.html>) were used to estimate the expression levels of all transcripts and perform mRNA expression levels by calculating fragments per kilobase million (FPKM). The differentially expressed genes (DEGs) were selected with fold change (FC) > 2 or FC < 0.5, and p-value < 0.05, using R package edgeR (Robinson et al., 2010) (<https://bioconductor.org/packages/release/bioc/html/edgeR.html>) or DESeq2 (Pertea et al., 2015) (<http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html>), after which GO enrichment and KEGG enrichment of the DEGs were analyzed. Some pathways enriched in KEGG that are not related to asthma such as rheumatoid arthritis (RA), legionellosis, and malaria were excluded.

2.8. mRNA purification and Real-Time quantitative PCR (qPCR)

qPCR analysis of key differential gene expression levels screened by transcriptome sequencing in the lung samples collected from the mice was performed in accordance with the manufacturer's recommendations. Total RNA isolated from each group of lung samples was reverse transcribed into the first strand of cDNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and a cDNA Reverse Transcription Kit (BioTNT, Shanghai, China). The qPCR amplification experiments were subsequently performed using a PCR amplification apparatus (QuantStudio 6 Flex Real-Time PCR Systems, Life Technologies, Carlsbad, CA, USA). We used the β -actin gene as an internal reference to normalize the expression of the target genes. CT (threshold cycle) was used to determine the target gene expression levels, and the results were presented as fold changes compared with one of the random samples in the control group according to the relative quantitative analysis of $2^{-\Delta\Delta CT}$ (Lin et al., 2021). The primer sequences used for IL-10, CCL2, CCL20, Rela, CXCL2, HIF-1 α , IL-4, TNF, IL-17A, and β -actin are shown in Fig. 7B.

2.9. Western blotting (WB)

The lung tissues were lysed by cold RIPA buffer (Beyotime, Shanghai, China), containing a protease and phosphatase inhibitor cocktail (Univ-bio, Shanghai, China), followed by centrifugation in a high-speed, low-temperature tissue grinding machine at 2000 rpm for 15 min at -30°C . Samples were separated by 10% SDS-PAGE and transferred onto PVDF membranes. After that, the membrane was blocked with high efficiency rapid blocking buffer (Genefist, Saiguo Bio, Guangzhou, Guangdong, China) to prevent nonspecific binding, incubated with primary antibodies (1:1000) overnight at 4°C , and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:15000) for 1.5 h, and finally exposed using a chemiluminescent imaging system (Image-Quant LAS 4000 mini, GE Healthcare, Little Chalfont, UK). The primary antibodies were obtained from Cell Signaling Technology, Inc. (Shanghai, China), including HIF-1 α rabbit mAb (#36169), NF- κ B p65 rabbit mAb (#8242), and phospho-NF- κ B p65 rabbit mAb (#3033).

2.10. Immunohistochemistry (IHC)

After fixation for 24 h in 4% paraformaldehyde, the tissues were dehydrated, embedded in paraffin, and sectioned into 5 μ m thick slices perpendicular to the direction of the airway. Afterwards, the slices were incubated for 12 h in primary antibody followed by secondary antibody with HRP. Subsequently, immunohistochemical staining was conducted using diaminobenzidine (DAB) and hematoxylin. Under an optical microscope, the lung tissue sections were scored according to the degree of staining (0–3 points divided into negative staining, pale yellow, light brown, and dark brown) and the range of positive staining. The results were normalized for statistical analysis. Primary monoclonal antibodies against HIF-1 α and NF- κ B p65 were obtained from Abcam (Cambridge, UK).

2.11. Statistical analysis

Data in the experiments were expressed as the mean $\pm$ standard deviation (SD). Differences among groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests. All statistical analyses were performed as indicated with GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Results with p-values less than 0.05, were accepted as statistically significant (* p < 0.05, ** p < 0.01, *** p < 0.001).

3. Results

3.1. Behavioral results

No behavioral changes were observed in the control group. The mice in the asthma model group frequently had signs of asthma attacks such as rubbing the nose, grasping ears, nodding, and standing with their forelimbs retracted. After the intervention with LKZP decoction and dexamethasone, there was improvement of symptoms in the asthma control group. This, however, did not result in an asymptomatic state such as in the blank control group. The mice in the dexamethasone group were found to have dull and dry fur 8 days after oral administration. The pelage of mice in the LKZP decoction group was shiny.

3.2. LKZP decoction extract improved the lung function of asthma model mice

The AHR of increasing Mch dose was assessed within 24 h after the last OVA challenge. In the experimental groups, mice exposed to OVA showed a dose-dependent increase in R_L (Mch = 3.125 mg/ml, p < 0.01; Mch = 6.25 mg/ml and Mch = 12.5 mg/ml, p < 0.001) and a decrease in Cdyn (p < 0.001 for all doses of Mch) compared with the normal control group (Fig. 1B/D), indicating the successful establishment of a murine allergic asthma model. Compared with the asthma model group, a markedly decreased R_L was observed in the mice treated with LKZP decoction and dexamethasone at Mch concentration of 12.5 mg/ml (p < 0.05, p < 0.001). In addition, mice in the LKZP (4.55, 9.1) and dexamethasone groups also had significantly decreased R_L at Mch concentration of 6.25 mg/ml (p < 0.001 or p < 0.01). Moreover, mice in the dexamethasone group showed significantly increased Cdyn at Mch concentrations of 3.125 and 6.25 mg/ml compared with the OVA-induced asthma model group (p < 0.05 and p < 0.01). Mice in the LKZP decoction group also exhibited a modest increase in Cdyn, but this was not statistically significant (Mch 3.125: p = 0.4774, p = 0.1690, p = 0.7483; Mch 6.25: p = 0.7840, p = 0.3164, p = 0.8532; Mch 12.5: p = 0.7720, p = 0.2975, p = 0.7069). Fig. 1C/E shows a histogram of the different levels of statistical significance between treatments at different Mch concentrations from 12.5 mg/ml.

3.3. Effect of LKZP decoction extract on the number of inflammatory cells in BALF

Analysis of inflammatory cells in BALF of each group showed that compared with the normal control group, mice sensitized and challenged with OVA showed a significant increase (p < 0.001) in the number of total white blood cells, neutrophils (Neu), lymphocytes (Lym), monocytes (Mon), eosinophils (Eos), and basophils (Bas) (Fig. 2A). The proportion of Eos increased most significantly (Fig. 2C). After treatment with different doses of LKZP decoction (2.275, 4.55 and 9.1 g/kg), we observed that total leukocytes (p < 0.001), Lym (p < 0.001), Mon (p < 0.05), Eos (p < 0.001) and Bas (p < 0.001) decreased to varying degrees, and in a dose-dependent manner. Fig. 2B/D shows a stacked histogram that compares the classified counts and percentages of inflammatory cells found in the BALF of different groups. Interestingly, LKZP decoction treatment reduced total inflammatory cells mainly by reducing Eos, Lym and Neu, but dexamethasone greatly increased the percentage of Neu and significantly reduced the number of Eos (Fig. 2A/C).

3.4. LKZP decoction extracts treatment regulated inflammatory mediators in serum and BALF of OVA-induced asthmatic mice

Significant increases were observed in the IgE (Fig. 3A), IL-4 (Fig. 3B), IL-5 (Fig. 3C), IL-13 (Fig. 3D), and TNF- α (Fig. 3E) levels in serum and BALF samples collected from the OVA-induced asthma mice model, as well as a decrease in their IFN- γ levels (Fig. 3F). Treatment with the LKZP decoction extracts resulted in a reduction in the levels of IgE, IL-4, IL-5, IL-13 and TNF- α in serum and BALF samples, and increased IFN- γ levels (Fig. 3). Overall, the inhibitory effects of the three different doses of LKZP decoction (2.275, 4.55, 9.1 g/kg/d) on IgE and pro-inflammatory cytokines (IL-4, IL-5, IL-13, and TNF- α) were weaker than those of dexamethasone (1 mg/kg/d).

3.5. LKZP decoction extracts alleviates the pathological changes of OVA-induced airway inflammation

OVA-induced airway inflammation was detected by three different staining methods. H&E staining revealed dense infiltration of inflammatory cells in the peribronchiolar and perivascular connective tissues of mice in the OVA-induced asthmatic model group compared with the control group (p < 0.001). In contrast, LKZP decoction significantly decreased airway inflammation at a middle dose of 4.55 g/kg (p < 0.01) and a high dose of 9.1 g/kg (p < 0.001), similar to the OVA/Dexamethasone 1 mg/kg group (p > 0.05) (Fig. 4A/B). In addition, PAS staining was positive in the OVA/saline group compared to the control group, indicating mucus hypersecretion and significantly increased goblet cell percentage (p < 0.001). LKZP decoction was found to attenuate mucus secretion and the number of PAS + cells in OVA/LKZP 2.275, 4.55 and 9.1 g/kg group compared with OVA/Saline group (Fig. 4A/C). Collagen deposition in the lung tissues of mice in the OVA/saline group was markedly increased by Masson's trichrome staining (p < 0.01). However, mice treated with LKZP 2.275, 4.55, 9.1 g/kg or Dexamethasone 1 mg/kg showed that collagen deposition in the peribronchial regions did not significantly decrease (Fig. 4A/D).

