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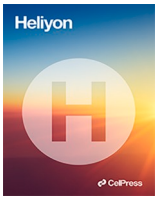

Research article

Mitigation of allergic asthma in mice: A compound mixture comprising luteolin, arbutin, and marmesin from Gerbera Piloselloides Herba by suppression of PI3K/Akt pathway

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ABSTRACT

Background: Gerberae Piloselloidis Herba (GPH) exhibits notable efficacy in alleviating allergic asthma. Previous studies in our research have identified a mixture of luteolin, arbutin, and marmesin as effective components of GPH in treating allergic asthma. However, the underlying mechanism remains unclear. This study aims to elucidate the molecular mechanism of these active components.

Method: Using an ovalbumin (OVA)-induced allergic asthma mouse model, various treatment groups were administered, including GPH, the active component mixture (termed "Mixture") containing luteolin, arbutin, and marmesin, and a positive drug (dexamethasone, DEX). Relevant indices were assessed, including behavioral characteristics, inflammatory cell counts, cytokine levels, histopathological examination of lung tissue, apoptosis, and expression of key proteins such as Caspase-3, Bax, Bcl-2, PI3K, p-PI3K, Akt, and p-Akt. The effect of the Mixture on the PI3K/Akt signaling pathway was further verified using the PI3K inhibitor LY294002.

Results: The Mixture significantly alleviated asthma symptoms, decreased IgE levels, cytokine levels (IL-4, IL-5, IL-13 and TNF-α), and the number of inflammatory cells in serum or bronchoalveolar lavage fluid (BALF), leading to the alleviation of lung pathological lesions. Additionally, the Mixture reduced the expression of Bax and Caspase-3 while increasing Bcl-2 expression, resulting in mitigated apoptosis in lung tissue. Furthermore, there appeared a decrease in the levels of PI3K and p-PI3K, as well as the ratio of p-Akt to Akt in the Mixture group, indicating the suppression of PI3K and Akt phosphorylation. Interestingly, the effects of the Mixture were comparable to those of GPH, LY294002, or the combination of LY294002 with the Mixture.

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Conclusion: The study confirms that the Mixture containing luteolin, arbutin, and marmesin indeed alleviates allergic asthma induced by OVA in mice by suppressing the PI3K/Akt signaling pathway. These findings highlight the potential of the GPH-derived Mixture as a novel therapeutic for the treatment of allergic asthma.

1. Introduction

Asthma is a complex and heterogeneous condition characterized by chronic airway inflammation, leading to symptoms such as airway hyperresponsiveness, inflammation, and remodeling. These manifestations arise from intricate interactions among various cell

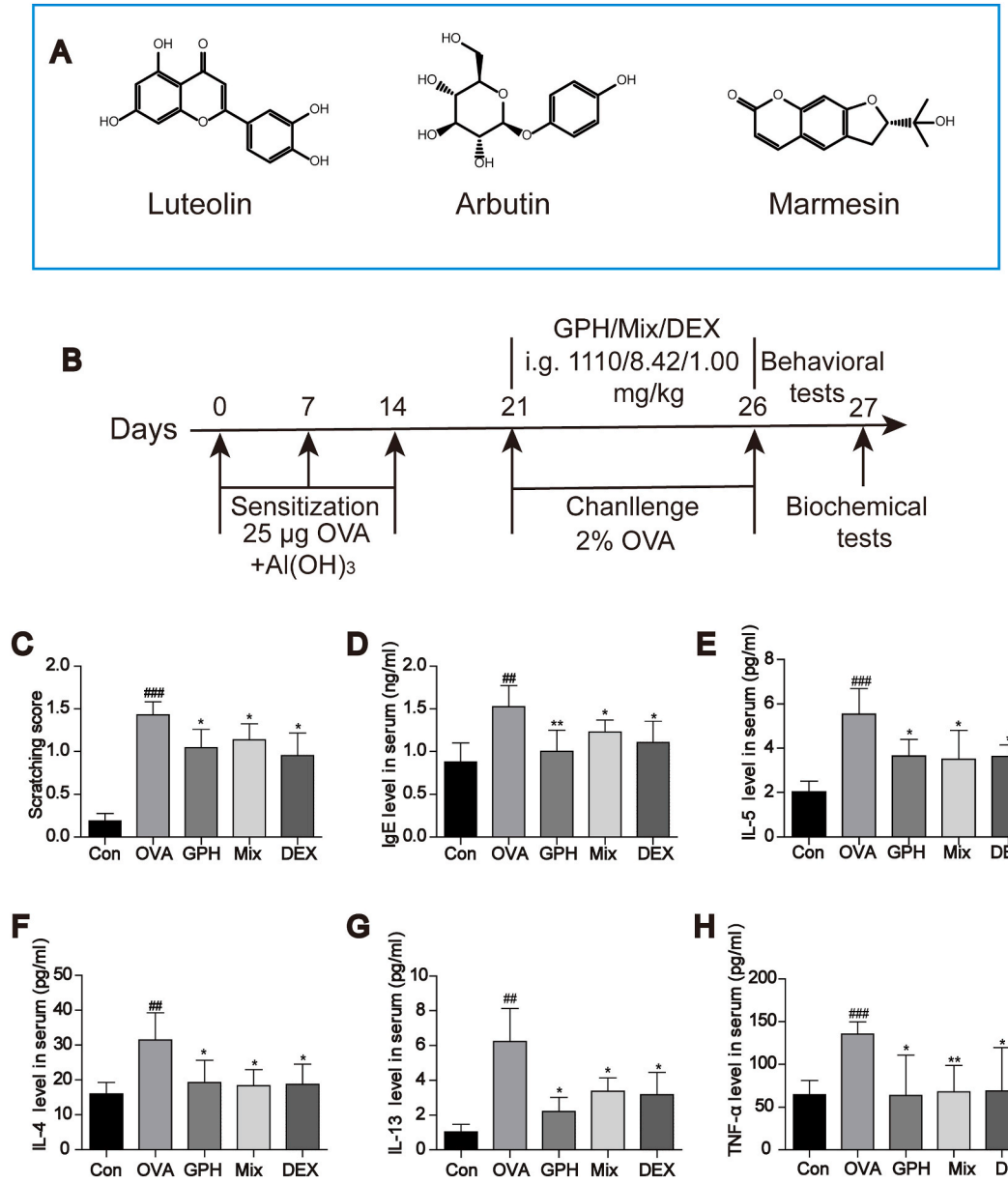

Fig. 1. Mixture ameliorated asthma symptoms and reduced levels of IgE, IL-5, IL-4, IL-13, and TNF-α in the serum of OVA-induced mice. A: Chemical structures of luteolin, arbutin, and marmesin; B: Workflow of asthma model establishment and treatment; C: Effects of different drugs on the behavior of OVA-induced asthma mice; D ~ H: The levels of IgE, IL-5, IL-4, IL-13 and TNF-α in the serum of mice. Compared with the Con group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared with the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$; mean ± SD, n = 6.

types, including epithelial cells [1]. To date, asthma's incidence is still escalating, with a prediction of 20–25 % annual increase, potentially affecting 400 million individuals by 2025, causing significant health and economic burdens [2,3]. Among asthma sufferers, allergies are predominant, constituting 66–83 % of cases [4]. Allergic asthma, associated with type II immune responses, is a chronic respiratory ailment involving the increase in Th2 differentiation from T cells and Th2 cytokine production (IL-4, IL-5, IL-13, etc.) [5–7]. Mitigating asthma attacks is achievable; however, whereas managing recurrent episodes remains challenging. Although anti-inflammatory therapy benefits most patients with allergic asthma, approximately 5–10 % require doses beyond the standard, with some progressing to severe asthma [8]. Conventional clinical medications like glucocorticoids, leukotriene receptor antagonists, cholinergic antagonists, and sustained-release theophylline are effective for treating allergic asthma, while they fall short in improving airway remodeling, and significant side effects, such as cataracts and tachycardia limit their application [8,9]. Hence, the development of novel therapeutic agents remains crucial for the treatment of allergic asthma.

Gerberae Piloselloidis Herba (GPH), also known as "jab bat nex jongx jud (加八喽龚旧)" in Miao medicine, is an ethnobotanical remedy prevalent in Guizhou Province, China. This herb is reputed for its heat-clearing, detoxifying, lung-benefiting, cough-relieving, qi-promoting, and blood-activating properties, and thus is primarily utilized for treating colds, coughs, and asthma [10]. GPH is derived from the dried whole plant of *Gerberae piloselloides* (Linn.) Cass of Compositae and widely distributed in southwest China (such as Sichuan, Guizhou and Yunnan provinces) [10]. Pharmacological research has revealed multiple biological activities of GPH, including anti-inflammatory, antibacterial, antitussive, antioxidative, immunoprotective, and expectorant effects [10]. Our previous study established the remarkable efficacy of GPH against allergic asthma and identified the active constituents, luteolin, arbutin, and marmesin (Fig. 1A) [11]. The comparative pharmacokinetics of these main components of GPH revealed that all the three compounds varied greatly in normal and asthmatic mice, indicating that these compounds might be the effective substances [12]. Furthermore, enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) implicated that GPH relieves the allergic asthma in mice probably through the PI3K/Akt pathway [11]. However, the precise active component of GPH responsible for ameliorating allergic asthma and modulating the PI3K/Akt pathway remains unclear. Therefore, the forthcoming study will assess the therapeutic impact of the active components of GPH on asthma and their influence on the PI3K/Akt signaling pathway, employing a mouse model of allergic asthma and a PI3K inhibitor.

