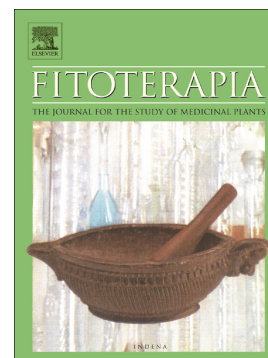


Tussilagone inhibits allergic responses in OVA-induced allergic rhinitis guinea pigs and IgE-stimulated RBL-2H3 cells

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Tussilagone inhibits allergic responses in OVA-induced allergic rhinitis guinea pigs and IgE-stimulated RBL-2H3 cells.

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Abstract

Farfarae Flos is the dried flower buds of *Tussilago farfara* L. which is widely used to treat allergic and inflammatory diseases in Chinese folk. Tussilagone (TSL), a sesquiterpene compound purified from Farfarae Flos, has been confirmed the main active component in the plant. However, its anti-allergic activity hasn't been reported yet. The purpose of this study is to investigate the anti-allergic effect of TSL in ovalbumin (OVA)-induced allergic rhinitis (AR) guinea pigs and immunoglobulin E (IgE)-stimulated RBL-2H3 cells. The AR symptoms such as nasal scratching, sneezing and runny nose were scored and the histological changes of nasal mucosa were observed by H&E staining. The levels of histamine, OVA-specific IgE, IL-6 and TNF- α in the serum were measured by ELISA. In IgE-stimulated RBL-2H3 cells, the

phosphorylation of Lyn, Syk, Akt, NF- κ B p65, ERK and p38 MAPK were investigated by western blot analysis. The results showed that intraperitoneal injection of TSL at doses of 25 and 50 mg/kg significantly alleviated the allergic symptoms and the histological changes of nasal mucosa in OVA-induced allergic rhinitis guinea pigs. Moreover, the levels of histamine, IgE and IL-6 in the serum decreased significantly ($p < 0.05$). In vitro, TSL suppressed the phosphorylation of Lyn, Syk, Akt, NF- κ B p65, ERK and p38 MAPK in IgE-stimulated RBL-2H3 cells. These results indicate TSL has therapeutic effect on allergic rhinitis in guinea pigs. The anti-allergic mechanism may be through the inhibition of allergic and inflammatory related pathways in mast cells.

Keywords: Tussilagone; Allergic rhinitis; Ovalbumin; RBL-2H3 cell

1. Introduction

Allergic rhinitis (AR) is an allergen-triggered and immunoglobulin (Ig) E-mediated inflammatory disease with the classic symptoms of nasal congestion, nasal itching, sneezing and rhinorrhea. Data analysis shows that people suffering from AR account for about 10% to 20% of the global population and the prevalence has been steadily increasing [1]. Anxiety, depression, sleep pattern changes or general malaise usually occur on patients with AR, therefore the quality of life will be reduced greatly [2]. At present, although various treatment measures such as allergens avoidance, drug treatment and allergen immunotherapy have been performed in the clinical management of AR, none of these treatment measures is ideal for alleviating symptoms. Oral antihistamines and intranasal corticosteroids are the two first line

drugs approved for the treatment of AR, however, their side effects still pose risk for patients. Oral antihistamines including both first-generation and new-generation drugs can cause CNS symptoms and intranasal corticosteroids can cause headache, throat irritation, epistaxis, stinging, burning and nasal dryness [1]. Therefore, to seek a more safe and effective anti-AR drug is still a worldwide urgent task.

Recently, a large number of natural compounds isolated from traditional medicinal plants arouse people's interest because of their safety and effectiveness. The dried flower buds of *Tussilago farfara* L. (Farfarae Flos) is a traditional Chinese medicine widely used to treat cough, bronchitis and asthma in clinical [3]. A variety of active components including sesquiterpenes, triterpenes, flavonoids and pyrrolizine alkaloids have been isolated from Farfarae Flos [4-6]. It was reported that these active components had antioxidant, anti-inflammatory, anti-bacterial, anti-cancer and neuron protective effects [7-10]. However, the anti-allergic components of Farfarae Flos have not been reported yet. Tussilagone (TSL), 14-acetoxy-7 β -(3'-ethyl crotonoyloxy)-noto-nopetranone (Fig. 1 A), is a sesquiterpene compound extracted and separated by petroleum ether from Farfarae Flos which has been confirmed the main anti-inflammatory component [8, 11]. As AR is a kind of inflammatory disease with amount of inflammatory mediators released from mast cells by IgE-dependent mechanisms, TSL with anti-inflammatory effect might have therapeutic effect for AR.