3.6. Network pharmacology predicts candidate targets and potential signaling pathways of LKZP for the treatment of asthma

A total of 408 asthma-related genes and 26 target genes intersecting LKZP compounds were identified by searching the DisGeNET, CTD, GeneCard databases (Fig. 5A/B). These genes were saved for use on the String website to construct protein-protein interaction (PPI) networks (Fig. 5C/D). GO and KEGG pathway enrichment were analyzed using the David database to understand the relationships between functional units and their potential significance in biological system networks. The top

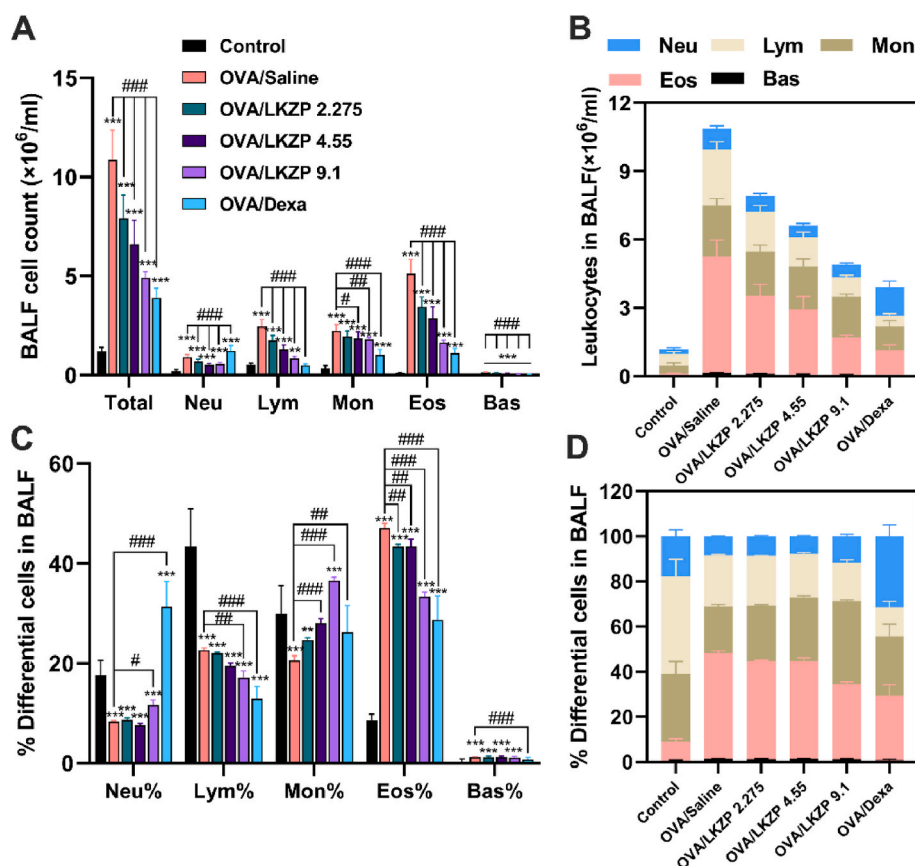


Fig. 2. Effects of LKZP decoction on inflammatory cells in the BALF. (A) The number of leukocytes in the BALF. (B) The stacked histogram shows the cell classification count. (C–D) Percentage of cell classification count. Data are shown as mean $\pm$ SD by one-way ANOVA. $N = 10$ mice/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs OVA/Saline group.

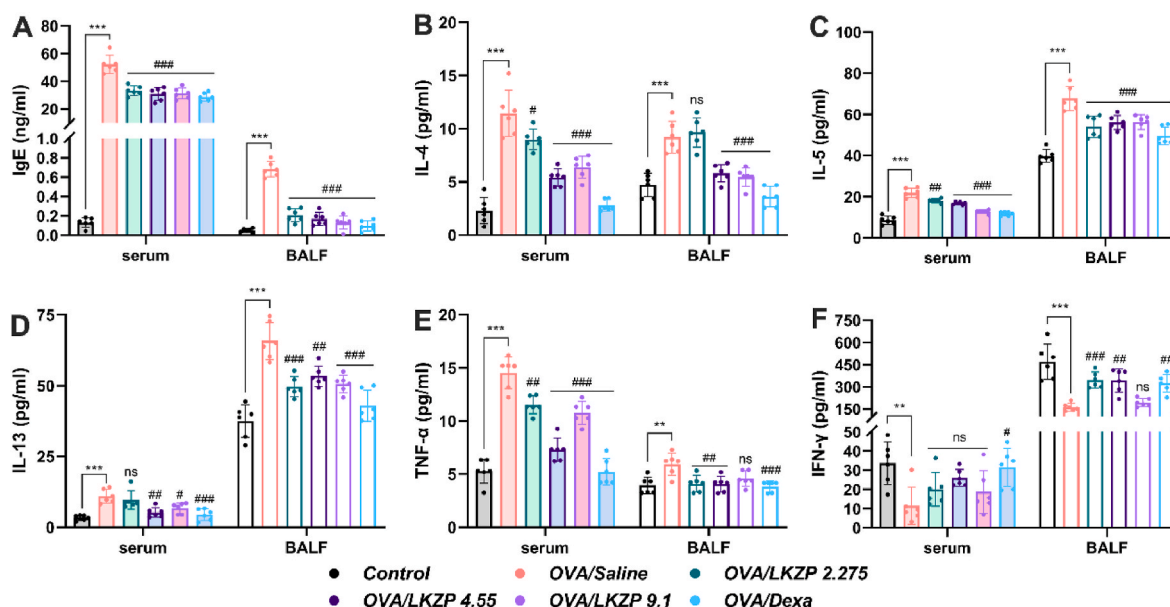


Fig. 3. LKZP decoction regulated the secretion of IgE and inflammatory cytokines in the serum and BALF. The IgE, IL-4, IL-5, IL-13, TNF- α , and IFN- γ levels in serum and BALF were measured by ELISA kits and the resulting data expressed as mean $\pm$ SD. (A) IgE level in the serum and BALF, (B) IL-4 level in serum and BALF, (C) IL-5 level in serum and BALF, (D) IL-13 level in serum and BALF, (E) TNF- α level in the serum and BALF, (F) IFN- γ level in the serum and BALF. $N = 6$ samples/group. ** $p < 0.01$, *** $p < 0.001$ vs Control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs OVA/Saline group; ns: no significant difference.