2. Material and methods

2.1. Reagents and antibodies

Arbutin (AF21041151, ≥98 %), marmesin (AF20071206, ≥98 %), and luteolin (AF20030854, ≥98 %) were all bought from Chengdu Efa Biotechnology Co., Ltd. (Chengdu, China). Sodium carboxymethyl cellulose (C104978) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Aluminum hydroxide adjuvant (TE267860) and PI3K inhibitor LY294002 (76957830) were purchased from Thermo Fisher Scientific (Massachusetts, USA). Ovalbumin (OVA, SLCB8249) was acquired from Sigma-Aldrich (St. Louis, Mo, USA). Dexamethasone (DEX, H03N5S43119) was obtained from Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Mouse ELISA kits (IgE, ZC-38496; IL-4, ZC-37986; IL-5, ZC-37987; IL-13, ZC-37967; TNF-α, ZC-39024) were obtained from Shanghai Zcibio Biotechnology Co., Ltd. (Shanghai, China). Bax (Bs-0127R, 1:200) and Bcl-2 (Bs-20351R, 1:100) were acquired from Beijing Boosen Biotechnology Co., Ltd. (Beijing, China). BCA protein concentration determination kit (20140722), protein phosphatase inhibitor (20151014), RIPA lysate (20141219) and Wright staining solution (20200910) were all purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Polyvinylidene fluoride (PVDF) was bought from Millipore (Merck Millipore, USA). Western blot Kit and High sensitivity Chemiluminescence Detection Kit (AC21141) were acquired from Beijing Kangwei Century Biotechnology Co., Ltd. (Beijing, China). Caspase-3 (19677-1-AP, 1:200), anti-β-actin (66009-1-lg, 1:5000), sheep anti-mouse IgG antibody (SA00001-1, 1:5000) and sheep anti-rabbit IgG antibody (SA00001-2, 1:5000) were bought from Proteintech Group. (Wuhan, China). Sheep anti-rabbit IgG (ab01151) , anti-Akt (ab179463, 1:10000), anti-p-Akt (ab38449, 1:800), anti-PI3K (ab191606, 1:1000) and anti-p-PI3K (ab182651, 1:1000) antibodies were all purchased from Abcam (Abcam plc, Cambridge, UK). PI3K inhibitor LY294002 (PHZ1144) was purchased from Thermo Fisher (Thermo Fisher Scientific Inc., USA). Other chemical reagents were of analytical purity.

Gerberae Piloselloidis Herba was procured from Duyun in the Guizhou Province and underwent identification by Associate Professor Zhang Xu, affiliated with the School of Pharmacy at Guizhou Medical University. The medicinal specimen (No. JX20170208) has been archived within the Guizhou Provincial Key Laboratory of Pharmaceutics, housed at Guizhou Medical University.

2.2. Preparation of GPH extract

The GPH extract used in present study was the same as that used in previous study [12], with a yield rate of 2.77 % and concentration of the main active compounds, arbutin, luteolin and marmesin, at 0.13 %, 0.08 % and 0.55 %, respectively.

2.3. Preparation of the mixture

Based on the contents of arbutin, luteolin and marmesin in the GPH extract, the Mixture was prepared by dissolving the corresponding standard substances in a 0.5 % sodium carboxymethyl cellulose solution.

2.4. Animals

Female Kunming mice (20 ± 5 g) were obtained from Changsha Tianqin Biotechnology Co., Ltd. (Changsha, China). All mice were acclimated for one week before the experiment in a standard laboratory (SPF lab) under controlled conditions: temperature 22 ± 2 °C, humidity 55 ± 5 %, and 12-h light-dark cycle. Also, they had free access to water and food. All experimental protocols for mice were approved by the Animal Experimentation Ethics Committee of Guizhou Medical University (the approval Number is 2000828, dated March 27, 2020), and complied with the Animal Protection Law of the people's Republic of China and the regulations on the Administration of Experimental Animals of Guizhou Medical University.

2.5. Experimental model of allergic asthma and treatment

The procedure was detailed in the prior report [11]. Briefly, except for the normal group, where the mice were administered with the same amount of normal saline, the other groups were intraperitoneally injected with 25 μg OVA together with 2 mg aluminum hydroxide adjuvants on the 0th, 7th and 14th day, respectively. The mice were challenged with 2 % OVA (1 mL/min for 20 min) for 6 consecutive days using nebulization starting on the 21st day, while the normal group was received normal saline instead. Thirty min before excitation, the mice in the treatment groups were given the corresponding drugs by intragastric administration, and the mice in model and normal groups were given the same dose of 0.5 % sodium carboxymethyl cellulose solution. The experiment schedule is presented in Fig. 1B.

Experiment 1. Sixty mice were randomly divided into five groups: normal group (Con); ovalbumin group (OVA); GPH extract group (GPH, 1.11 g/kg); active components groups (Mixture, arbutin: luteolin: marmesin = 1.44 mg/kg: 0.89 mg/kg: 6.09 mg/kg, based on their amount in GPH extract); dexamethasone group (DEX, 1.00 mg/kg); The mice in the normal and OVA group were given the same amount of 0.5 % sodium carboxymethyl cellulose.

Experiment 2. To verify that Mixture regulates the PI3K/Akt pathway to exert the effect on the treatment of allergic asthma, PI3K inhibitor (LY294002) was used in this study. This experimental segment comprised five distinct groups: normal group (Con), ovalbumin group (OVA), Mixture group (administered at the same dosage as in Experiment 1), PI3K inhibitor (LY294002) group (LY), and Mixture + PI3K inhibitor group (LY + Mixture). LY294002 (1 mg/kg) was administered to mice in the LY and LY + Mixture groups 1 h prior to nebulization.

2.6. Behavioral characteristics

The behaviors associated with asthma symptoms were observed for 10 min after mice inhaled 2 % ovalbumin or saline during the challenged period. Then, they were scored based on the specific scoring criteria mentioned in previous research [11,13].

2.7. Collection of blood and bronchoalveolar lavage fluid (BALF)

Blood was drawn from the orbital sinus of mice after anesthetization, 24 h after the last aerosol challenge. Serum samples were obtained from whole blood by centrifugation at 4 °C and 3000 rpm for 10 min, and all were stored at −80 °C prior to analysis of IgE, IL-8 and TNF- α. BALF samples were obtained using tracheal intubation, during which the bronchus was lavaged with PBS twice (1 mL of PBS each time). After centrifugation at 4 °C and 2500 rpm for 10 min, the collected supernatants were stored at −80 °C before detection of TNF- α and IL-5 using ELISA kits. Meanwhile, the precipitated cells were suspended in 1 mL of cold PBS buffer, stained with Wright's staining solution, and finally counted under an optical microscope according to the shape differences of inflammatory cells. Different visual fields were randomly selected to count 300 stained cells, and the proportion of eosinophils was calculated [13].

2.8. Histomorphological examination

The left lungs were fixed in 4 % paraformaldehyde at room temperature for more than 24 h, then embedded in paraffin. Subsequently, tissue sections (4-μm thick) were prepared, dewaxed with xylene, and finally stained with hematoxylin-eosin (H&E). After these processes, pathological alterations such as alveolar structure and inflammatory cell infiltration were observed under an optical microscope, and the degree of injury in lung tissues was scored on the basis of the specific scoring criteria mentioned in previous study [14].

In addition, the tissue sections were also subjected to periodic acid-Shiff (PAS) staining and Masson staining and observed under an optical microscope [15]. To determine the degree of goblet cell hyperplasia, PAS-positive goblet cells were scored according to the specific scoring system: 0 points, no PAS-positive goblet cells; 1 point, PAS-positive area less than 25 %; 2 points, staining area 25 %–50 %; 3 points, staining area 50 %–75 %; 4 points, staining area greater than 75 %. Simultaneously, the thickness of bronchial wall and collagen deposition in the airway were evaluated by the Masson-positive area using a semi-quantitative method. In each group, the slices of 4 mice were randomly selected for observation and image acquisition by a Pannoramic250 digital slice scanner (3DHISTECH Hungary).

2.9. TdT-mediated dUTP nick end labeling (TUNEL) staining

The left lung tissue was also fixed, embedded in paraffin and sliced (4-μm thick). The tissue sections were then stained using a TUNEL (TdT-mediated dUTP nick-end labeling) Apoptosis Assay Kit (49330900, Roche Group, Switzerland) according to its instructions. The cell nuclei were stained using DAPI. With the TUNEL signals observed using fluorescence microscopy (× 400), the proportion of TUNEL-positive cells was calculated in three fields of view that were randomly selected [16].