This study aims to evaluate the anti-allergic effects of TSL in OVA-induced AR

guinea pigs. Furthermore, the (RBL)-2H3 cell line was used to explore the mechanism of anti-allergic effect of TSL. This study will provide data support for further development of TSL as a new drug for the treatment of allergic rhinitis in the future.

2. Materials and methods

2.1 Reagents

Tussilagone (purity: 98.0%) was purchased from Shanghai Yuanye Biotechnology Co. Ltd (Shanghai, China). Ovalbumin (OVA) and anti-2,4-dinitrophenyl IgE (anti-DNP IgE) antibody were purchased from Sigma Chemical Co. (MO, USA); Clarityne was purchased from Shanghai Schering Baoyao Pharmaceutical Co (Shanghai, China). Aluminum hydroxide gel was purchased from Tianjin Komeo Reagent Co. Ltd (Tianjin, China). The guinea pig histamine, IgE, IL-6, TNF- α ELISA kit were purchased from Shanghai Yuanye Biotechnology Co. Ltd (Shanghai, China). DNP-human serum albumin (DNP-HAS) antibody were purchased from Alpha Diagnostic company (USA). Antibodies against Lyn, phospho-Lyn, ERK, phospho-ERK, Syk, phospho-Syk, p38, phospho-p38, AKT, phospho-AKT, NF- κ B p65, phospho-NF- κ Bp65 and β -actin were purchased from Cell Signaling Technology (USA). Goat Anti-Rabbit IgG peroxidase conjugate was purchased from Bio-Rad Laboratories (USA). RIPA lysis buffer, BCA protein assay kit and clarityTM western ECL substrate were purchased from Beyotime Biotechnology (Shanghai, China).

2.2 Animals and cell culture

80 guinea pigs (300 ± 5 g), half male and half female, were purchased from the

Experimental Animal Center of Zhejiang Chinese Medical University (Hangzhou, China). Guinea pigs were bred in a specific pathogen-free environment with a temperature of 24 ± 1 °C, humidity of $50 \pm 10\%$, and 12 h light/dark cycle. All the procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals and with the guide-lines established by the Ethics Committee of Laboratory Animal Care and Welfare, Zhejiang Chinese Medical University. Mast cell rat basophilic cell line RBL-2H3 purchased from Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China) was cultured in MEM (100 U/mL penicillin, 100 µg/mL streptomycin, NaHCO_3 1.5 g/L, Sodium Pyruvate 0.11 g/L) containing 15% FBS under standard cell culture conditions (37 °C in 95% humidified air and 5% CO_2).

2.3 AR animal preparation and drug administration

The guinea pigs were randomly divided into two groups: one is normal group consisted of 10 guinea pigs and another is OVA-sensitized/challenged group consisted of 70 guinea pigs. The experimental procedures for allergic sensitization were summarized in Fig. 1 B. After a week of adaptive feeding, animals in the OVA-sensitized/challenged group were sensitized by intraperitoneal injected with OVA (2 mg/animal) and aluminum hydroxide (4 mg/animal) on days 1, 3, 5, 7, 9, 11 and 13. The animals in the normal group were given the same dose of aluminum hydroxide gel without OVA. On the day 15 to day 40, the animals in the OVA-sensitized/challenged group were challenged by dripping 2% OVA solution (50 µl/nostril) into bilateral nasal cavities using a micro-injector. The animals in the normal

group was dripped with saline solution instead of OVA solution. From day 21 to day 27, the symptoms of scratching the nose, sneezes and runny nose were observed and scored immediately after dripping OVA within 30 min. If the total score > 5, it indicated that the AR model was successful. The evaluation criteria are shown in Table 1. On the day 27, the successfully modeled AR guinea pigs were picked out and randomly divided into four groups: model control group, TSL at dose of 25 mg/kg or 50 mg/kg group, clarityne at 10 mg/kg as a positive control group. The animals were intraperitoneal injected with TSL and clarityne once a day one hour before OVA challenge for 14 consecutive days. The normal group and model group were given equal volume of distilled water.