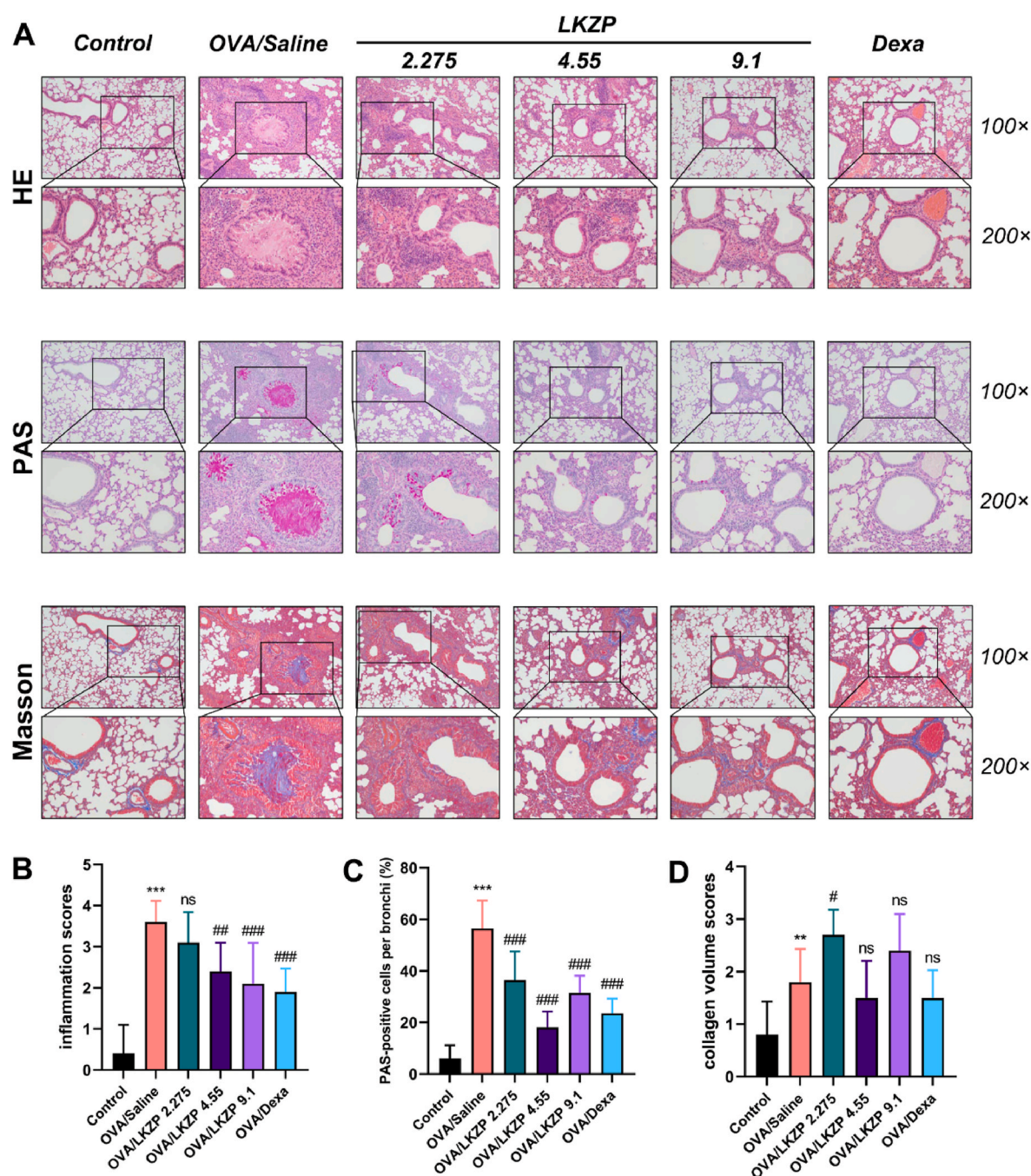


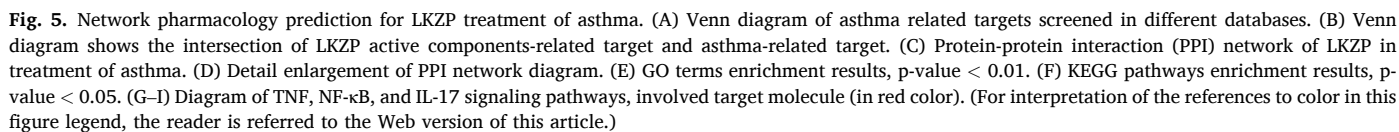
Fig. 4. Pathological features of LKZP decoction in attenuating airway inflammation in OVA-induced asthmatic mice. (magnification: $\times 100$ and $\times 200$). (A) Hematoxylin and eosin (H&E), periodic acid-schiff (PAS), Masson's trichrome stained images of control, OVA/Saline, OVA/LKZP with doses of 2.275, 4.55, 9.1 g/kg/d, and dexamethasone treated lung tissue of mice. (B) Inflammation scores acquired with H&E staining. (C) The percentage of PAS-positive cells per bronchiole. (D) Collagen volume scores acquired with Masson's trichrome. Data depicted as mean $\pm$ SD analyzed by one-way ANOVA. $N = 10$ mice/group. ** $p < 0.01$, *** $p < 0.001$ compared to control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline group; ns: no significant difference.

35 GO enrichment terms and top 20 KEGG pathways are shown in Fig. 5E and F, respectively. KEGG pathway analysis showed that many target genes were strongly associated with pathways related to inflammation and immunity, such as the "PI3K-Akt signaling pathway," "TNF signaling pathway," "IL-17 signaling pathway," and "MAPK signaling pathway". Moreover, we selected three signaling pathways, the TNF signaling pathway, NF- κ B signaling pathway, and IL-17 signaling pathway, for annotation (Fig. 5G/H/I). The results indicated that the mechanism of action during the treatment of asthma with LKZP decoction may be diverse, involving a variety of biological processes and different signaling pathways. The multiple molecular pathways and

potential therapeutic targets in asthma may explain the compatibility of LKZP for asthma treatment.

3.7. Transcriptomics reveals the relevant biological processes and signaling pathways of LKZP decoction in the treatment of asthma

With the results of animal pharmacodynamics experiments, we observed that LKZP decoction at 4.55 g/kg had a more consistent therapeutic efficacy in the asthma model mice. Hence, this concentration was selected for further experiments. The mRNA profiles of lung tissue samples from healthy, OVA-induced asthmatic mice and LKZP



v1.0.12 and hclust v3.5.0 function of R package were used for mapping and clustering analysis of the DEGs based on the FPKM values, which suggested that there were significant differences in gene expression between the LKZP group and the asthma group (Fig. 6C).

To further confirm the reliability of the transcriptome data, we performed an enrichment analysis to ascertain whether these genes were biologically related to asthma by the Gene Set Enrichment Analysis (GSEA) software (version 4.1.0) (Subramanian et al., 2005). The results showed that the “Asthma” group transcriptome data was obviously enriched by genes related to those found in asthma compared with the “Control” group [Enrichment Score (ES) = 0.8376948, Normalized ES