2.10. Immunohistochemical analysis

Using the same protocol of processing, paraffin-embedding, sectioning and dewaxing as H&E staining, tissue sections were incubated in citric acid buffer for antigen retrieval at 97 °C for 20 min, then blocked with 3 % H_2O_2 to reduce activity of endogenous peroxidase. Subsequently, these sections were incubated with goat serum for 20 min at room temperature, then incubated overnight using anti-Caspase-3 (1:200), anti-Bax (1:200) and anti-Bcl-2 (1:100) at 4 °C. After incubation with the primary antibodies, they were washed with PBS (pH = 7.4) three times, and then these sections were incubated using secondary antibodies. After washing with PBS three times, the sections were stained with 3,3-diaminobenzidine (DAB, Beijing, China) and counterstained with hematoxylin. The cellular immune positive degree was visualized under a microscope, in which the nuclei stained by hematoxylin were blue and the DAB-positive expression was brown [17].

2.11. Western blotting

As described previously [18], the right lung tissues of mice were collected to extract the total protein using radioimmunoprecipitation (RIPA) lysate, the concentration of which was determined using a BCA protein assay kit (Solebo, Beijing, China). A 30 μg protein sample was separated on 8 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the protein was electrically transferred onto PVDF membranes (Millipore, USA). These membranes were then blocked using 5 % BSA, and incubated with specific primary antibodies overnight at 4 °C, including anti-Akt (1:10000, abcam, UK), anti-PI3K (1:1000, abcam, UK), anti-p-Akt (1: 800, abcam, UK), anti-p-PI3K (1: 1000, abcam, UK) and anti-β-actin (1:5000, proteintech, USA), respectively. After several washes, the membranes were then incubated with sheep anti-mouse secondary antibody (1:5000, proteintech, USA) and sheep anti-rabbit secondary antibody (1:5000, proteintech, USA) for 2 h at room temperature. After washing with TBST, ECL reagent (Beijing Soleibao Technology Co., Ltd., China) was added on the membranes to visualize and photograph the immunoreactive bands using a gel imager (Bio-Rad, USA). The semi-quantitative analysis was then performed using the Quantity one gel image analysis system based on the gray values of the target protein and the internal control protein. Anti-β-actin was used as the internal reference and there were three repetitions in the experiments.

2.12. Statistical analysis

Statistical analyses were carried out using SPSS 20.0 statistical software and GraphPad Prism 8.0. The data were presented as mean ± SD, analyzed by *t*-test when comparing two groups and for comparison among multiple groups one-way ANOVA was used. Non-parametric statistics (Kruskal-Wallis test) was applied to the analysis of score data. When the *P* values were <0.05, it was considered statistically significant.

3. Results

3.1. Effects of mixture on behavioral characteristics in allergic asthma mice

Among clinical symptoms of allergic asthma are wheezing, dyspnea, and cough. However, the symptoms in mice are characterized by cyanosis of lips, scratching of nose, shortness of breath, obvious abdominal agitation, restlessness, and so on [19]. The results (Fig. 1C) showed that the comprehensive score of behavior was obviously higher in OVA-induced asthma mice than those in Con group. Compared with OVA group, the score in the groups treated with GPH, Mixture, or DEX significantly decreased. These results suggested that Mixture could significantly mitigate the behavioral characteristics of OVA-induced asthma in mice.

3.2. Effect of mixture on levels of cytokines and IgE in serum in allergic asthma mice

The concentration of IL-4, IL-5, IL-13, TNF- α and IgE in serum was detected using ELISA. It was found that in OVA group, the levels of IgE, IL-4, IL-5, IL-13 and TNF- α in serum of mice significantly increased compared to Con group (Fig. 1D–H). Nevertheless, with the treatment of GPH, Mixture, or DEX, their levels significantly reduced relative to OVA group (Fig. 1D–H). These data indicated that Mixture might inhibit TNF- α generation, thereby modulating Th2 cytokine secretion.

3.3. Effect of mixture on lung inflammation in allergic asthma mice

Pathological alterations of lung tissue in each group were observed (Fig. 2A). The results from H&E staining showed several features in Con group: the structure of bronchi and lungs was intact; the size of alveoli and the thickness of bronchial smooth muscle

were normal; no inflammatory cell infiltration was seen around the bronchi. In the OVA group, there appeared a range of alterations, including bronchial stenosis, increased airway mucus, thickened bronchial smooth muscle, and numerous inflammatory cells round the airway in allergic asthma mice. Intriguingly, the above pathological changes were significantly alleviated after treatment with GPH, Mixture, or DEX. With the data analyzed, there was a finding that the degree of lung lesion in the OVA group was significantly

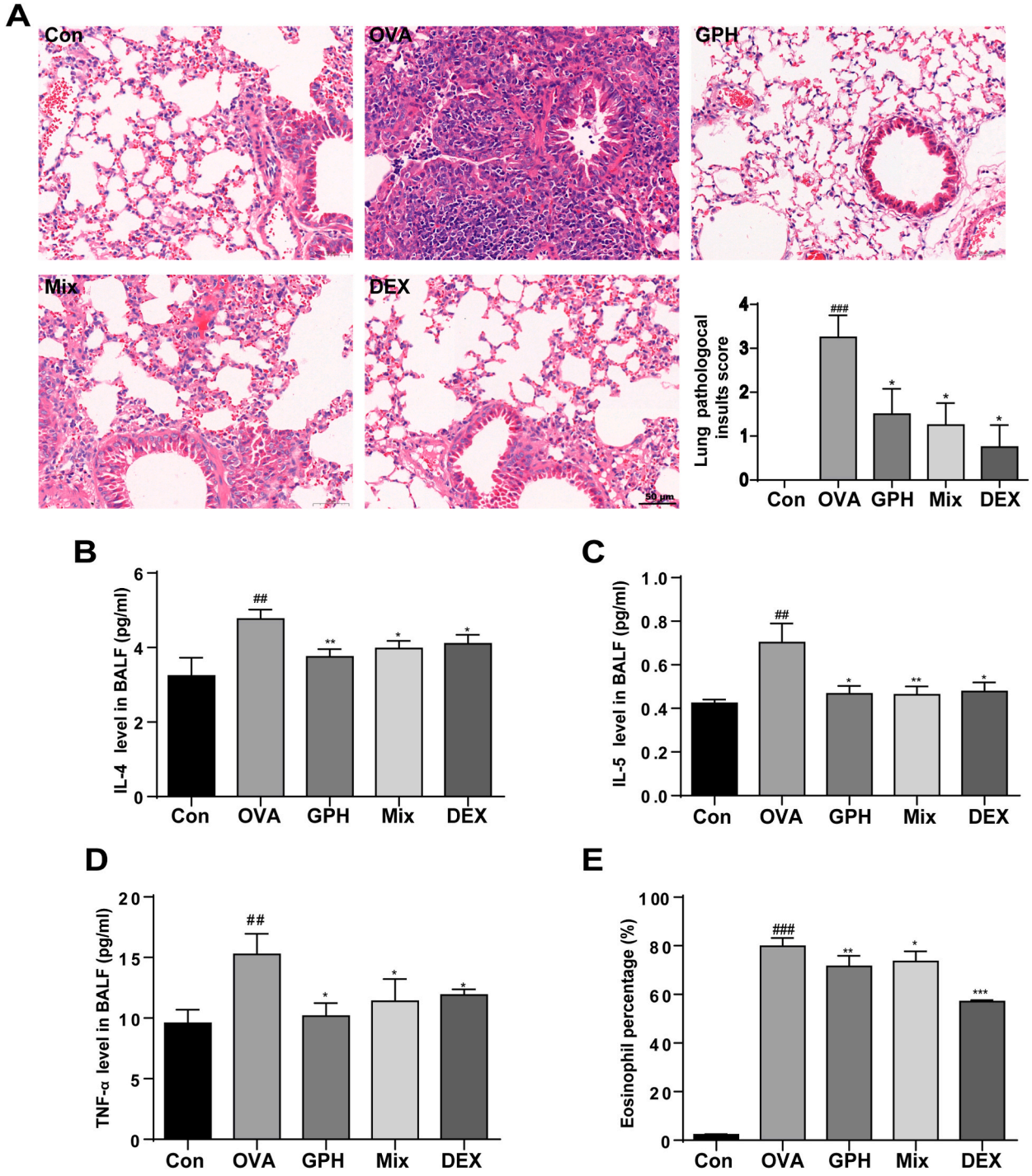

Fig. 2. Mixture ameliorated pathological lesions in the lungs, decreased levels of IL-4, IL-5, and TNF-α, and reduced the number of eosinophils in BALF in OVA-induced asthma mice. A: Light microscopic appearance of histopathological morphology of the lung (H&E staining, scale bar = 50 μm); B ~ D: Levels of IL-4, IL-5, and TNF-α in BALF. E: The number of eosinophils in BALF. Compared to the Con group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; mean ± SD, n = 4.

larger than that in Con group, while it was significantly lower in GPH, Mixture or DEX group relative to the OVA group.

Also, the levels of the proinflammatory cytokine TNF- α and Th2 cytokines in BALF of mice in each group were detected (Fig. 2B–D). These results displayed that the expression levels of IL-5, IL-4and TNF- α in the OVA group dramatically augmented compared to the Con group, whereas their expression obviously declined in the GPH, Mixture, or DEX groups. Simultaneously, the total number of eosinophils in BALF (Fig. 2E) was significantly higher in the OVA group compared to the Con group. However, the total number of eosinophils in mice treated with GPH, Mixture, or DEX significantly reduced relative to the OVA group, illuminating that Mixture might mitigate lung inflammation caused by allergic asthma.