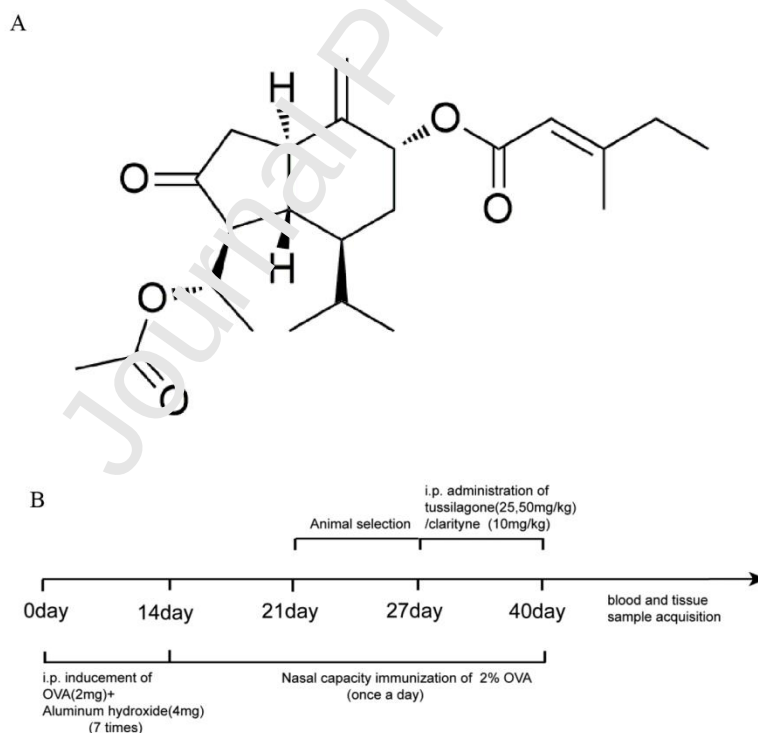


Fig. 1 Chemical structure of TSL and the process of modeling allergic rhinitis in

guinea pigs. A. Chemical structure of TSL; B. Sensitization and challenge protocol for the experimental guinea pig allergic rhinitis model.

Symptom	1 points	2 points	3 points
Scratching the nose	gently 1/2 times	frequently	non-stop
Sneeze	1-3	4-10	>11
Runny nose	left to the nostrils	beyond the anterior nostril	turbulent

Table 1 The evaluation criteria of allergic rhinitis symptoms.

2.4 Evaluation of AR symptoms

Before the administration of the tested drug, the score of the nasal symptoms of the guinea pigs challenged by OVA on day 27 was taken as the pre-treatment symptoms. After the last treatment of the tested drug on day 40, the score of nasal symptoms was taken as the post-treatment symptoms.

2.5 Histological analysis of nasal mucosal tissues

24 h after the last OVA challenge and drug administration, the animals in each group were weighed and anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium. The nasal mucosa was removed and fixed with 10% buffered formalin fixative solution for 48 h. The fixed tissue was dehydrated, hyalinized, paraffin embedded and made into 4 μ m tissue sections and stained with hematoxylin-eosin (H&E). After that, the epithelial tissue and the infiltration of inflammatory cells were

observed under optical microscope.

2.6 Measurement of concentrations of IgE, histamine, IL-6 and TNF- α in the serum

24 h after the last challenge and drug administration, the animals in each group were weighed and anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium. Blood were collected by intraorbital vein puncture. Blood samples were placed at room temperature for 2-3 h and centrifuged for 10 min at 3000 rpm/min. Blood serum were collected and stored at - 20 °C. Serum frozen at - 20 °C was taken out and thawed. Histamine, OVA-specific IgE, IL-6 and TNF- α levels of the serum were measured by using enzyme-linked immunosorbent assay (ELISA) according to the manufacture's instruction.

2.7 Analysis of phosphorylation of Lyn, Syk, Akt, NF- κ B and MAPKs in IgE-stimulated RBL-2H3 cells

RBL-2H3 cells (2.5×10^5 cells/ml) were seeded into a 24-well flat-bottom plate. 24 h later, 0.25 μ g/ml anti-DNP IgE was used to stimulate the cells for 48 h. Then the cells were washed three times with PIPES (+) buffer and treated with 1, 10, 100 and 500 μ M of TSL. Cells with (model control) or without (normal control) anti-DNP IgE were treated with the same amount of medium. After 6 h, the cells were washed 3 times with PIPES (+) buffer and further stimulated with 30 ng/ml DNP-HAS for 30 min. In the normal control group, the cells was treated by the same amount of buffer for 30 min. After that, the supernatant of 24-well flat-bottom plate was discarded, washed once and immediately lysed in RIPA lysis buffer for 30 min. After lysis, all

