

Screening of *Sceptridium ternatum* for antitussive and antiasthmatic activity and associated mechanisms

Ping Huang^{1,2}, Wenxiu Xin², Xiaowei Zheng²,
Fang Luo², Qinglin Li² and Guiyuan Lv¹

Abstract

Objectives: *Sceptridium ternatum* is an expectorant in traditional Chinese medicine and is prescribed for the treatment of asthma. The study aim was to screen *Sceptridium ternatum* for ingredients with antitussive and antiasthmatic effects and to study their associated mechanisms.

Methods: Cough in mice was induced using ammonia. Cough latency and the number of coughs within 3 minutes were determined. Airway responsiveness was assessed using ovalbumin as a sensitizer and characteristic asthma indicators were measured.

Results: Chloroform and ethyl acetate extracts significantly reduced the number of coughs within 3 minutes, tidal volume, and the percentage of eosinophilic granulocytes, lymphocytes and neutrophils. All extracts decreased airway responsiveness in asthmatic mice compared with the untreated group. Petroleum ether, chloroform and n-butanol extracts lowered the Penh values of asthmatic mice. Petroleum ether and ethyl acetate extracts greatly reduced interleukin-4 expression and the interleukin-4/interferon gamma ratio. Compared with the model group, all extracts reduced mRNA expression of the cysteinyl leukotriene receptor-1 (CysLT₁).

Conclusions: Chloroform extract and ethyl acetate extract displayed obvious antitussive effects and reduced airway inflammation. Thus, these two extracts contain the effective ingredients of *Sceptridium ternatum*. The active mechanism was ascribed to inhibition of mRNA expression of the CysLT₁ receptor in mice with bronchial asthma.

Keywords

Sceptridium ternatum, effective ingredients, antitussive, antiasthmatic, asthma, acting mechanism

Date received: 5 January 2017; accepted: 30 June 2017

Introduction

Asthma is a chronic inflammatory disorder characterized by airway hyperresponsiveness, obstruction, hyperproduction of mucus, persistent inflammation and infiltration of the

¹College of Pharmacy, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, PR China

²Department of Pharmacy, Zhejiang Cancer Hospital, Hangzhou, PR China

Corresponding author:

Guiyuan Lv, Zhejiang Chinese Medical University, Hangzhou, Zhejiang 310053, PR China.

Email: lv.gy@263.net



airways with airway wall remodeling, and histological changes. Airway remodeling, characterized by thickening of the airway wall, can have profound consequences for the mechanics of airway narrowing and can contribute to the chronic progression of the disease.¹⁻⁵ The clinical hallmarks of asthma include airway hyperresponsiveness and inflammation. Allergic asthma is a clinical syndrome characterized by T helper cell (Th)1/Th2 imbalance. Allergies are caused by characteristic immune responses to allergens, including the production of interferon gamma (IFN- γ) and interleukin (IL)-12, which is primarily secreted by Th1 cells. The cytokines released by Th2 cells modulate airway inflammation, which induces airway remodeling. Th2 cells synthesize high levels of IL-4 and IL-5. Research has shown that an imbalance between Th1 and Th2 cells leads to the clinical expression of allergic asthma disease.^{6,7} Cysteinyl leukotrienes (CysLTs), known historically as “the slow-reacting substance of anaphylaxis,” are primary inflammatory lipid mediators of several inflammatory diseases, including allergic asthma. CysLTs are produced predominantly by macrophages, eosinophils and mast cells in response to a variety of stimuli.⁸⁻¹⁰ These receptor antagonists have been widely used clinically with well-demonstrated therapeutic effectiveness.