3.4. Effect of mixture on airway remodeling in allergic asthma mice

The key factors of pathological change in asthma include airway wall thickening, collagen deposition and goblet cell proliferation, which are often used to evaluate how severe airway remodeling is. In this study, goblet cell proliferation was evaluated by periodic

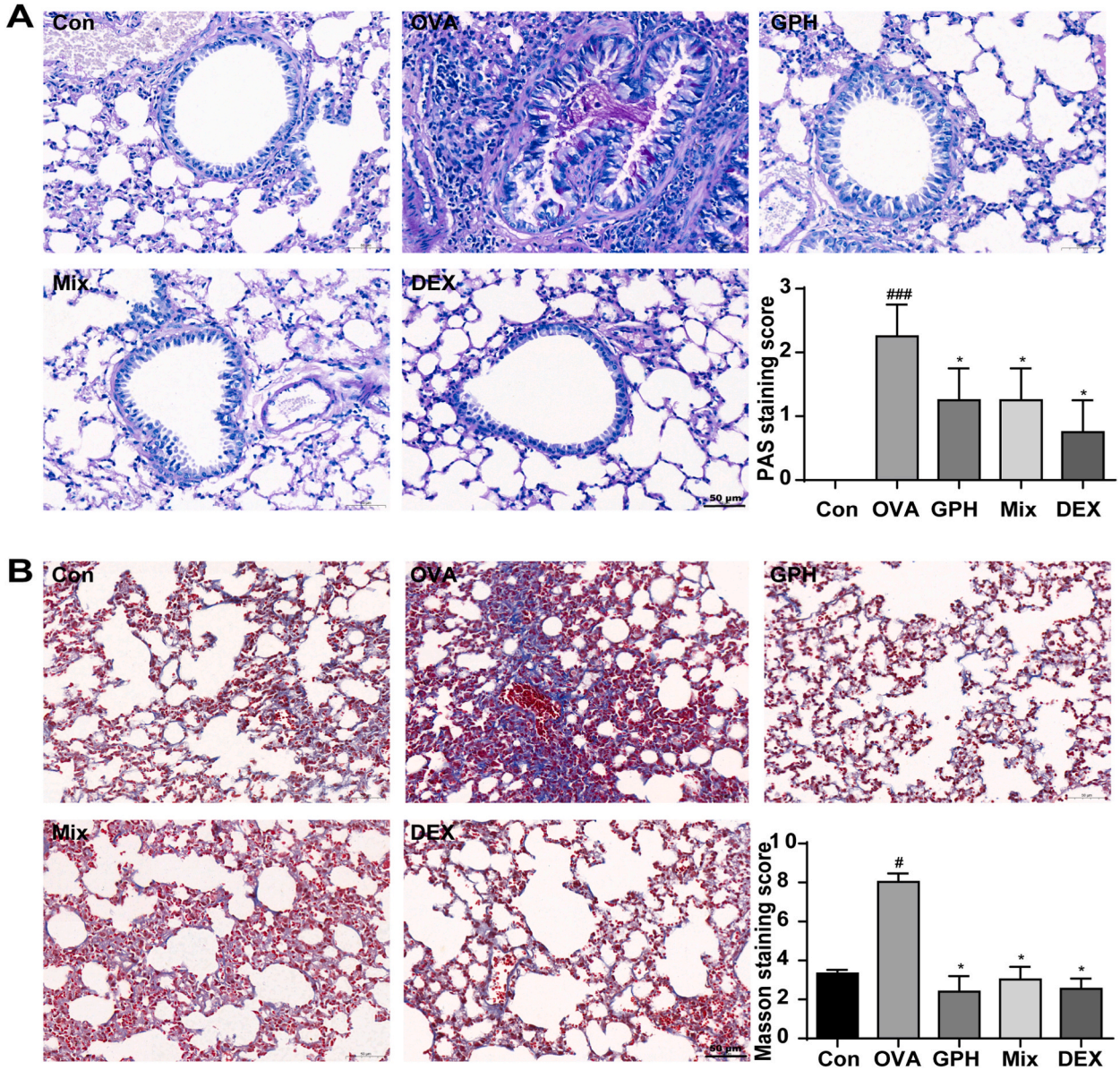

Fig. 3. Mixture alleviated airway remodeling in lung tissue in OVA-induced asthma mice. A: Detection of goblet cells and mucus in lung tissue by PAS staining (PAS staining, scale bar = 50 μm). B: Masson staining revealed reduced collagen deposition and airway wall thickening in lung tissue (Masson staining, scale bar = 50 μm). Compared to the Con group, $^{###}P < 0.001$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; × 400, mean ± SD, n = 4. (PAS: Periodic acid-Schiff).

acid-Schiff (PAS) staining. As presented in Fig. 3A, the PAS-positive area of bronchial and airway goblet cells in the OVA group was markedly larger than those in the Con group. Additionally, to further assess the degree of collagen deposition and airway wall thickening, Masson staining was employed in the present study. It was demonstrated (Fig. 3B) that compared to the Con group, the positive area in the OVA group dramatically increased, while it was significantly reduced in the GPH, Mixture, or DEX-treated groups, which indicated that Mixture improved airway remodeling caused by allergic asthma.

3.5. *Effect of mixture on apoptosis in allergic asthmatic mice*

TUNEL staining was applied to detect whether Mixture could reduce the apoptosis degree in lung tissue in mice with allergic asthma induced by OVA. There appeared to be a significantly larger number of TUNEL-positive cells in the OVA group relative to the Con group, but the GPH, Mixture, or DEX obviously reduced the number compared to the OVA group (Fig. 4A∼B). Additionally, for the purpose of evaluating whether Mixture could regulate apoptosis-related proteins such as Caspase-3, Bax and Bcl-2, their expressions in lung tissue were detected using immunohistochemistry. It was found that the expression of Bcl-2 significantly decreased in the OVA group compared with those in the Con group, while the expressions of Bax and Caspase-3 were significantly higher. However, there

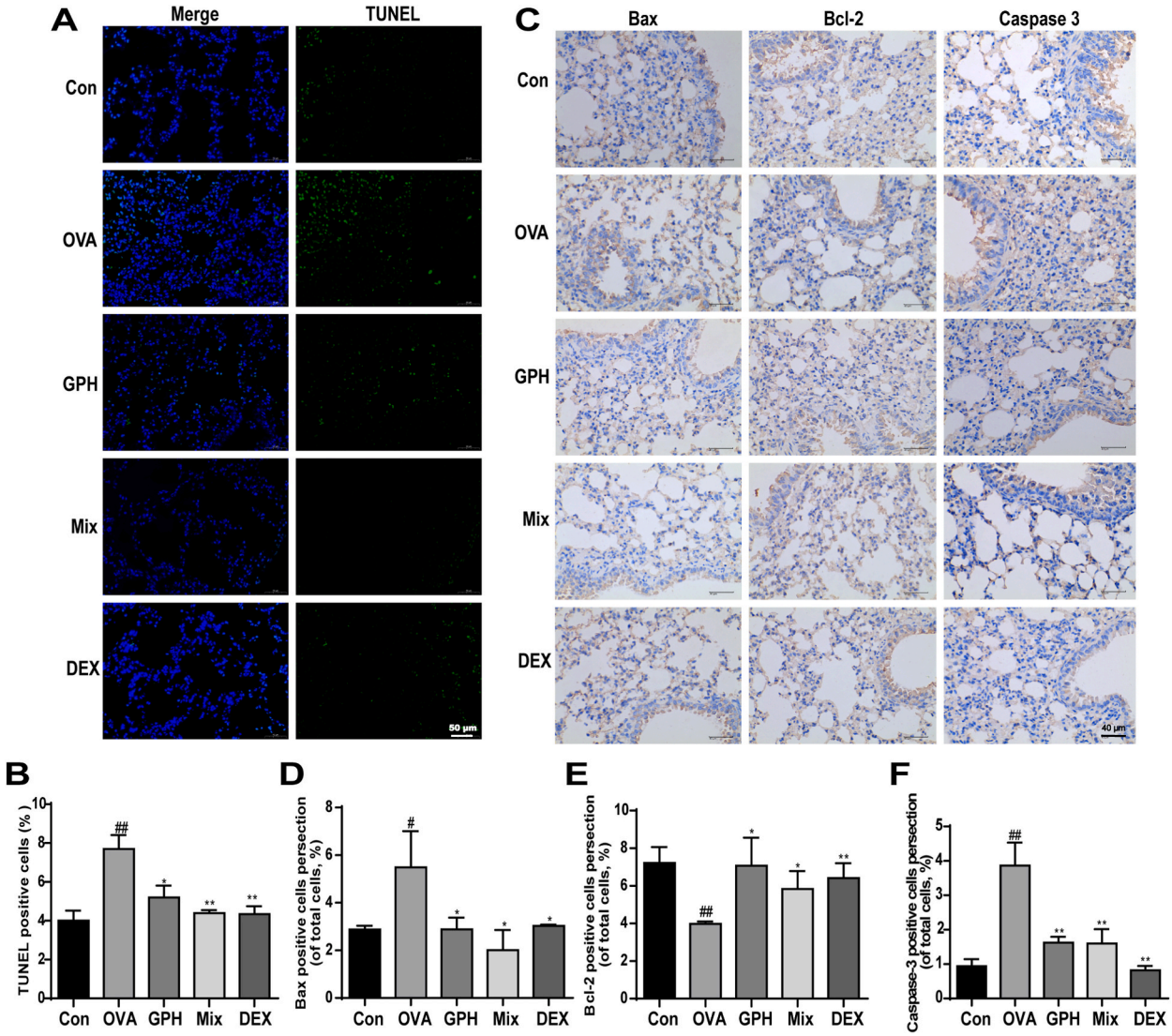

Fig. 4. Mixture alleviated apoptosis in lung tissue in OVA-induced asthma mice. A: Representative images of apoptosis in lung tissue (TUNEL staining, scale bar = 50 μm). B: Proportion of TUNEL-positive cells. C: Immunohistochemical images of Bax, Bcl-2, and Caspase-3 in each group (scale bar = 40 μm). D: Statistical chart of Bax-positive area proportion. E: Statistical chart of Bcl-2-positive area proportion. F: Statistical chart of Caspase-3-positive area proportion. Compared to the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$; × 400, mean ± SD, n = 4.