the liquids were absorbed and centrifuged at 12 000 rpm/min for 20 min at 4 °C. The supernatant was absorbed and the protein concentration was determined by BCA protein assay kit. Samples were mixed with 5 x loading Buffer at 4:1, denatured at 95 °C for 10 min, and stored at – 80 °C. Equal amounts of protein (20 µg) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked at room temperature for 2 h by TBST containing 5% skim milk. After blocking, membranes were washed by TBST and incubated with different primary antibodies overnight at 4 °C. Next, the PVDF membranes were incubated with secondary antibody (Goat Anti-Rabbit IgG peroxidase conjugate) for 1 h at 37 °C. The primary antibodies include Rabbit monoclonal antibodies for Lyn, phospho-Lyn, ERK, phospho-ERK, Syk, phospho-Syk, p38, phospho-p38, Akt, phospho-Akt, NF-κB p65, phospho-NF-κB and β-actin. Finally, the membranes were washed again and the protein bands were visualized using clarityTM western ECL substrate and the gray level analysis of protein bands was also carried out.

2.8 Statistical analysis

The data were expressed as mean $\pm$ standard deviation (SD) from at least three independent experiments. Comparisons between different groups were performed using one-way ANOVA, * p < 0.05, ** p < 0.01, *** p < 0.001 were considered to be statistically significant.

3. Results

3.1 Effect of TSL on allergic symptoms in OVA-induced AR guinea pigs

Nasal symptoms of guinea pigs within 30 min were evaluated after the challenge on day 27 and the last administration on day 40. After challenge by OVA, the symptoms of nasal scratching, sneezing and runny nose in guinea pigs were obvious, and the superimposed scores were more than 5 points, which indicated that the model was successful. As shown in Fig. 2, before drug administration (day 27), the total symptom scores of both AR model group and drug administration group were significantly higher compared with the normal group ($p < 0.01$), which proved that the AR model was successful. After drug administration (day 40), the scores of nasal symptoms in TSL (50, 25 mg/kg) and Clarityne group (10 mg/kg) were significantly lower than those in the model group ($p < 0.01$). Compared with normal control group, the scores of nasal symptoms in TSL groups and Clarityne group were still higher, but the difference was not statistically significant. The results showed that TSL and Clarityne could significantly improve the nasal allergic symptoms of OVA-induced allergic rhinitis guinea pigs.

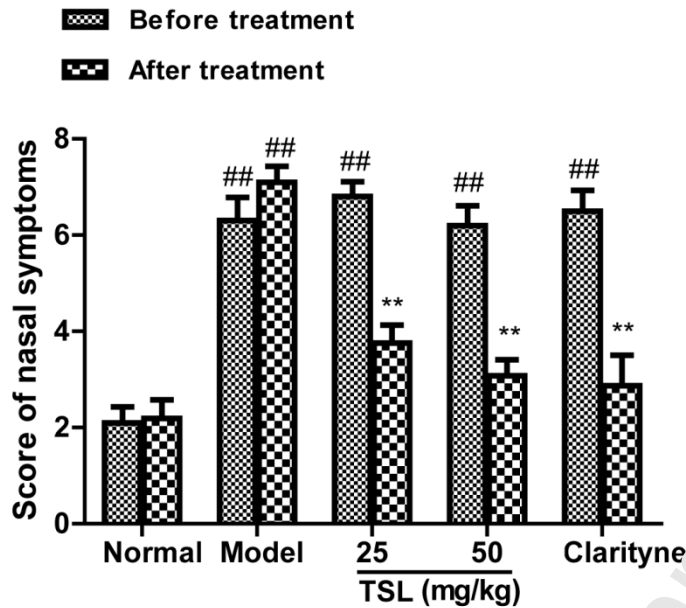


Fig. 2 Effect of TSL on allergic symptoms of OVA-induced allergic rhinitis guinea pigs. Nasal symptoms including nasal scratching, sneezing and runny nose were measured and scored according to the evaluation criteria. The data were presented as mean $\pm$ SD, $n = 7$ per group. ^{##} $p < 0.01$, compared with normal group; ^{**} $p < 0.01$, compared with model group.

3.2 Effects of TSL on histological changes of nasal mucosa

To evaluate the histological changes of nasal mucosa in OVA-induced AR guinea pigs, the nasal mucosa samples were stained with H&E and observed under light microscope. As shown in Fig. 3 A, the nasal epithelial cells of normal guinea pigs were intact and there is few inflammatory cell infiltration. The infiltration of inflammatory cells such as eosinophils after treatment with OVA alone was significantly increased compared to the normal group (Fig. 3 B). In the OVA-sensitized guinea pigs, TSL administrated at doses of both 50 and 25 mg/kg

decreased the inflammatory cell infiltration (Fig. 3 D, E). It was notable that the effect of TSL at 50 mg/kg was similar to the positive control Clarityne (Fig. 3 C).