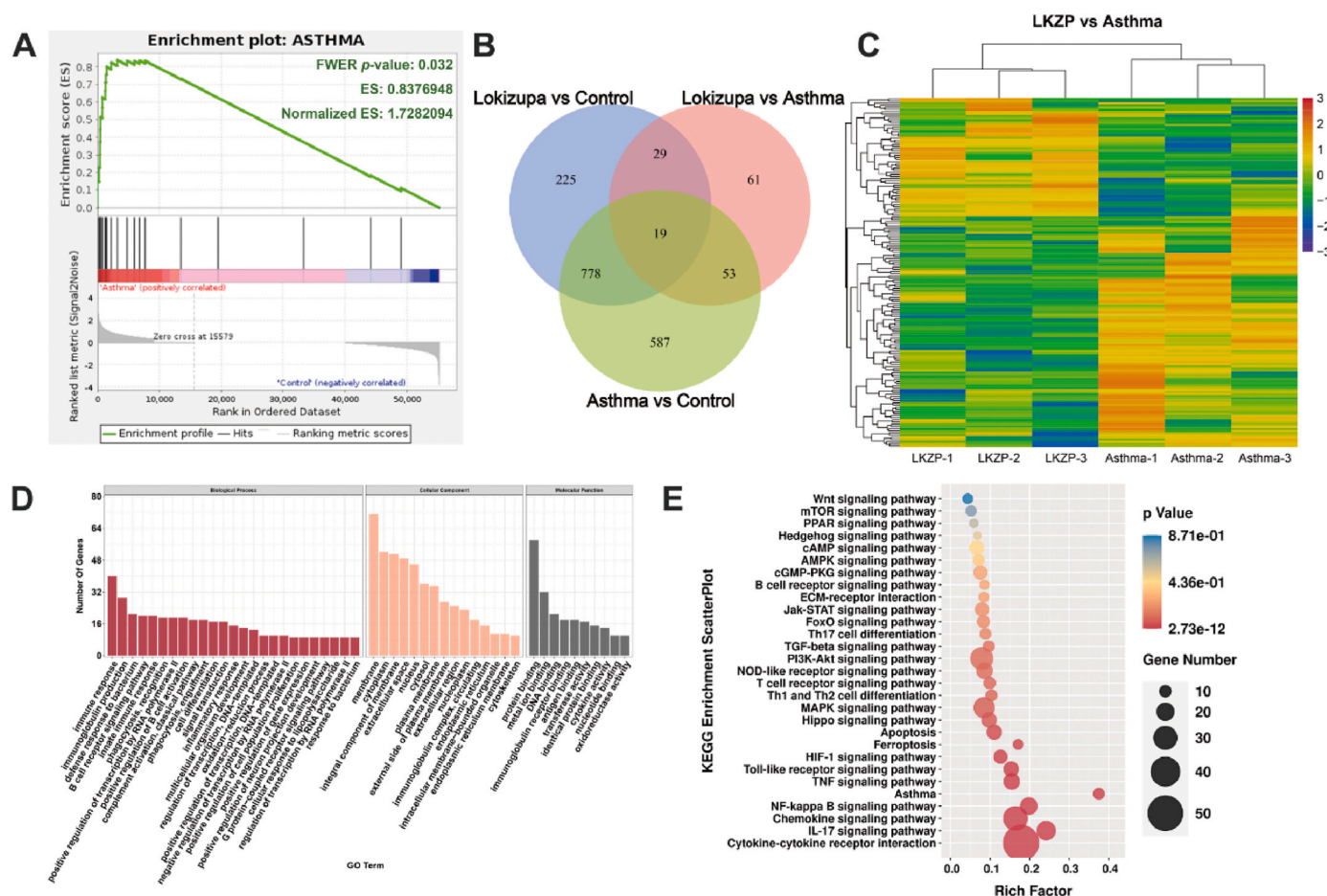


Fig. 6. Transcriptomics analysis of LKZP-treated OVA-induced asthma mice. (A) GSEA enrichment plot: Asthma. Profile of running ES score and positions of GeneSet members on the rank ordered list. (B) DEGs Venn diagram. Different colors represent different combinations of comparisons. (C) The clustering correlation heatmap of DEGs between LKZP and Asthma groups. Color scheme: Rainbow. (D) GO Enrichment BarPlot for DEGs of LKZP vs Asthma. (E) KEGG Enrichment ScatterPlot for DEGs of LKZP vs Asthma. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(NES) = 1.7282094, Nominal p -value = 0.0, FDR q -value = 0.0034580806, FWER p -value = 0.032], as shown in Fig. 6A. To identify potentially affected LKZP pathways, we obtained the following main signaling pathways by introducing DEGs into the KEGG pathway (Fig. 6E) and GO enrichment analysis (Fig. 6D). These results indicate that LKZP functions as an anti-asthma therapy by affecting various biological processes, such as cytokine-cytokine receptor interactions, IL-17 signaling pathway, chemokine signaling pathway, NF-kappa B signaling, TNF signaling pathway, Toll-like receptor signaling pathway, and HIF-1 signaling. Interestingly, some KEGG pathways of DEGs overlapped with candidate network pharmacological target genes, implying that some pivotal target genes might exist, and the GO enrichment results of the functional analysis were also essentially in agreement with LKZP network pharmacology.

These results illustrate that the LKZP decoction plays a significant role in the immune regulation of inflammatory processes. The LKZP decoction may influence IL-17, NF-kB, TNF, and HIF-1 signaling pathways, thus reducing asthma symptoms in model mice.

3.8. Verification of selected differentially expressed genes and associated pathway proteins

According to a combined analysis of network pharmacology and transcriptomics, *Il10*, *Ccl2*, *Ccl20*, *Rela*, *Cxcl2*, *Hif1a*, *Il4*, *Tnf*, and *Il17a* were selected for further validation. We first produced a heat map to show the relative expression of these genes in the control, asthma, and LKZP-treated groups based on mRNA-seq FPKM (Fig. 7A). These genes

were selected because of their association with the inflammatory response, type 2 cell polarization, and inflammatory cell chemotaxis of asthma, and the enrichment of the KEGG pathway that suggests that they may be the key targets of LKZP decoction in the treatment of asthma. We performed qPCR analysis of the nine genes to confirm the results of the DEGs in the lungs. As shown in Fig. 7C/D/E/F, a significant increase in the expression of these nine genes was observed in the asthma group compared with the control group, whereas LKZP treatment completely or partially reversed asthma-induced expression changes in *IL-4* (*Il4*), *IL-17A* (*Il17a*), *TNF- α* (*Tnf*), *NF-kB p65* (*Rela*), *HIF-1 α* (*Hif1a*), *CCL2* (*Ccl2*), *CCL20* (*Ccl20*) and *CXCL2* (*Cxcl2*). It is interesting to note that LKZP can improve the expression of *IL-10*, a cytokine that inhibits inflammation. As anticipated, the qPCR results were consistent with those from mRNA-seq, suggesting that the DEGs resulting from RNA-seq were credible.

To further validate gene expression data at the protein level, we selected two of the above-mentioned nine genes, *NF-kB p65* (*Rela*) and *HIF-1 α* (*Hif1a*) for WB and immunohistochemical analysis. WB analysis showed that the protein expression of *HIF-1 α* and *NF-kB p65* in lung tissue was significantly increased with OVA stimulation ($p < 0.001$). In contrast, the expression of these proteins with LKZP 4.55 and dexamethasone treatments showed significant decreases ($p < 0.05$). Analogously, the results showed that *NF-kB* phosphorylation was remarkably activated in the asthmatic group mice compared with the control group mice. Conversely, LKZP and dexamethasone treatments suppressed the phosphorylation status of *NF-kB* (Fig. 8A/B/C). The IHC analyses showed trends similar to those in the WB analyses. Upregulation of *HIF-*

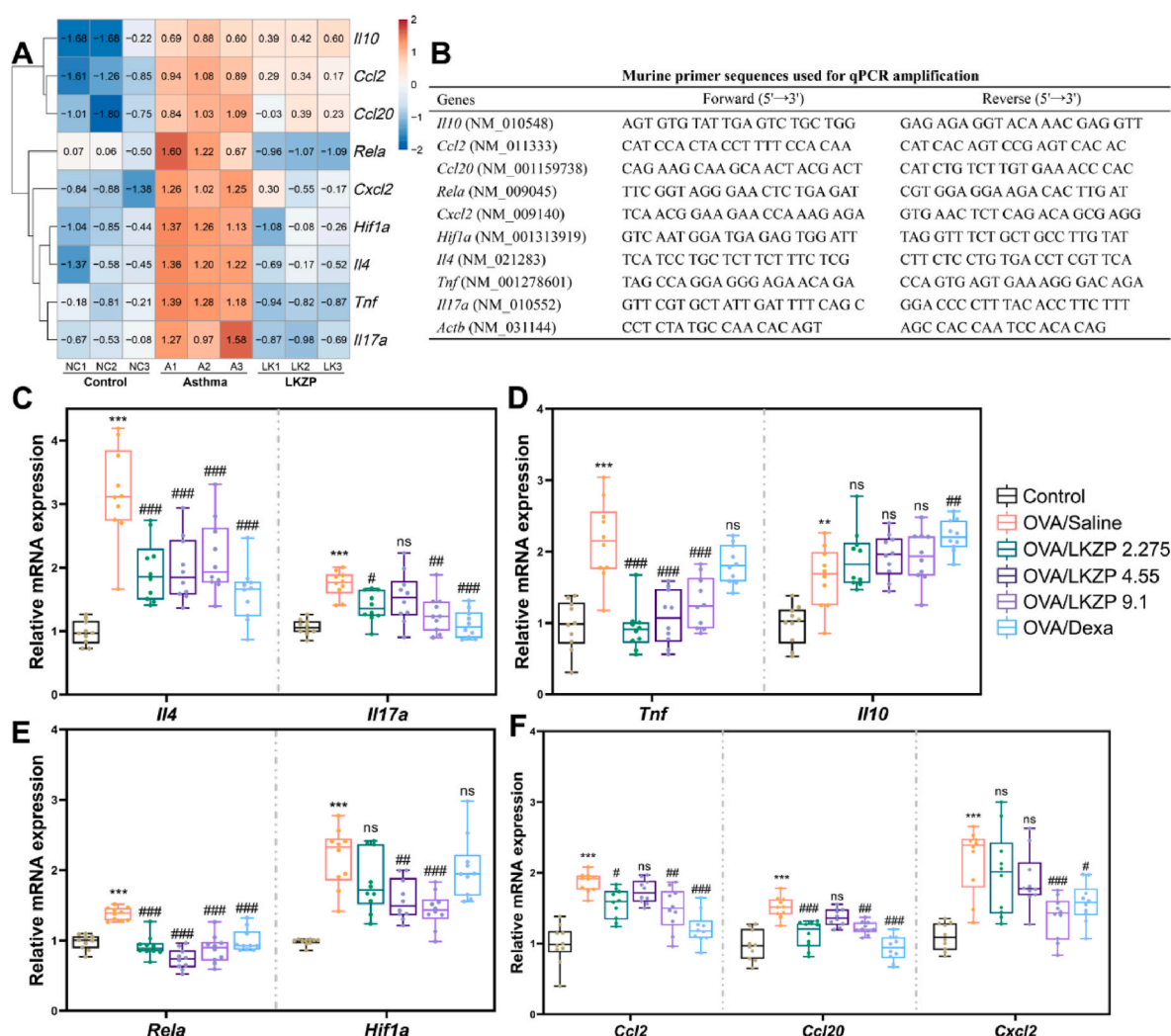


Fig. 7. Assay of gene expression via qPCR confirmed mRNA-seq results. (A) A heat map was created to visualize the data for the relative expression abundance of nine genes screened by RNA-seq. (B) Primer sequences used for qPCR amplification. (C–F) The results of qPCR were performed to analyze IL-4, IL-17A, TNF- α , IL-10, NF- κ B p65, HIF-1 α , CCL2, CCL20 and CXCL2 mRNA expression in the lung tissues. ** $p < 0.01$, *** $p < 0.001$ compared with control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with OVA/Saline group; ns: no significant difference. N = 10 mice/group.

1 α and NF- κ B p65 was observed in the OVA-induced mouse asthma model ($p < 0.001$, respectively), and three doses of LKZP and dexamethasone resulted in significant downregulation of HIF-1 α and NF- κ B p65 protein expression ($p < 0.05$, $p < 0.01$, or $p < 0.001$, Fig. 8D/E/F/G).