existed an increase in expression of Bcl-2 and a decline in the expressions of Caspase-3 and Bax in these mice after treatment with GPH, Mixture, or DEX (Fig. 4C–F), suggesting that Mixture could attenuate OVA-induced apoptosis in lung tissue in mice with allergic asthma.

3.6. Effect of mixture on PI3K/Akt signaling pathway in allergic asthma mice

As shown in Fig. 5A∼D, the expressions of PI3K, p-PI3K, and the ratio of p-Akt to Akt in the OVA group remarkably increased compared to the Con group. However, the expressions obviously reduced in GPH, Mixture, and DEX groups relative to OVA group. This illuminated that Mixture could inhibit the activation of PI3K/Akt signaling pathway, through which Mixture could alleviate OVA-induced lung injury in allergic asthmatic mice.

3.7. Effect of LY294002 and mixture on allergic asthma in mice

To further explore the mechanism behind Mixture's effect against allergic asthma, LY294002, an inhibitor of PI3K, was employed to pretreat OVA-induced allergic asthma mice before they were administered Mixture (Fig. 5E). After treatment with LY294002, there was a significant decrease in the expressions of PI3K, p-PI3K, and the ratio of p-Akt to Akt in lung tissue relative to the OVA group, similar to the effect of Mixture, and so did the combination of LY294002 with Mixture do (Fig. 5F ∼ I). Regarding apoptosis in pulmonary tissues, both LY294002 and Mixture enabled the expression of Bcl-2 to increase and Caspase-3 and Bax to decrease, with their combination producing the same alteration (Fig. 6C ∼ F). Also, they all made the TUNEL-positive cells reduced (Fig. 6A∼B). In addition, with the mice pre-treated with LY294002, there was a decrease in immunoinflammatory related indicators, including IL-4, IL-5, IL-13, TNF-α and IgE in serum or BALF (Fig. 7B-F; Fig. 8B ∼ D). It was also found that lung tissue lesions - such as elevated airway mucus, inflammatory cells around the airway, airway wall thickening, and collagen deposition - were reduced in mice that received

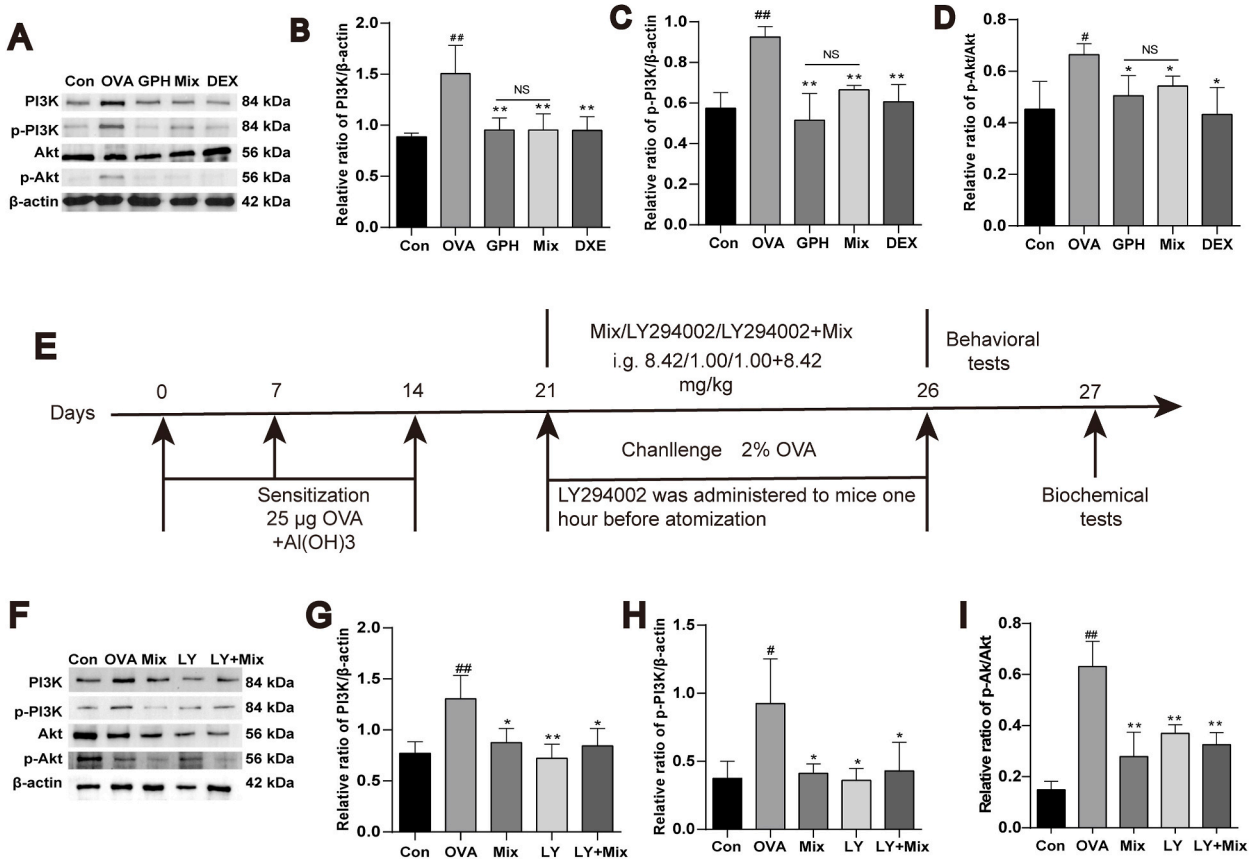

Fig. 5. Mixture and LY294002 inhibited the phosphorylation of PI3K and Akt in lung tissue of OVA-induced asthma mice detected by Western blot analysis. A: Representative bands of PI3K, p-PI3K, Akt, and p-Akt in lung tissue of mice treated with GPH and Mixture. B ∼ D: Statistical charts of PI3K, p-PI3K, and p-Akt/Akt in lung tissue of mice treated with GPH and Mixture. E: Workflow of asthma model establishment and pretreatment with LY294002; F: Representative bands of PI3K, p-PI3K, Akt, and p-Akt in lung tissue after pretreatment with LY294002 and Mixture. G ∼ I: Statistical charts of PI3K, p-PI3K and p-Akt/Akt in lung tissue of mice treated with LY294002 and Mixture. Compared to the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$; mean ± SD, n = 4.

LY294002, Mixture or their combination (Fig. 8A; Fig. 9). Consistent with other indexes, LY294002 pretreatment, along with Mixture, reduced the proportion of EOS and the score of asthma behavior (Fig. 7A; Fig. 8E). These results suggested that the efficacy of Mixture on the improvement of allergic asthma is similar to that exerted by LY294002, revealing the mechanism behind this effect indeed involves in inhibition of PI3K/Akt signaling pathway.

4. Discussion

Allergic asthma, a heterogeneous condition caused by different predisposing factors [20], has been recognized as a global public health problem in recent years, posing a serious threat to the health of millions of people. Despite great strides in diagnosis and treatment, allergic asthma remains a serious challenge to public health. Traditional Chinese medicine (TCM) is an effective complementary and alternative medical therapy. Increasing scientific evidence supports the use of TCM to treat allergic asthma. Additionally, the treatment of allergic asthma with plant active components of TCM has garnered widespread attention [21]. Therefore, it is a potential strategy to deeply investigate active component groups derived from herb drugs for the control of allergies.