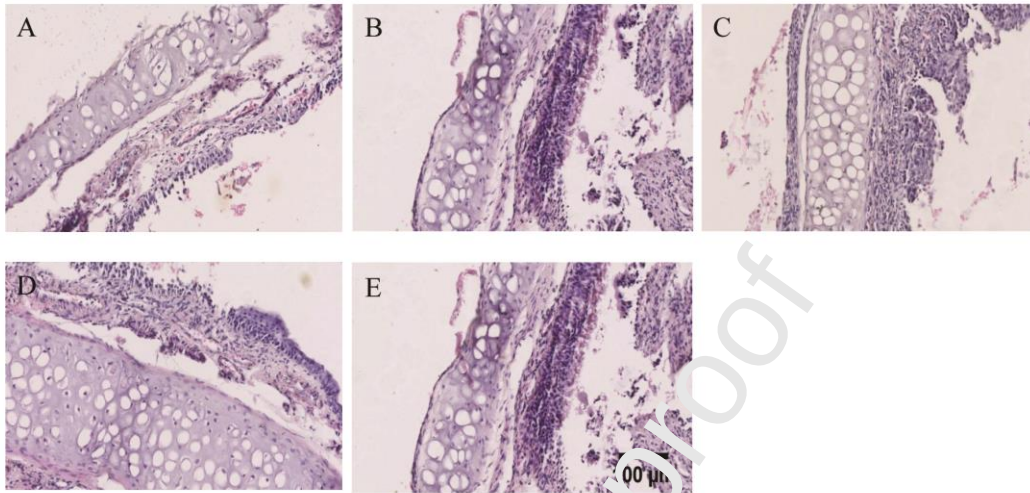


Fig. 3 Effect of TSL on inflammatory cells infiltration in the nasal mucosa tissue of allergic rhinitis. The histological changes of nasal mucosa were observed by light microscope after stained with H&E. A. Normal; B. Model; C. Clarityne (10 mg/kg); D. TSL (50 mg/kg); E. TSL (25 mg/kg). The data were presented as mean $\pm$ SD, n= 7 per group.

3.3 Inhibitory effect of TSL on the levels of OVA-specific IgE, histamine, IL-6 and TNF- α in the serum from OVA-induced AR guinea pigs

The concentrations of histamine, OVA-specific IgE, IL-6, and TNF- α in the serum were determined by ELISA kit. Compared with normal group, the concentrations of histamine, OVA-specific IgE, IL-6 and TNF- α increased significantly after OVA sensitization (Fig. 4, $p < 0.05$). However, the levels of histamine, OVA-specific IgE and IL-6 decreased obviously after intraperitoneal injection of TSL at the doses of 50

and 25 mg/kg. It was notable that the effect of 50 mg/kg TSL was as good as the positive control drug Clarityne. Unexpectedly, the secretion of TNF- α increased after administration of 25 mg/kg of TSL. Even at the higher dose of 50 mg/kg, TSL didn't show significant inhibition of the secretion of TNF- α .