4. Discussion

As a classic prescription in the Xinjiang region of western China, LKZP has been extensively used to treat a variety of respiratory diseases, especially chronic airway inflammatory diseases such as bronchial asthma and COPD. Several studies have indicated that LKZP and its constituent herbs can effectively restrain inflammatory processes and regulate immune responses in asthma (Wei et al., 2016; Wuniquiemu et al., 2021). However, due to the complexity and variety of the ingredients involved, the mechanisms underlying the protective effect of LKZP in asthma treatment remain unclear. The development of peripheral disciplines such as systems pharmacology and transcriptomics has made it possible to screen core gene targets associated with LKZP and its components that are relevant to asthma treatment.

In the process of animal experiments, we noticed that LKZP decoction could improve the overall state and respiratory symptoms of acute allergic asthma model mice, but it is a comparatively subjective

judgment. In this study, pulmonary function and pathological results were used as the gold standard to confirm the anti-asthmatic effect of LKZP. Our data indicated LKZP could ameliorate lung function and bronchial hyperreactivity, reduce the number of inflammatory cells in the BALF, especially the percentage of Eos and Lym, and attenuate airway inflammation and mucus hypersecretion by regulating the type 2 immune response in OVA-induced asthmatic mice. LKZP treatment downregulated the expression of multiple cytokines related to immunity and inflammation, such as IgE, IL-4, IL-5, IL-13, and TNF- α , both in the BALF and serum. Hence, our results provide evidence of a novel anti-inflammatory role of LKZP that results in symptomatic improvement and downregulation of inflammatory responses among mice. Next, we conducted network pharmacology and mRNA-seq analysis and preliminarily verified that LKZP decoction may exert its therapeutic function by regulating the expression of HIF-1 α and NF- κ B p65 in the lung tissue of OVA-induced asthmatic mice.

When OVA was injected into mice, its soluble antigen component stimulated the body to produce a high IgE titer, which put the body in a sensitized state. A few days later, the experimental animals were re-exposed to the antigen, mediating an antigen-antibody reaction that degranulated mast cells and released active chemicals such as histamines, cysteinyl leukotrienes (CysLTs), and eosinophil chemokines. These act on the bronchus to cause AHR and inflammatory cell

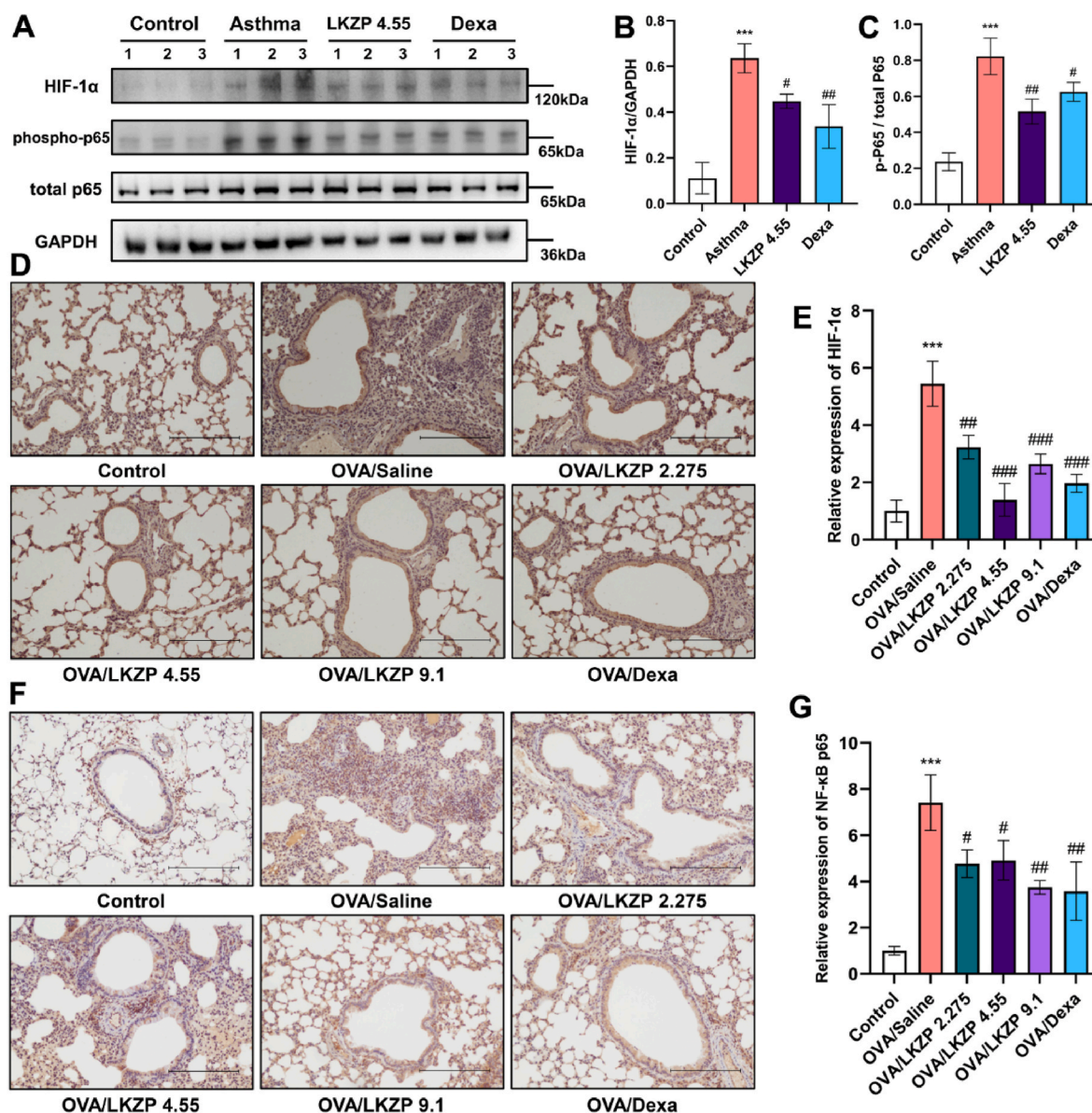


Fig. 8. Further clarification on the mechanism of LKZP in reducing airway inflammation in OVA-induced asthmatic mice at the protein level. (A) The protein expression of HIF-1α and NF-κB p65 was determined by means of Western blotting. (B) The relative density quantification of HIF-1α. The results were expressed as the ratio of HIF-1α to GAPDH. (C) The relative density quantification of NF-κB p65. The results were expressed as the ratio of phosphorylated p65 to total p65. (D, E) Immunohistochemical analysis of HIF-1α in the lung slices in each group, magnification: $\times 100$; scale bar: 200 μ m. (F, G) Immunohistochemical analysis of NF-κB p65 in the lung slices in each group, Bar = 200 μ m. Data were shown as mean $\pm$ SD analyzed by one-way ANOVA. N = 3 mice/group. ***p < 0.001 vs control group, #p < 0.05, ##p < 0.01, ###p < 0.001 vs Asthma group.

infiltration, which represent the characteristics of allergic asthma (Casaro et al., 2019). Reversible airway obstruction, bronchial hyper-responsiveness, and Eos-rich pulmonary inflammation are the most striking features of allergic asthma, continuously demonstrated by increased R_L , decreased lung Cdyn, and a significant increase in the infiltration of leukocytes, especially Eos, *in vivo*. This explains the use of bronchodilators and glucocorticoids as the standard regimen for asthma treatment (Mims, 2015). The preparation process used in our previous report in the treatment of asthmatic mice (Mohammadtursun et al., 2020; Wei et al., 2016) and patients (Lv et al., 2018) using LKZP was a decocting method of TCM, but in the current study we used a water extraction and alcohol precipitation method based on the screening of several different preparation processes, so as to facilitate the large-scale standardized production of “LKZP oral liquid” in the future. Here, our results confirmed that LKZP decoction extract could diminish AHR, inflammatory infiltration, and mucus hypersecretion, but not collagen

deposition in OVA-induced asthmatic model mice. Although three dosages of LKZP ameliorated AHR, they had little effect on Cdyn, particularly when the concentration of methacholine was increased to 12.5 mg/ml. Treatment with LKZP resulted in a dose-dependent decrease in the number of total white blood cells and Eos in the BALF. Another fascinating finding was that LKZP reduced the count of Eos and Neu simultaneously, while dexamethasone increased the percentage of Neu but reduced Eos, indicating that the anti-asthmatic mechanisms of LKZP and Dexamethasone would likely be different. LKZP decoction extract with 4.55 g/kg dosage exerted a more pronounced reduction in the number of leukocyte subpopulations in the BALF, as well as mucus secretion mitigation using PAS staining, while 9.1 g/kg dosage of LKZP manifested a curative effect in lung tissue resident inflammatory cells via H&E staining. Moreover, LKZP and dexamethasone treatment groups tested in the current study had no significant effect on airway collagen deposition. This might be due to the fact that airway remodeling and

fibrosis were not evident in the acute asthma model (Alessandrini et al., 2020).