In our previous studies, it was found that GPH exerted an obvious effect on anti-allergic asthma [13], and its main active components (Mixture) were identified using a method to integrate plasma pharmacochemistry and network pharmacology [11], indicating the potency of Mixture to develop into a drug for treatment of allergic asthma. In this study, it was proved that the active components could indeed significantly reduce inflammatory cell infiltration, airway hyperresponsiveness, tissue apoptosis, and asthma symptoms

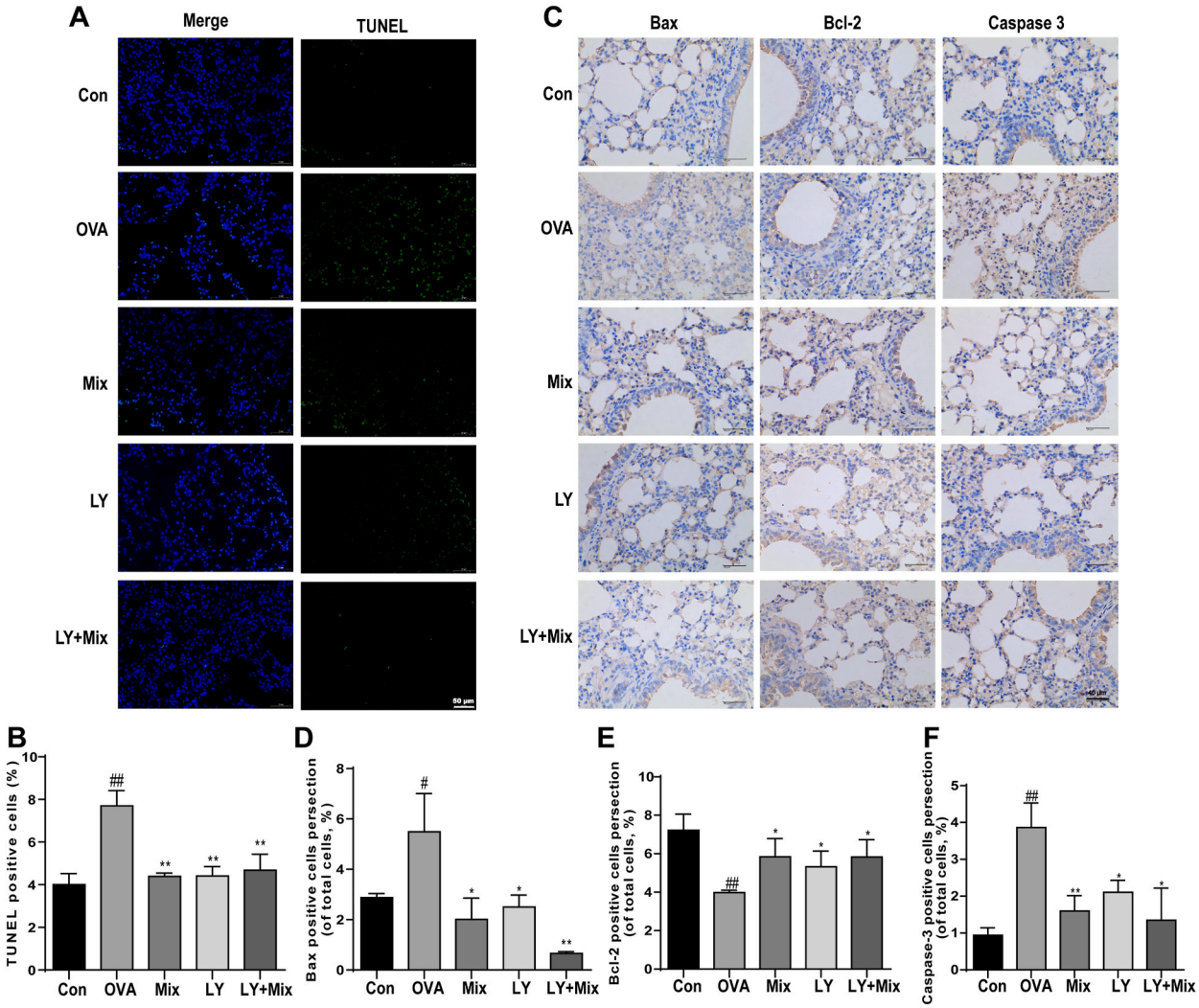

Fig. 6. LY294002 and Mixture alleviated apoptosis in lung tissue in OVA-induced asthma mice. A: Representative images of lung tissue apoptosis (TUNEL staining, scale bat = 50 μm). B: the proportion of TUNEL positive cells. C: Immunohistochemical images of Bax, Bcl-2 and Caspase-3 in each group (scale bat = 40 μm). E: Statistical chart of Bax-positive area; F: Statistical chart of Bcl-2-positive area; D: Statistical chart of Caspase-3-positive area. Compared to the Con group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$; × 400, mean ± SD, n = 4.

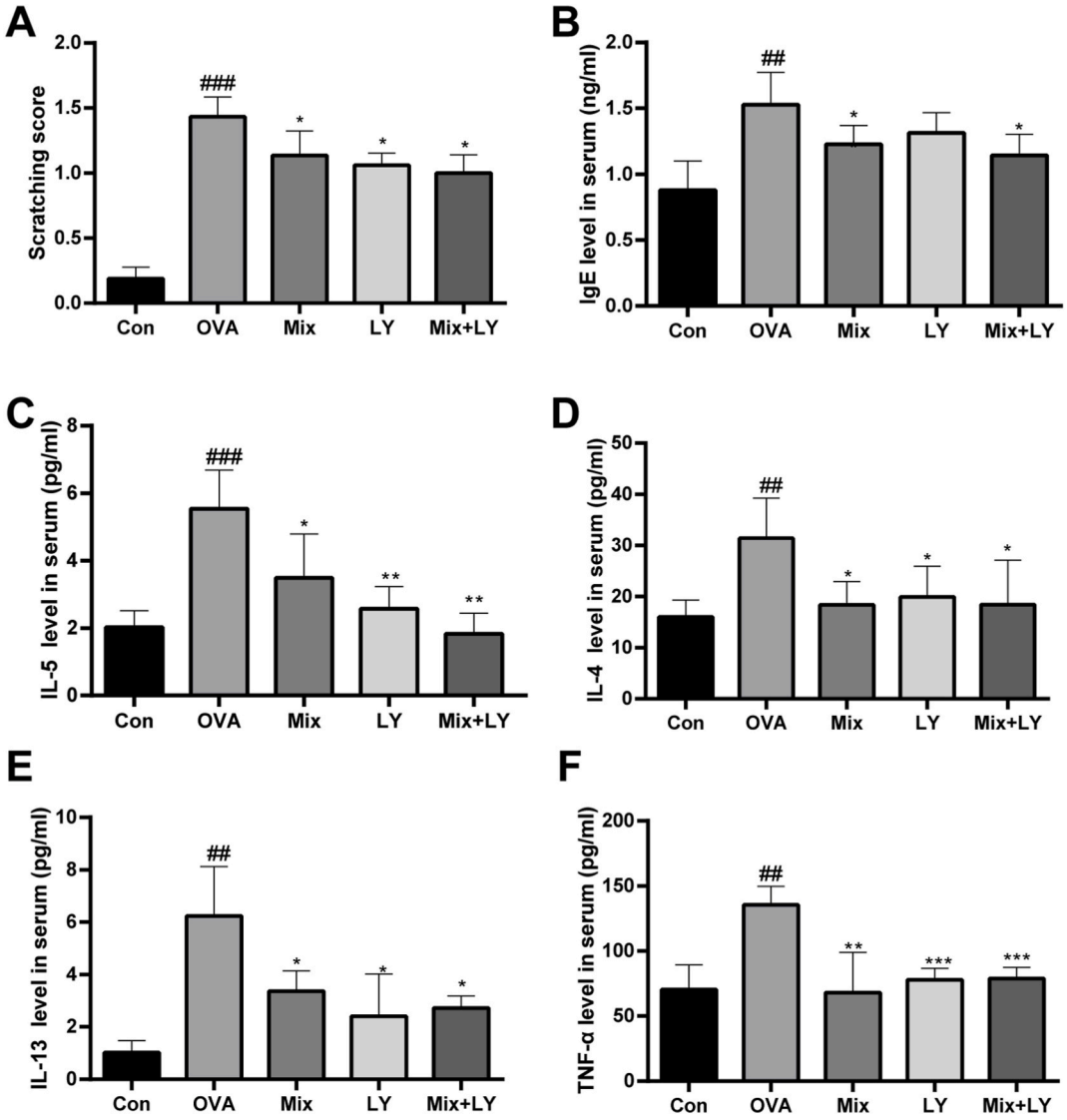

Fig. 7. LY294002 and Mixture ameliorated asthma symptoms and reduced levels of IgE, IL-5, IL-4, IL-13, and TNF-α in the serum of OVA-induced mice. A: Effects of different treatments on behavior in OVA-induced mice; B ~ F: The levels of IgE, IL-5, IL-4, IL-13 and TNF-α in serum. Compared to the Con group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; mean ± SD, n = 6.

in allergic asthma mice induced by OVA. Intriguingly, the efficacy of Mixture was similar to that of GPH and dexamethasone. In addition, the results from the previous pharmacokinetic study [12] also showed that the AUC values of luteolin, arbutin, and marmesin in blood were higher, suggesting that their absorption into plasma might be greater *in vivo*. These data indicated that they may have a good bioavailability, which is conducive to further application and development of Mixture. Clinical studies have shown that during allergic asthma, there appeared alveolar structure loss, inflammatory cell infiltration, alveolar wall thickening, airway epithelial mucus hypersecretion, goblet cell proliferation, and collagen deposition [20]. In this study, OVA could induce significant pathological changes in the mouse's lung tissue. For example, PAS staining showed mucus hypersecretion and goblet cell proliferation in the OVA group, and Masson staining also showed increased collagen deposition in the OVA group, which are the main markers for allergic asthma [19,22]. Remarkably, Mixture was able to mitigate the above pathological features, revealing its protective effect on allergic asthma.