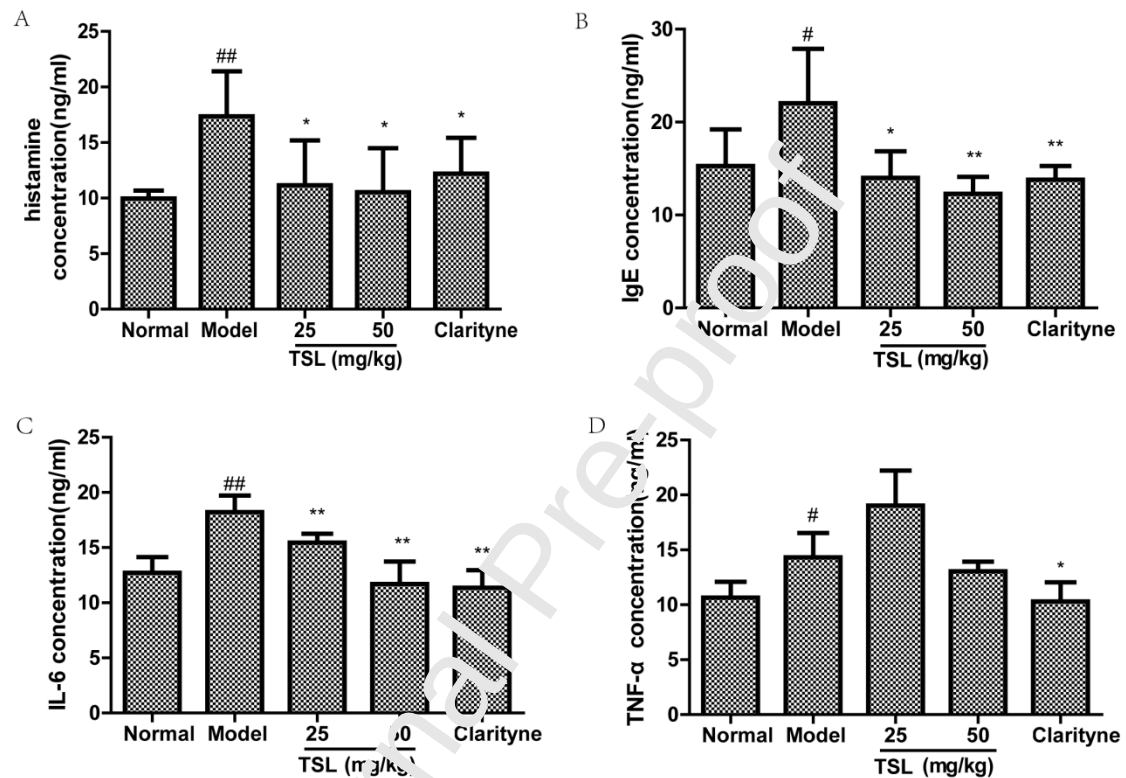


Fig. 4 Effect of TSL on the levels of histamine, OVA-specific IgE, IL-6 and TNF- α in OVA-induced guinea pigs. The levels of histamine (A), OVA-specific IgE (B), IL-6 (C), TNF- α (D) were measured by ELISA. The data were presented as mean $\pm$ SD, $n=7$ per group. [#] $p < 0.05$, ^{##} $p < 0.01$, compared with normal group; ^{*} $p < 0.05$, ^{**} $p < 0.01$, compared with model group.

3.4 Inhibitory effect of TSL on the phosphorylation of Lyn, Syk, Akt, NF- κ B and MAPKs in IgE-stimulated RBL-2H3 cells

To determine whether TSL relieves AR pathological changes through allergic and

inflammatory related signaling pathways, the phosphorylation of Lyn, Syk, Akt, NF- κ B and MAPKs were analyzed in IgE-stimulated RBL-2H3 cells. We tested the cell viability after TSL treatment by MTT assay in many cell lines including RBL-2H3 and found TSL 1 - 500 μ M show little cytotoxicity (data not shown). Therefore, we use these concentrations for drug treatment in this experiment. As shown in Fig. 5, the phosphorylation of Lyn, Syk, Akt, NF- κ B p65, ERK and p38 MAPK in RBL-2H3 cells sensitized with anti-DNP IgE (0.25 μ g/mL) for 48 h were up-regulated than those in normal cells ($p < 0.01$). After treatment with TSL at the doses of (1, 10, 100, 500 μ M), the phosphorylation levels of Lyn, Syk, Akt, NF- κ B p65, ERK and p38 MAPK was obviously suppressed in a concentration-dependent manner.