IgE and cytokines play crucial roles in allergic responses and asthmatic inflammation. Increased secretion of the type 2 cytokines IL-4, IL-5, and IL-13 were reported in allergic asthma, promoting airway eosinophilia, mucus overproduction, AHR, and IgE synthesis (Lambrecht et al., 2019). Our study demonstrated that IgE, IL-4, IL-5, IL-13, and TNF- α levels in both serum and the BALF declined with LKZP treatment, which in part explained the efficacy of LKZP in treating asthma. As shown in Fig. 9A (Chung, 2015), airway type 2 immune responses are mainly mediated by airway epithelial cells, Th2 cells, group 2 innate lymphoid cells (ILC2s), Eos, mast cells, Bas, and IgE-producing B cells, the mechanism of which is understood in detail (Fahy, 2015; Porsbjerg et al., 2020). Interactions between anaphylactogens (e.g., OVA) and innate and adaptive immune and inflammatory responses begin with airway epithelial cells, alveolar macrophages, and dendritic cells (DCs) with the release of thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 (Hong et al., 2020). Epithelial cytokines broadly stimulate innate type 2 cells such as ILC2s and boost DC function to polarize CD4⁺ T (Th0) cells and promote Th2 cells that express the GATA3, IL-33R, and chemoattractant receptor homologous molecule of Th2 cells (CRTH2). Th2 cells and ILC2s produce cytokines that induce goblet cell metaplasia (IL-13), tissue eosinophilia (IL-5), bronchial smooth muscle contraction (IL-13), polyclonal IgE synthesis via allergen-specific B cell activation (IL-4), and mast cell degranulation (IL-9). Fig. 9A shows the speculated cytokine network that LKZP regulates to reduce the expression of inflammatory mediators, thereby reducing airway inflammation in asthmatic mice.

Since TCM is well known for its unique treatment model, such as the synergistic effect of multiple components acting on multiple pathways and targets, treatment based on syndrome, and regulation of immune balance, clarifying the specific targets of LKZP in modern medical terms remains a major challenge. Network pharmacology was first proposed by Andrew et al., in 2007 (Hopkins, 2007). It is a new interdisciplinary field based on the theory of system biology that uses bioinformatics and

network analysis methods to study the mechanism of drug action and design multi-target drug molecules. The core problem we solved using network pharmacology is to evaluate the multi-component and multi-target synergy of LKZP in the context of asthma-related networks. In order to further explore the mechanism of pharmacological effects of LKZP, we first collected the target proteins of the chemical compounds in LKZP from multiple databases through network pharmacological analysis. Subsequently, the LKZP-component-target-asthma network was constructed to predict the potential anti-inflammatory mechanism of LKZP. In the KEGG enrichment assay, we observed that the HIF-1 α and NF- κ B signaling pathways are two important target pathways. The similar result was further verified in the KEGG enrichment of DEGs in mRNA-seq. We used qPCR, WB, and immunohistochemical staining to measure the expression of HIF-1 α and NF- κ B p65 in lung tissues. HIF-1 α and NF- κ B were found to play a role in the anti-asthma effects of LKZP *in vivo*. In general, network pharmacology has certain benefits in understanding drug-body interactions that improve or restore the balance of biological networks and revealing the mechanism of multi-component synergistic treatment of diseases. However, in the current network pharmacology research of TCM, the selection of chemical components and gene targets mainly depends on the analysis of literature and databases which lacks pertinence. It is also impossible to distinguish whether drugs activate or inhibit target pathways and receptor molecules (Zhu et al., 2021). Transcriptome sequencing can compensate for these shortcomings. The transcriptome is the necessary link between the genetic information of the genome and the biological function of the proteome. The regulation of transcription levels is the most studied and pivotal regulation pattern in organisms. Transcriptomic analysis of lung tissues using mRNA-seq could obtain high-throughput and detailed gene expression profile data at the tissue level. mRNA-seq results suggested that 246 DEGs were identified among the OVA/LKZP vs. the OVA/saline group, indicating multiple gene targets for LKZP. We also verified the nine DEGs selected through qPCR and confirmed that their variation trends were consistent with the transcriptomic analysis. Similarly, we performed KEGG enrichment analysis on the differentially expressed

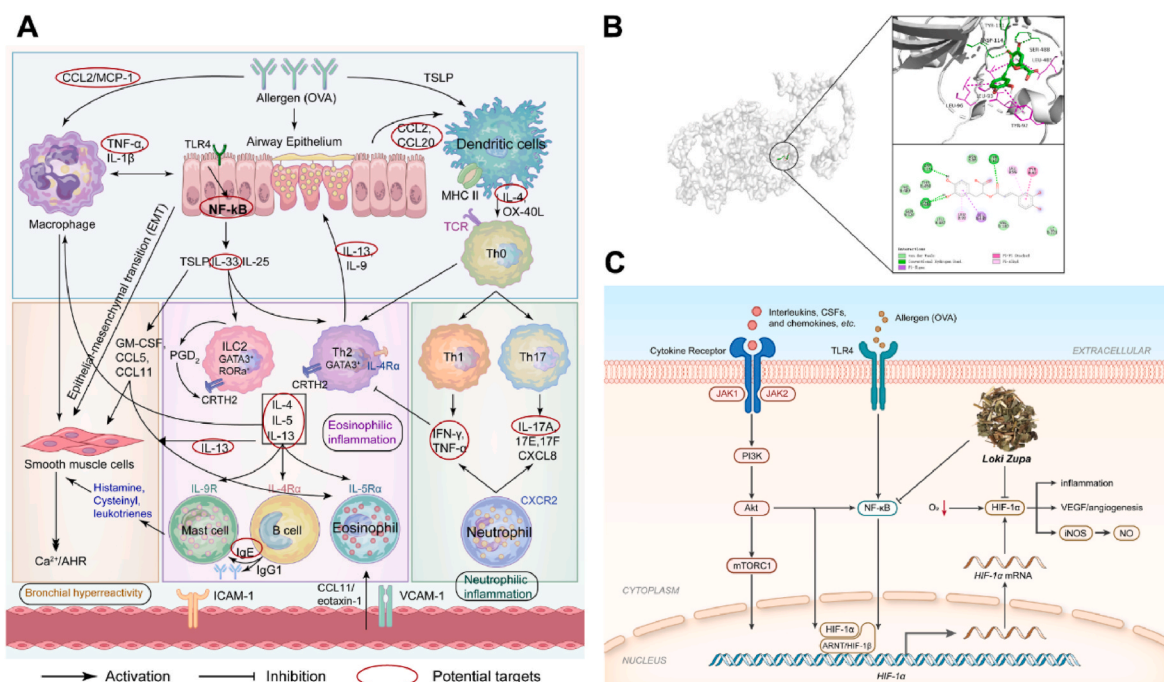


Fig. 9. Schematic illustration of targets prediction of LKZP in the treatment of asthma. (A) Pathophysiological mechanisms underlying OVA-induced allergic asthma and targets for LKZP therapy. TLR = Toll-like receptor. TSLP = thymic stromal lymphopoietin. ILC2 = type 2 innate lymphoid cells. Th0/1/2/17 = T-helper-0/1/2/17 cell. PGD₂ = prostaglandin D₂. ICAM-1 = intercellular adhesion molecule 1. VCAM-1 = vascular cell adhesion molecule 1. GM-CSF = granulocyte-macrophage colony-stimulating factor. (B) A docking model of rosmarinic acid (ball-and-stick) binding to the domain of HIF-1 α (shown in the white cartoon). (C) Diagrammatic sketch of LKZP acting on NF- κ B and HIF-1 signaling pathways.

genes obtained by transcriptome sequencing. As previously mentioned, combined with the KEGG enrichment analysis results of network pharmacology and mRNA-seq, we screened two pathways closely related to asthma, the NF- κ B pathway, and the HIF-1 α pathway.