When allergic asthma occurs, a variety of inflammatory cells are involved in airway inflammation, such as eosinophils, lymphocytes and neutrophils [23,24]. In this study, the cells in BALF was counted using Wright's staining. It was showed that Mixture could prevent inflammatory cells from infiltrating into the lungs, especially eosinophils. Eosinophils are the main participants in the occurrence and development of allergic asthma, participating in the induction and remodeling of airway hyperresponsiveness [25]. IgE plays a vital role in the whole allergic inflammation. There are studies showing that reducing IgE is effective during the treatment of allergic asthma [26], suggesting that targeting IgE may be a new treatment means. In present study, Mixture could significantly reduce

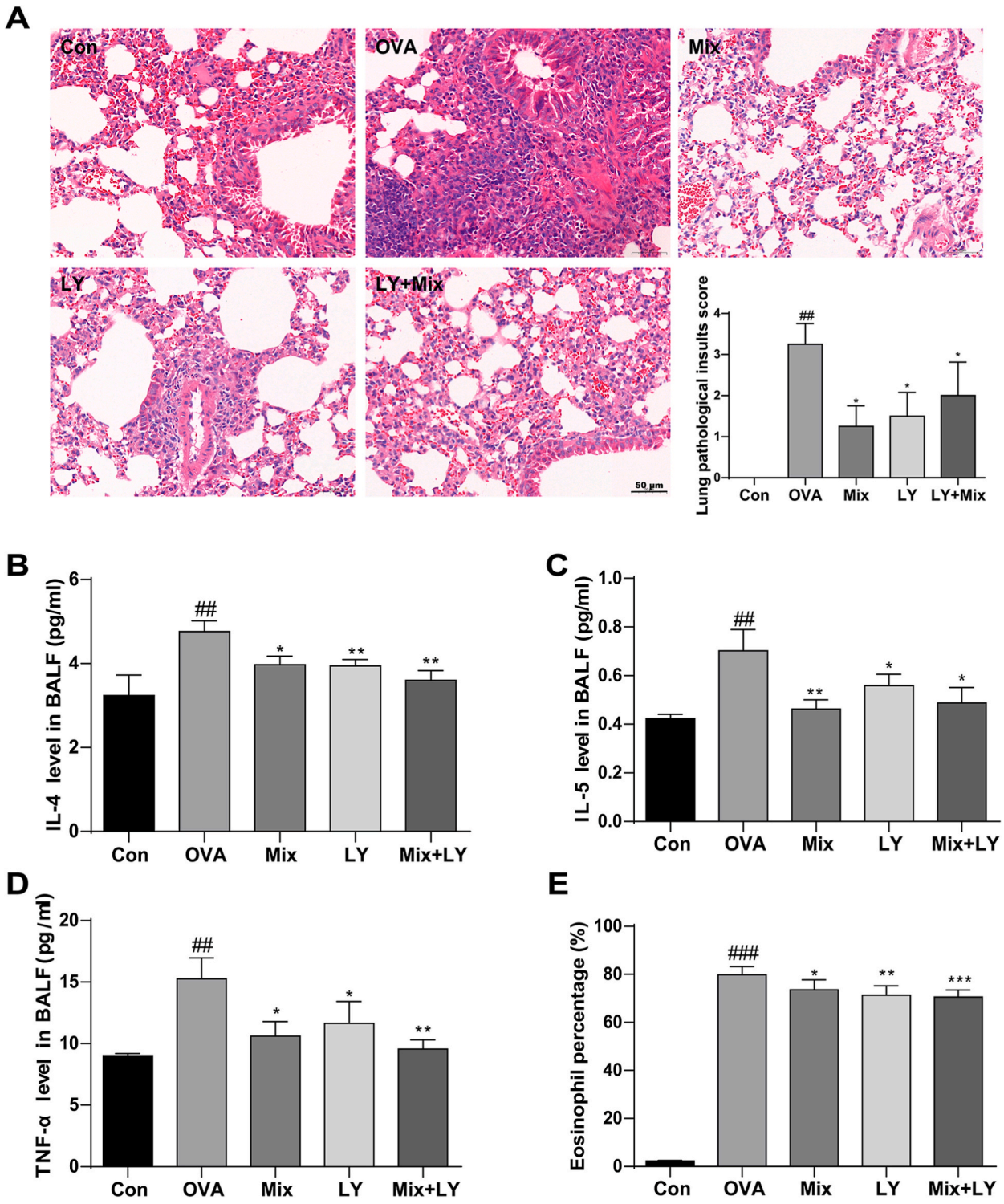

Fig. 8. LY294002 and Mixture ameliorated pathological lesions in the lungs, decreased levels of IL-4, IL-5, and TNF-α, and the number of eosinophils in BALF in OVA-induced asthma mice. A: Light microscopic appearance of histopathological morphology of lung (H&E staining, scale bar = 50 μm); B ~ D: Levels of IL-4, IL-5 and TNF-α in BALF. E: The number of eosinophils in BALF. Compared to the Con group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; mean ± SD, n = 4.

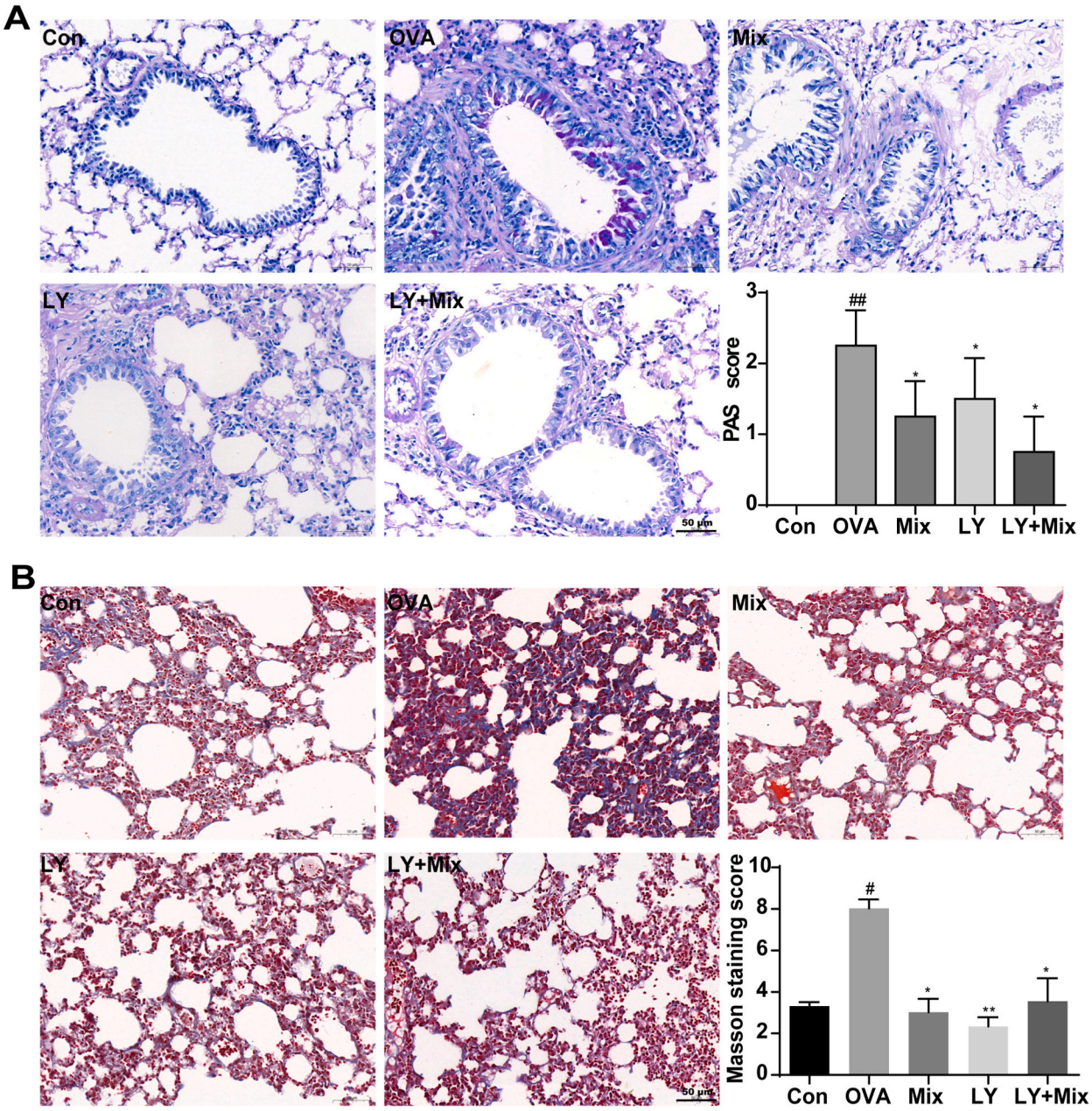

Fig. 9. LY294002 and Mixture alleviated airway remodeling in lung tissue in OVA-induced asthma mice. A: Detection of goblet cells and mucus in lung tissue by PAS staining (PAS staining, scale bar = 50 μm). B: Collagen deposition and airway wall thickening in lung tissue were detected by Masson staining (Masson staining, scale bat = 50 μm). Compared to the Con group, $^{###}P < 0.001$; compared to the OVA group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; × 400, mean ± SD, n = 4.