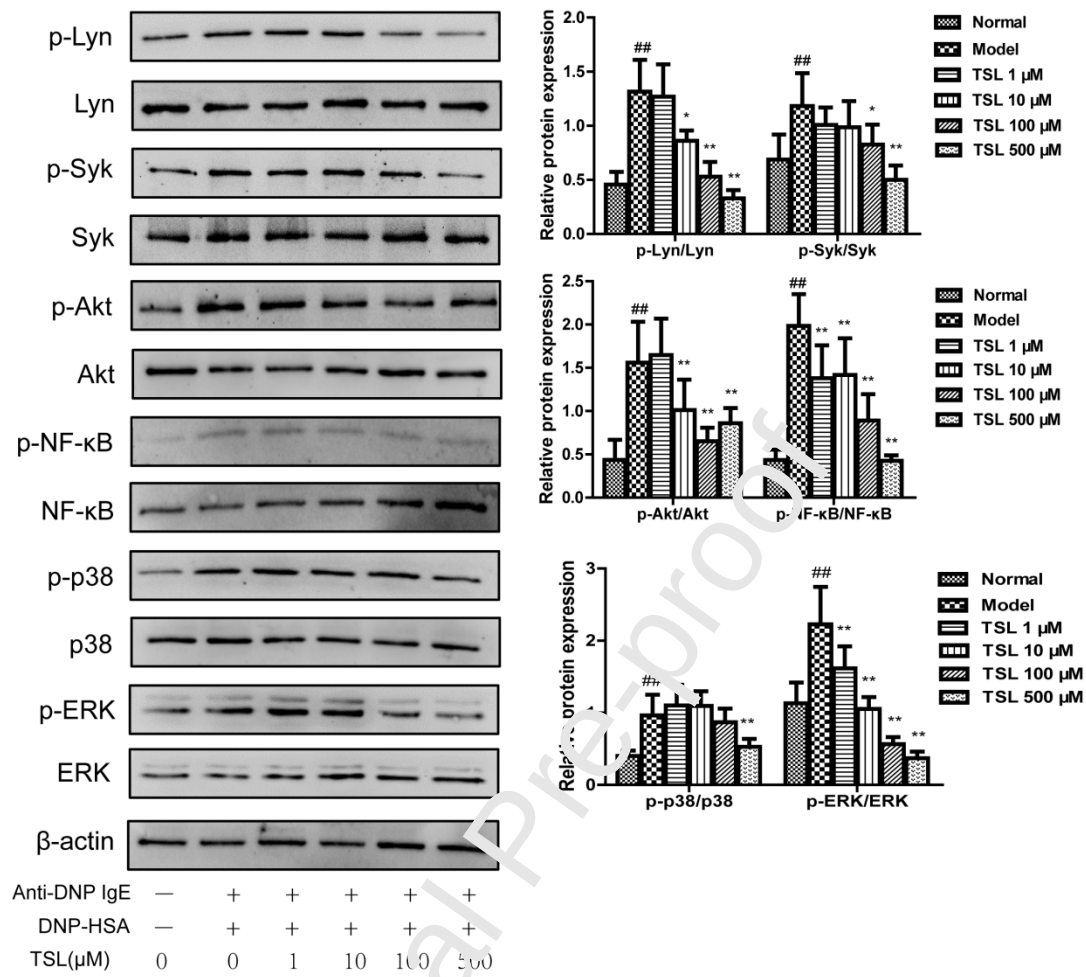


Fig. 5 Effect of TSL on the phosphorylation of Lyn, Syk, Akt, NF- κ B p65, Erk and p38 MAPK signaling pathways in anti-DNP IgE-sensitized RBL-2H3 cells. The protein expression of Lyn, Syk, Akt, NF- κ B p65, ERK and p38 MAPK was analyzed by western blot. The results were expressed as mean $\pm$ SD, $^{##}p < 0.01$, compared with normal group; $^{*}p < 0.05$, $^{**}p < 0.01$, compared with model group.

4. Discussion

Farfarae Flos is a traditional Chinese medicine widely used to treat inflammatory and allergic diseases in clinical. Although many active components have been isolated

from the plant, the anti-allergic component is still unknown. The main finding in the present study is TSL, an important active component of *Farfarae Flos*, has obvious anti-allergic effect in OVA-induced allergic rhinitis guinea pigs. The mechanism may be through the inhibition of Lyn, Syk, NF- κ B and MAPK pathways in the mast cells.

AR belongs to the category of type I allergic diseases and its pathological features are airway remodeling of the lung and allergic inflammation [12]. In AR, the body first responds to the inhalation antigen and produces IgE antibodies in the nasal mucosa tissues. In sensitized individuals, the simulated IgE binds to the high affinity IgE receptor (Fc ϵ R I) expressed on the surface of mast cells when re-exposed to the antigen. This cross binding then activates mast cells to release inflammatory mediators such as histamine and leukotrienes (LTs) which further stimulates local mucosal nerve endings and blood vessels to exhibit sneezing, runny nose, nasal mucosal edema and other AR symptoms. After these pathological processes, various inflammatory cells such as eosinophils will be recruited to the local nasal mucosa by cytokines such as TNF- α and IL-6 released by mast cells, which further aggravate the inflammatory symptoms [1, 13]. It is known that OVA can be used to successfully model AR in guinea pigs [14]. In this study, our results showed the symptoms of scratching nose, sneezing and runny nose increased after OVA stimulation (Fig. 2). The tissue pathology changes of the infiltration of eosinophils of the nasal mucosa were also seen (Fig. 3). After TSL treatment, both the nasal allergic symptoms and the pathological changes relieved in a dose-dependent manner. Moreover, our results showed the elevation of histamine, IgE and IL-6 in the serum of OVA-sensitized

guinea pigs were obviously decreased by TSL (Fig. 4). It is unexpected that $\text{TNF-}\alpha$, an important inflammatory cytokine in the pathological process of AR, was observed increased after TSL administration at the dose of 25 mg/kg. We speculated that there might be a feedback regulation in the body of mouse for the inhibitory effect of TSL. However, our results still indicate the definite therapeutic effect of TSL for AR. TSL is a main active component with a variety of biological activities. The significance of this finding is to supplement the anti-allergic effect on the basis of the reported biological activities of TSL.