NF- κ B, an important transcription factor, is known to play a critical role in inflammation. Phosphorylation of NF- κ B RelA (p65) is highly activated during pulmonary inflammation (Alharbi et al., 2021). Our study consistently found that OVA exposure accelerated the activation of NF- κ B in asthmatic mice compared to the control group (Mishra et al., 2018; Yi et al., 2020). In this study, PCR, WB, and IHC demonstrated that upregulated NF- κ B signaling was pinned down by LKZP, whose curative effect was similar to that of dexamethasone. In addition, we found that hypoxia may be an important potential factor that induces asthma. HIF-1 is a heterodimer composed of HIF-1 α , a protein expressed in an oxygen-regulated manner, and a constitutively expressed factor, HIF-1 β (Moslehi and Rathmell, 2020). The lung is highly sensitive to oxygen concentration. Under hypoxic conditions, inflammatory mediators are released and upregulate the expression of HIF-1 α through the PI3K/Akt, MAPK, and NF- κ B pathways in the lungs. Although a growing body of evidence suggests that HIF-1 α plays an important role in immune and inflammatory responses, its underlying mechanism remains controversial (Huerta-Yepez et al., 2011; Ning et al., 2019). Therefore, we also verified in further molecular biology experiments that LKZP may influence the asthma inflammatory response induced by OVA by regulating HIF-1 α . According to the results of HPLC, rosmarinic acid and IH-1 were identified as the main index components of *Hyssopus cuspidatus* Boriss (Shenxiangcao) and *Iris halophila* Pall, respectively. Recent studies have suggested that rosmarinic acid, a water-soluble phenolic compound extracted from *Hyssopus cuspidatus* Boriss (Shenxiangcao) of LKZP (Shomirzoeva et al., 2020; Zhao et al., 2020), is a natural antioxidant with therapeutic effects against oxidative stress *in vivo* and *in vitro* (Chung et al., 2020; Khojasteh et al., 2020; Luo et al., 2020; Petersen and Simmonds, 2003). Thus, we can assume that rosmarinic acid exerts anti-oxidant and anti-inflammatory effects in lung tissue by suppressing the activity of HIF-1 α . This hypothesis also lies at the root of new ideas for molecular docking simulations *in silico* to confirm the binding of rosmarinic acid with HIF-1 α (Pinzi and Rastelli, 2019). As shown in Fig. 9B, the results of the virtual calculations were carried out using AutoDock Vina (version 1.1.2) (Trott and Olson, 2010), followed by AutoDock Tools (version 4.2.6) (Morris et al., 2009). The predicted binding affinity between rosmarinic acid and HIF-1 α was -7.1 kcal/mol. In short, rosmarinic acid was stabilized by four strong hydrogen bonds with residues Ser 488, Asp 114, Tyr 111, one π - σ bond with Leu 93, two π -alkyl bonds with Leu 93 and Leu 96, one π - π stacked hydrophobic interaction with Tyr 92, and several van der Waals contacts at the catalytic site of the enzyme. Accordingly, we conjectured that rosmarinic acid had good binding activities to HIF-1 α , whether it played an inhibitory or activating role required comprehensive consideration of binding site information.

Although systems pharmacology and transcriptomics can effectively make up for the defects of single target and low selectivity in the previous research of TCM, they still face some challenges in practical application. On the one hand, LKZP is a compound composed of two herbs. Its efficacy is based on the efficacy of a single herb, but it is not simply the sum of the efficacy of a single herb. There is a complex interaction between the two herbs in the process of preparation and extraction, and their efficacy is the result of the coordinated and comprehensive action of a variety of chemical components. On the other hand, whether in system pharmacology or transcriptomics, target prediction is based on the existing database, so its accuracy largely depends on network server and computer algorithm. Therefore, the mechanisms of LKZP in the treatment of allergic asthma still needs to be further confirmed by *in vivo*, *in vitro* experiments, and clinical trials.

5. Conclusion

The present study focused on a new pharmacodynamics mechanism of LKZP based on the balanced regulation theory of pro-inflammation and anti-inflammation. Using systems pharmacology and transcriptomics, we revealed that LKZP could effectively alleviate asthma-induced AHR and airway inflammation. Our preliminary conclusion is that LKZP regulates NF- κ B p65 and HIF-1 α protein expression (Fig. 9C), which may underlie the anti-inflammatory effects of the active components in LKZP. However, the precise active ingredients and corresponding targets in LKZP need to be further investigated.

CRedit authorship contribution statement

Cong Xie: Validation, Investigation, Formal analysis, Resources, Data curation, Writing – original draft, Visualization. **Aman Gul:** Software, Investigation, Formal analysis. **Hang Yu:** Resources, Investigation. **Xi Huang:** Investigation. **Lingling Deng:** Investigation. **Yue Pan:** Resources, Investigation. **Shuangshuang Ni:** Resources. **Mammat Nurahmat:** Resources. **Muhammadjan Abduwaki:** Resources. **Qingli Luo:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Funding acquisition. **Jingcheng Dong:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Acknowledgments

This work was financially supported by grants from the National Key R&D Program of China (No. 2018YFC1708300), the National Natural Science Foundation of China (No. 82004318), the Special Project for INTERNATIONAL COOPERATION in TCM of the National Administration of Traditional Chinese Medicine (No. 0610-2140NF020631), and the Innovative Research Team of High-level Local Universities in Shanghai (Clinical and Basic Research on the Prevention and Treatment of Some Inflammatory Diseases by Integrative Medicine).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2022.115351>.

References

- Alessandrini, F., Musiol, S., Schneider, E., Blanco-Perez, F., Albrecht, M., 2020. Mimicking antigen-driven asthma in rodent models-how close can we get? *Front. Immunol.* 11, 575936.
- Alharbi, K.S., Fuloria, N.K., Fuloria, S., Rahman, S.B., Al-Malki, W.H., Javed Shaikh, M. A., Thangavelu, L., Singh, S.K., Rama Raju Allam, V.S., Jha, N.K., Chellappan, D.K., Dua, K., Gupta, G., 2021. Nuclear factor-kappa B and its role in inflammatory lung disease. *Chem. Biol. Interact.* 345, 109568.
- Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. *The Gene Ontology Consortium. Nat. Genet.* 25 (1), 25–29.
- Aun, M.V., Bonamichi-Santos, R., Arantes-Costa, F.M., Kalil, J., Giavina-Bianchi, P., 2017. Animal models of asthma: utility and limitations. *J. Asthma Allergy* 10, 293–301.
- Caramori, G., Nucera, F., Mumby, S., Lo Bello, F., Adcock, I.M., 2021. Corticosteroid resistance in asthma: cellular and molecular mechanisms. *Mol. Aspect. Med.*, 100969.
- Casaro, M., Souza, V.R., Oliveira, F.A., Ferreira, C.M., 2019. OVA-induced allergic airway inflammation mouse model. *Methods Mol. Biol.* 1916, 297–301.
- Chung, C.H., Jung, W., Keum, H., Kim, T.W., Jon, S., 2020. Nanoparticles derived from the natural antioxidant rosmarinic acid ameliorate acute inflammatory bowel disease. *ACS Nano* 14 (6), 6887–6896.