Sceptridium ternatum (ST) is a common herb from Zhejiang Province, China. ST is considered to have an antitoxic effect on the body and has been prescribed for the treatment of asthma. Several studies have found that ST also has expectorant and anti-inflammatory effects.¹¹ However, the effective compounds and intrinsic mechanism of its antiasthmatic activity are unclear. In this study, we investigated the effect of the petroleum ether, chloroform, ethyl acetate and n-butanol layers on Th1/Th2 balance, the mRNA levels of several CysLTs and pathophysiological changes in lung tissues in an allergic asthma mouse model.^{11,12}

Materials

Animals

We purchased 60 female C57BL/6 mice and 70 female BALB/c mice (weight: 18–22 g) from Shanghai B & K Universal Group Limited (Shanghai, China) and raised them in specific pathogen-free conditions in the Laboratory Animal Center of Zhejiang University.

This study conformed to the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. The use of animals and all procedures in the study comply with relevant animal welfare acts and were approved and overseen by the Institutional Animal Care and Use Committee (application number: AEACU-14-018).

Medicines and reagents

ST herb (Figure 1) was collected from Lishui, Zhejiang Province. Voucher specimens were identified by Professor Xilin Chen of Zhejiang Chinese Medicine University, China. A voucher specimen of ST was deposited in the herbarium of the College of Pharmacy, Zhejiang Chinese Medical University, China.



Figure 1. The entire plant of *Sceptridium ternatum* Herba.

We used ammonia (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China, lot no. 201111); petroleum ether (AR grade, Tianjin Yong Ltd., Tianjing, China, lot no. 20141117); ethyl acetate (AR grade, Tianjin Yong Ltd., lot no. 20141018); chloroform (AR grade, Tianjin Yong Ltd., lot no. 20140404); n-butanol (AR grade, Tianjin Yong Ltd., lot no. 20140828); sodium carboxymethyl cellulose (Xian Yuhua Biotechnology Co., Ltd., Xian, China, lot no. 20140911); montelukast (Merck Pharmaceutical Company Co., Ltd, Jiangsu, China, lot no. 120360); ovalbumin (OVA, J & K Technology Co., Ltd., Shanghai, China, lot no. 1184101); adjuvant liquid aluminum (Imject Alum, Al(OH)₃/Mg(OH)₂, Pierce, USA); methacholine chloride (Mch, Sigma-Aldrich, St. Louis, MO, USA, lot no. 1396364); IL-4 ELISA kit (Bang Yi Biotechnology Co., Ltd, Shanghai, China, lot no. BYE30064); IFN- γ ELISA kit (Bang Yi Biotechnology Co., Ltd, Shanghai, China, lot no. BYE30038); Giemsa dye (Giemsa Stain, enzyme-linked assay, Biotechnology Co., Ltd., Shanghai, China, lot no. 015305); hematoxylin (J & K Technology Co., Ltd., Shanghai, China, lot no. 266819); eosin Y (Eosin Y disodium salt, J & K Technology Co., Ltd., Shanghai, China, lot no. 53019); reverse transcriptase (AMV Reverse Transcriptase, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China, lot no. B500999-0200); ribonucleotide mixture (DNTP mixture solution, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China, lot no. A610057); RNA inhibitor (RNase inhibitor, Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China, lot no. B600008); PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Shiga, Japan, lot no. 6110A) and One Step SYBR[®] PrimeScriptTM PLUS RT-PCR Kit (Perfect Real Time) (Takara Bio Inc., Japan, lot no. RR096A).

Laboratory apparatus

We used a double-chamber body plethysmograph (Buxco, Wilmington, NC, USA); optical microscope (Olympus, Tokyo, Japan); AL204 electronic analytical balance (ppm, Mettler Toledo, Greifensee, Switzerland); 3L rotary evaporator (RE-3000A, Asia Rong Biochemical Instrument Factory, Shanghai, China); 10 μ L, 200 μ L, 1 mL pipette (Eppendorf, Hamburg, Germany); full wavelength microplate reader (Thermo Fisher Scientific Inc., Rockford, IL, USA); enzyme-linked immunosorbent assay (Synergy 2, Asia Rong Biochemical Instrument Factory, Shanghai, China); nucleic acid protein analyzer (ND-2000, Thermo Fisher Scientific Inc., Rockford, IL, USA); centrifuge (5702, 5415R, Eppendorf, Hamburg, Germany); polymerase chain reaction (PCR; Mastercycler Gradient, Eppendorf, Hamburg, Germany); gel imaging system (Gel Doc 2000, Bio-Rad, Hercules, CA, USA); 96 quantitative PCR instrument (Mastercycler ep realplex, Eppendorf, Hamburg, Germany) and agarose gel electrophoresis (Mini-Sub[®] Cell GT Cell, Bio-Rad, Hercules, CA, USA).