Mast cell is a key effector cell in AR and would release three types of pro-inflammatory mediators under the stimulation of antigen-antibody reaction: chemical mediators stored in cytoplasmic granules such as histamine, arachidonic acid metabolites like leukotrienes, and pro-inflammatory cytokines such as $\text{TNF-}\alpha$ and IL-6 [15]. (RBL)-2H3 cells is the rat basophilic leukemia cell line which represents many of the characteristics of the mast cell and has been widely used in the study of allergy diseases. In addition, it is especially a good tool for studying IgE and IgE receptor ($\text{Fc}\epsilon\text{R I}$) initiated signaling pathways [16]. Accordingly, we choosed (RBL)-2H3 cells to observe the direct effect of TSL on mast cells to further explore the mechanism of its anti-AR effect. The assembly of heterotetramer structure of $\text{Fc}\epsilon\text{R I}$ is initiated by the phosphorylation of tyrosine activated motifs of immune receptors on the β and γ subunits by Src family kinases (such as Lyn and Fyn). The phosphorylation of γ subunit then further recruits and activates Syk and inducing the

activation of a variety of downstream signaling molecules [15]. Our results showed the phosphorylation of Lyn and Syk increased significantly in RBL-2H3 cells when sensitized by anti-DNP IgE. Notably, after treated with TSL at doses of 1, 10, 100 and 500 μ M, the phosphorylation of Lyn and Syk was significantly reduced in a concentration dependent manner (Fig. 5). NF- κ B and MAPK pathways are two important inflammatory related signaling pathways in mast cells. NF- κ B is a transcription factor which can induce the production of various inflammatory cytokine including IL-6 and TNF- α . MAPKs include c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase 1/2 (ERK1/2). The phosphorylation of MAPKs can activate another transcription factor AP-1 which can also increase the gene expression of multiple inflammatory cytokines. Akt is a molecule which can regulate the activation of NF- κ B by phosphorylation of I κ B kinases, an inhibitory protein of NF- κ B [17]. Akt was also found a downstream molecule of Lyn-Syk mediating AMPK inhibition in (RBL)-2H3 cells [18]. Our results showed TSL significantly inhibited the phosphorylation of NF- κ B p65, ERK1/2, p38 MAPK and Akt (Fig. 5). All of these results in (RBL)-2H3 cells indicate TSL exerts anti-allergic effect through inhibiting allergic and inflammatory related pathways. In the previous studies, TSL was also found inhibiting the NF- κ B signaling pathway in airway epithelial cells [19]. Another study showed that TSL significantly decreased LPS-induced production of IL-1 β , and IL-6 mRNA by inhibiting MAPK and NF- κ B pathways in BV2 Microglia [20]. These results are consistent with our results found in mast cell line, which indicate TSL can produce similar anti-inflammatory effects in different types of cells.

Although the anti-inflammatory activities of TSL have been reported in some other cells in the previous study, there are few studies on the effect of TSL on Lyn-Syk pathway. The present results confirmed the inhibition of TSL on Lyn-Syk axis, which indicated the anti-allergic and anti-inflammatory mechanisms of TSL is through multiple targets.

5. Conclusion

The present study demonstrated that TSL has a protective effect on OVA-induced allergic guinea pigs by improving rhinitis symptoms, alleviating pathology changes of the nasal mucosa and decreasing the production of IgE, histamine, and IL-6. One of the mechanisms of anti-AR effect of TSL is the inhibition of Lyn/Syk, NF- κ B and p38 MAPK signaling pathways in activated mast cells. These results suggest that TSL is an important anti-allergic component of the Chinese traditional medicine *Farfarae Flos* and it has the potential of being developed into a new natural drug for treating AR in the future.

Acknowledgments

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Declarations of Interests: None.

Supplementary data**Supplementary material****References**

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Sneeze	1-3	4-10	>11
Runny nose	left to the nostrils	beyond the anterior nostril	turbulent

Author Statement

All authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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Declaration of interest

The authors declare that there are no conflict of interests **involved in the** manuscript

“Tussilagone inhibits allergic responses in OVA-induced allergic rhinitis guinea pigs and IgE-stimulated RBL-2H3 cells.”