- Chung, K.F., 2015. Targeting the interleukin pathway in the treatment of asthma. *Lancet* 386 (9998), 1086–1096.
- Davis, A.P., Grondin, C.J., Johnson, R.J., Sciaky, D., Wieggers, J., Wieggers, T.C., Mattingly, C.J., 2021. Comparative toxicogenomics database (CTD): update 2021. *Nucleic Acids Res.* 49 (D1), D1138–D1143.
- Derendorf, H., Nave, R., Drollmann, A., Cerasoli, F., Wurst, W., 2006. Relevance of pharmacokinetics and pharmacodynamics of inhaled corticosteroids to asthma. *Eur. Respir. J.* 28 (5), 1042–1050.
- Fahy, J.V., 2015. Type 2 inflammation in asthma—present in most, absent in many. *Nat. Rev. Immunol.* 15 (1), 57–65.
- Gene Ontology, C., 2021. The Gene Ontology resource: enriching a Gold mine. *Nucleic Acids Res.* 49 (D1), D325–D334.
- Glaab, T., Daser, A., Braun, A., Neuhaus-Steinmetz, U., Fabel, H., Alarie, Y., Renz, H., 2001. Tidal midexpiratory flow as a measure of airway hyperresponsiveness in allergic mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280 (3), L565–L573.
- Hong, H., Liao, S., Chen, F., Yang, Q., Wang, D.Y., 2020. Role of IL-25, IL-33, and TSLP in triggering united airway diseases toward type 2 inflammation. *Allergy* 75 (11), 2794–2804.
- Hopkins, A.L., 2007. Network pharmacology. *Nat. Biotechnol.* 25 (10), 1110–1111.
- Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4 (1), 44–57.
- Huang, L., Xie, D., Yu, Y., Liu, H., Shi, Y., Shi, T., Wen, C., 2018. Tcmid 2.0: a comprehensive resource for TCM. *Nucleic Acids Res.* 46 (D1), D1117–D1120.
- Huerta-Yepetz, S., Baay-Guzman, G.J., Bebenek, I.G., Hernandez-Pando, R., Vega, M.I., Chi, L., Riedl, M., Diaz-Sanchez, D., Kleerup, E., Tashkin, D.P., Gonzalez, F.J., Bonavida, B., Zeidler, M., Hankinson, O., 2011. Hypoxia inducible factor promotes murine allergic airway inflammation and is increased in asthma and rhinitis. *Allergy* 66 (7), 909–918.
- Jin, H., Luo, Q., Zheng, Y., Nurahmat, M., Wu, J., Li, B., Lv, Y., Wang, G., Duan, X., Dong, J., 2013. CD4+CD25+Foxp3+ T cells contribute to the antiasthmatic effects of Astragalus membranaceus extract in a rat model of asthma. *Int. Immunopharm.* 15 (1), 42–49.
- Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res.* 44 (D1), D457–D462.
- Khojasteh, A., Mirjalili, M.H., Alcalde, M.A., Cusido, R.M., Eibl, R., Palazon, J., 2020. Powerful plant antioxidants: a new biosustainable approach to the production of rosmarinic acid. *Antioxidants* 9 (12).
- Lambrecht, B.N., Hammad, H., Fahy, J.V., 2019. The cytokines of asthma. *Immunity* 50 (4), 975–991.
- Lin, J., Yang, X., Liu, S., Luo, Z., Chen, Q., Sun, Y., Ding, Z., Chen, J., 2021. Long non-coding RNA MFAT1 promotes skeletal muscle fibrosis by modulating the miR-135a-5p-Tgfb β 2/Smad4 axis as a ceRNA. *J. Cell Mol. Med.* 25 (9), 4420–4433.
- Liu, Z., Guo, F., Wang, Y., Li, C., Zhang, X., Li, H., Diao, L., Gu, J., Wang, W., Li, D., He, F., 2016. BATMAN-TCM: a bioinformatics analysis tool for molecular mechanism of traditional Chinese medicine. *Sci. Rep.* 6, 21146.
- Luo, C., Zou, L., Sun, H., Peng, J., Gao, C., Bao, L., Ji, R., Jin, Y., Sun, S., 2020. A review of the anti-inflammatory effects of rosmarinic acid on inflammatory diseases. *Front. Pharmacol.* 11, 153.
- Lv, Y., Wei, Y., Abduwaki, M., Jurat, T., Li, F., Wang, H., Wu, Y., Li, Z., Liu, B., Yin, H., Cao, Y., Nurahmat, M., Tang, Z., Dong, J., 2018. A multicenter, randomized, double-blind, placebo-controlled study of the effects of Loki zupa in patients with chronic asthma. *Front. Pharmacol.* 9, 351.
- Mims, J.W., 2015. Asthma: definitions and pathophysiology. *Int. Forum Allergy Rhinol.* 5 (Suppl. 1), S2–S6.
- Mishra, V., Banga, J., Silveyra, P., 2018. Oxidative stress and cellular pathways of asthma and inflammation: therapeutic strategies and pharmacological targets. *Pharmacol. Ther.* 181, 169–182.
- Mohammadursun, N., Li, Q., Abduwaki, M., Jiang, S., Zhang, H., Sun, J., Dong, J., 2020. Loki zupa alleviates inflammatory and fibrotic responses in cigarette smoke induced rat model of chronic obstructive pulmonary disease. *Chin. Med.* 15, 92.
- Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., Olson, A.J., 2009. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. *J. Comput. Chem.* 30 (16), 2785–2791.
- Mosleh, J., Rathmell, W.K., 2020. The 2019 Nobel Prize honors fundamental discoveries in hypoxia response. *J. Clin. Invest.* 130 (1), 4–6.
- Ning, F., Takeda, K., Schedel, M., Domenico, J., Joetham, A., Gelfand, E.W., 2019. Hypoxia enhances CD8(+) TC2 cell-dependent airway hyperresponsiveness and inflammation through hypoxia-inducible factor 1 α . *J. Allergy Clin. Immunol.* 143 (6), 2026–2037 e2027.
- Papi, A., Brightling, C., Pedersen, S.E., Reddel, H.K., 2018. Asthma. *Lancet* 391 (10122), 783–800.
- Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T., Salzberg, S.L., 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 33 (3), 290–295.
- Petersen, M., Simmonds, M.S., 2003. Rosmarinic acid. *Phytochemistry* 62 (2), 121–125.
- Pinero, J., Ramirez-Anguaita, J.M., Sauch-Pitarch, J., Ronzano, F., Centeno, E., Sanz, F., Furlong, L.I., 2020. The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Res.* 48 (D1), D845–D855.
- Pinzi, L., Rastelli, G., 2019. Molecular docking: shifting paradigms in drug discovery. *Int. J. Mol. Sci.* 20 (18).
- Porsbjerg, C.M., Sverrild, A., Lloyd, C.M., Menzies-Gow, A.N., Bel, E.H., 2020. Anti-alarmers in asthma: targeting the airway epithelium with next-generation biologics. *Eur. Respir. J.* 56 (5).
- Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26 (1), 139–140.
- Ru, J., Li, P., Wang, J., Zhou, W., Li, B., Huang, C., Li, P., Guo, Z., Tao, W., Yang, Y., Xu, X., Li, Y., Wang, Y., Yang, L., 2014. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. *J. Cheminf.* 6, 13.
- Shomirzoeva, O., Li, J., Numonov, S., Atolikhoeva, S., Aisa, H.A., 2020. Chemical components of *Hyssopus cuspidatus* Boriss.: isolation and identification, characterization by HPLC-DAD-ESI-HRMS/MS, antioxidant activity and antimicrobial activity. *Nat. Prod. Res.* 34 (4), 534–540.
- Stelzer, G., Rosen, N., Plaschkes, I., Zimmerman, S., Twik, M., Fishilevich, S., Stein, T.I., Nudel, R., Lieder, I., Mazor, Y., Kaplan, S., Dahary, D., Warshawsky, D., Guan-Golan, Y., Kohn, A., Rappaport, N., Safran, M., Lancet, D., 2016. The GeneCards suite: from gene data mining to disease genome sequence analyses. *Curr. Protoc. Bioinfo.* 54, 13031–313033.
- Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* 102 (43), 15545–15550.
- Sulaiman, I., Tan, K., Mohtarrudin, N., Lim, J.C.W., Stanslas, J., 2018. Andrographolide prevented toluene diisocyanate-induced occupational asthma and aberrant airway E-cadherin distribution via p38 MAPK-dependent Nrf2 induction. *Pulm. Pharmacol. Therapeut.* 53, 39–51.
- Tachdjian, R., Al Khatib, S., Schwingshackl, A., Kim, H.S., Chen, A., Blasioli, J., Mathias, C., Kim, H.Y., Umetsu, D.T., Oettgen, H.C., Chatila, T.A., 2010. In vivo regulation of the allergic response by the IL-4 receptor α chain immunoreceptor tyrosine-based inhibitory motif. *J. Allergy Clin. Immunol.* 125 (5), 1128–1136 e1128.
- Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31 (2), 455–461.
- Wang, J., Wu, J., Kong, L., Nurahmat, M., Chen, M., Luo, Q., Li, B., Wu, X., Dong, J., 2014. BuShenYiQi Formula strengthens Th1 response and suppresses Th2-Th17 responses in RSV-induced asthma exacerbated mice. *J. Ethnopharmacol.* 154 (1), 131–147.
- Wei, Y., Abduwaki, M., Li, M., Luo, Q., Sun, J., Lv, Y., Nurahmat, M., Dong, J., 2016. Loki zupa (Luoukeuzupa) decoction reduced airway inflammation in an OVA-induced asthma mouse model. *Chin. Med.* 11, 22.
- Wuniquiem, T., Qin, J., Teng, F., Nabijan, M., Cui, J., Yi, Tang, W., Zhu, X., Abduwaki, M., Nurahmat, M., Wei, Y., Dong, J.C., 2021. Quantitative proteomic profiling of targeted proteins associated with Loki Zupa Decoction Treatment in OVA-Induced asthmatic mice. *J. Ethnopharmacol.* 266, 113343.
- Ye, H., Ye, L., Kang, H., Zhang, D., Tao, L., Tang, K., Liu, X., Zhu, R., Liu, Q., Chen, Y.Z., Li, Y., Cao, Z., 2011. HIT: linking herbal active ingredients to targets. *Nucleic Acids Res.* 39, D1055–D1059. Database issue.
- Yi, Cui, J., Wang, W., Tang, W., Teng, F., Zhu, X., Qin, J., Wuniquiem, T., Sun, J., Wei, Y., Dong, J., 2020. Formononetin attenuates airway inflammation and oxidative stress in murine allergic asthma. *Front. Pharmacol.* 11, 533841.
- Zhao, L., Ji, Z., Li, K., Wang, B., Zeng, Y., Tian, S., 2020. HPLC-DAD analysis of *Hyssopus cuspidatus* Boriss extract and mensuration of its antioxygenation property. *BMC Complement Med. Ther.* 20 (1), 228.
- Zhu, H., Wang, S., Shan, C., Li, X., Tan, B., Chen, Q., Yang, Y., Yu, H., Yang, A., 2021. Mechanism of protective effect of xuan-Bai-cheng-qi decoction on LPS-induced acute lung injury based on an integrated network pharmacology and RNA-sequencing approach. *Respir. Res.* 22 (1), 188.