the level of IgE, indicating that Mixture is likely to act on the regulation of IgE to exert the effect on allergic asthma. When exposed to allergens, antigen-presenting dendritic cells could make immature T cells sensitive to allergens, resulting in their development into Th2 cells [27]. They can induce the generation of such inflammatory cytokines as IL-13 and IL-4, which could trigger B cells to produce allergen-specific IgE [28]. In turn, IgE has direct impact on the function of eosinophils, including eosinophils activation and TNF- α release [29]. At the same time, IgE could also activate airway smooth muscle to generate IL-13, IL-5, IL-4 and TNF-α, affecting the contraction and proliferation of airway smooth muscle, ultimately leading to airway remodeling [30]. The IL-13 in the airway can also promote the survival and migration of eosinophils, the activation of macrophages, and the permeability and viscosity of airway epithelial cells. Moreover, it can also stimulate airway hyperresponsiveness [31,32]. IL-5 involve in eosinophils recruitment and chemotaxis, together with other anti-apoptotic factors, making the survival of eosinophils prolonged and the stickiness between eosinophils and endothelial cells increased [8]. Simultaneously, IL-4 and IL-13 can induce the conversion of immunoglobulin classes to

IgE during its process of synthesis in B cells [31]. They also participate in recruitment of mast cells and up-regulate the expression of vascular cell adhesion molecule-1 (VCAM-1), thus facilitating the migration of eosinophils and other inflammatory cells induced by mast cell-derived chemokines [32]. TNF-α is a peptide inflammatory mediator secreted by macrophages, a cytokine that could induce adhesion and infiltration of inflammatory cells and cause inflammatory response [33]. Therefore, monitoring inflammatory cytokines such as TNF-α, IL-13, IL-5 and IL-4 can be regarded as a way to evaluate whether the drugs work for allergic asthma. In this study, there was a finding that Mixture could inhibit the production of the above cytokines in mice with allergic asthma, indicating Mixture could exert an anti-inflammatory effect during allergic asthma.

The PI3K/Akt signaling pathway plays a crucial role in the pathogenesis of allergic asthma [34]. Functional studies showed that PI3Ks could regulate several key events in the inflammatory response [35]. For example, the activation of PI3K poses a central position in the development of pulmonary inflammation and tissue remodeling [36]. Owing to the involvement of the PI3Ks pathway in the aberrant activation of immune cells as well as pulmonary fibroblasts, PI3Ks are considered as potential therapeutic targets in asthma. So far, some scientists have developed a range of PI3K inhibitors to improve lung function in asthma and fibrosis [34,36]. In this study, LY294002, a relatively nonspecific inhibitor of PI3K, was applied to further confirm the action of Mixture for treatment of asthma. It is worth noting that Akt is a very important regulatory kinase downstream of PI3K, whose activation requires phosphorylation of threonine-308 and serine-473 located at PI3K [37]. Once phosphorylated, Akt transfers into the nucleus, a process that affects the activity of transcription factors, and ultimately results in the phosphorylation and activation of downstream NF-κB [38]. In this experiment, there appeared increased expression of p-PI3K, p-Akt, PI3K and Akt in the OVA-induced allergic asthma mouse model, while Mixture significantly decreased their expression. Our previous research also found that the main active ingredients of GPH are likely to directly act on the Akt/PI3K pathway using network pharmacology and molecular docking. In present study the experimental results corroborate these findings. In addition, there is ample evidence that inhibiting the Akt/PI3K pathway in mouse models of asthma can suppress the infiltration of inflammatory cell, the production of mucus, and the occurrence of airway hyperresponsiveness [39]. Therefore, it was established that the main mechanism behind Mixture against asthma is to inhibit PI3K/Akt signaling pathway, thereby regulating inflammation, apoptosis and airway hyperresponsiveness. After the mice attacked by allergic asthma were pre-treated with LY294002, a range of indexes were indeed improved, including the concentration of inflammatory mediators (like TNF-α, IL-5, IL-4, and IL-13) and IgE in BALF or plasma, as well as the number of eosinophils and inflammatory cell invasion in lung tissues. Intriguingly, the functional intensity of Mixture was similar to that LY294002 exerted, further indicating that the effect of Mixture on allergic asthma might be indeed through acting on the PI3K/Akt signaling pathway.

In addition to airway inflammation, airway remodeling is another vital characteristic of allergic asthma. Airway remodeling can cause structure alterations and damage to the airway wall, finally resulting in airway obstruction and exacerbation in patients who suffer from allergic asthma [40]. Included among airway remodeling mainly are epithelial fibrosis, airway epithelial destruction, goblet cell hyperplasia, collagen fiber deposition, increased mucus secretion, and smooth muscle thickening, etc [41]. In this study, there appeared hyperplasia of goblet cells and mucus secretion in mice with allergic asthma induced by OVA, while after pretreatment with LY294002, the phenomena were improved. In addition, LY294002 could also reduce collagen deposition around the airway in mice with allergic asthma. Meanwhile, pretreatment with Mixture also mitigated goblet cell proliferation, mucus secretion, and collagen accumulation around the airways. It was reported that LY294002 could reduce production of Th2 cytokines, eosinophil infiltration, airway mucus secretion and airway structure changes in OVA allergic asthma model mice [42]. In this study, the results were consistent with the above published discovery. At the same time, it was found that the effect of Mixture on inflammation, apoptosis, and airway hyperresponsiveness in OVA-induced allergic asthma model mice was also similar to that of the PI3K inhibitor LY294002.

There is evidence suggesting that luteolin improves pulmonary vascular remodeling and right ventricular hypertrophy by reducing the expression of caspase-9 and caspase-3 and inhibiting transduction of the PI3K/Akt signaling pathway, and suppresses the migration and proliferation of smooth muscle cells in the pulmonary artery [43]; arbutin improves renal function after lipopolysaccharide challenge and reduces apoptosis and inflammation by regulating the PI3K/Akt/Nrf2 signaling pathway transduction [44]; marmesin could inhibit the PI3K/Akt pathway to exert anti-cancer activity in EC cells [45]. These findings also supported that these three compounds in Mixture can regulate the Akt/PI3K pathway and possess the potency in anti-inflammation and improvement of airway remodeling. As mentioned above, the effect of Mixture on asthma was similar to LY294002, illuminating that Mixture indeed can inhibit the activation of the PI3K/Akt signaling pathway to attenuate inflammation, apoptosis and airway structure changes in OVA-induced allergic asthma mice.

In conclusion, the current study suggests that the active compounds of GPH, collectively referred to as the Mixture, exhibit significant protective effects against allergic asthma. This therapeutic benefit is associated with the inhibition of the PI3K/Akt signaling pathway. These findings establish a theoretical foundation indicating that the Mixture may function as a potent inhibitor of the PI3K/Akt pathway, thereby harboring potential for advancement into a novel pharmacological agent for the treatment of diverse pathologies governed by this signaling cascade.

Institutional review board statement

The Guizhou Medical University Institutional Animal Care and Use Committee reviewed and approved the animal study.

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Informed consent statement

Not applicable.

Ethics statement

The all procedures of animal study were carried out in compliance with the China National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, reviewed and approved by the Institutional Animal Care and Use Committee of Guizhou Medical University (approval number: 2000828, dated March 27, 2020).

Data availability statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Chunhua Liu: Writing – review & editing, Funding acquisition, Data curation, Conceptualization. **Yu He:** Writing – original draft, Methodology, Formal analysis, Data curation. **Kun Zhou:** Writing – original draft, Formal analysis, Data curation. **Hong Wang:** Formal analysis, Data curation. **Meng Zhou:** Writing – review & editing. **Jia Sun:** Formal analysis, Data curation. **Yuan Lu:** Formal analysis. **Yong Huang:** Funding acquisition, Formal analysis. **Yonglin Wang:** Writing – review & editing, Funding acquisition. **Ting Liu:** Writing – review & editing, Conceptualization. **Yongjun Li:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors have declared that there is no conflict of interest among them.

Abbreviations

AKT	protein kinase B
Bcl-2	B-cell lymphoma-2
Bax	Bcl-2-Associated X
BALF	bronchoalveolar lavage fluid
Con	normal group
CREB	cAMP-response element binding protein
DEX	dexamethasone group
ELISA	enzyme linked immunosorbent assay
GPH	Gerberae Piloselloidis Herba
H&E	hematoxylin-eosin
IgE	Immunoglobulin E
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
LY	LY294002
Mixture	Groups of active components of GPH
NF-κB	E2F and nuclear factor kappa-B
OVA	ovalbumin
PAS	periodic acid-Shiff
PI3K	phosphatidylinositol 3 kinase
RIPA	radioimmunoprecipitation
ROCC	receptor-operated calcium channel
SABA	short-acting β2 receptor agonist
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF-α	Tumor Necrosis Factor-α
TUNEL	terminal dexynucleotidyl transferase(TdT)-mediated dUTP nick end labeling
TCM	Traditional Chinese medicine
VCAM-1	vascular cell adhesion protein 1
VDCC	voltage-dependent calcium channel

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37632.

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