Methods

Extraction of *ST* herb

ST herb (weight: 15 kg) was heated in 10 times the volume of 70% ethanol and extracted by reflux. Extraction was performed three times for 2 h each time. The filtrates were combined and concentrated under reduced pressure to obtain the extract. After dissolution in water, equal volumes of petroleum ether, chloroform, ethyl acetate and n-butanol were added successively for repeated extraction. Finally, 231 g petroleum ether extract (1.53%), 86.7 g chloroform extract (0.57%), 70 g ethyl acetate extract (0.46%) and 293.5 g n-butanol extract (1.94%) were obtained (Figure 2).

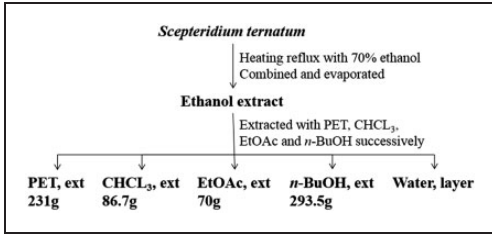


Figure 2. Flow chart of the extraction process for *Scepteridium ternatum*. PET: petroleum ether; CHCl₃: chloroform; EtOAc: ethyl acetate; n-BuOH: n-butanol.

Table 1. Chromatographic conditions of the high-performance liquid chromatography-tandem mass spectrometric method.

| Time (minutes) | A% | B% |
|----------------|----|----|
| 0 | 90 | 10 |
| 10 | 90 | 10 |
| 25 | 60 | 40 |
| 45 | 30 | 70 |
| 65 | 30 | 70 |
| 65.01 | 10 | 90 |
| 85 | 10 | 90 |

A: water with 0.5% formic acid; B: acetonitrile.

Analysis of ST extracts

All analysis of extracts was performed using high-performance liquid chromatography-tandem mass spectrometric methods. Chromatographic separations were achieved using a Waters Acquity BEHC18 column (2.1 mm × 150 mm, particle size 1.7µm, Waters, Wexford, Ireland). The chromatographic conditions are shown in Table 1. The total ion chromatogram graphs are shown in Figure 3.

Pharmacodynamic screening of ST for effective ingredients: cough induction

(1) *Animal grouping*

Sixty female C57BL/6 mice were randomly divided into six groups: control group, montelukast positive group, petroleum ether layer group, chloroform layer group, ethyl acetate layer group and n-butanol layer group (n = 10 per group).

(2) *Drug administration*

The ST extract for the different layers was dissolved using 0.5% sodium carboxymethyl cellulose. The petroleum ether layer, chloroform layer, ethyl acetate layer and n-butanol layer doses were 30 mg/kg, 12 mg/kg, 10 mg/kg and 40 mg/kg, respectively. The montelukast group dose was 10 mg/kg. Doses were administered for 7 days once daily by continuous gavage.

(3) *Cough induction with ammonia*

At 30 minutes after the last drug administration, 0.5 ml of ammonia was drawn with a pipette into the air-compressed atomizer. Ammonia atomization was performed for 2 minutes and cough was induced in mice by the breathing in of atomized ammonia for 30 s. The response of the mice was observed and the latency to the first cough and the number of coughs within 3 minutes were recorded. Mild cough was defined as abdominal muscle contraction and severe cough as the cough sound. One severe cough was counted as two mild coughs.

Pharmacodynamic screening of ST for effective ingredients: asthma indicators

(1) *Animal grouping*

Seventy female BALB/c mice were randomly divided into seven groups: control group, model group, montelukast positive group, petroleum ether layer group, chloroform layer group, ethyl acetate layer group and n-butanol layer group (n = 10 per group).

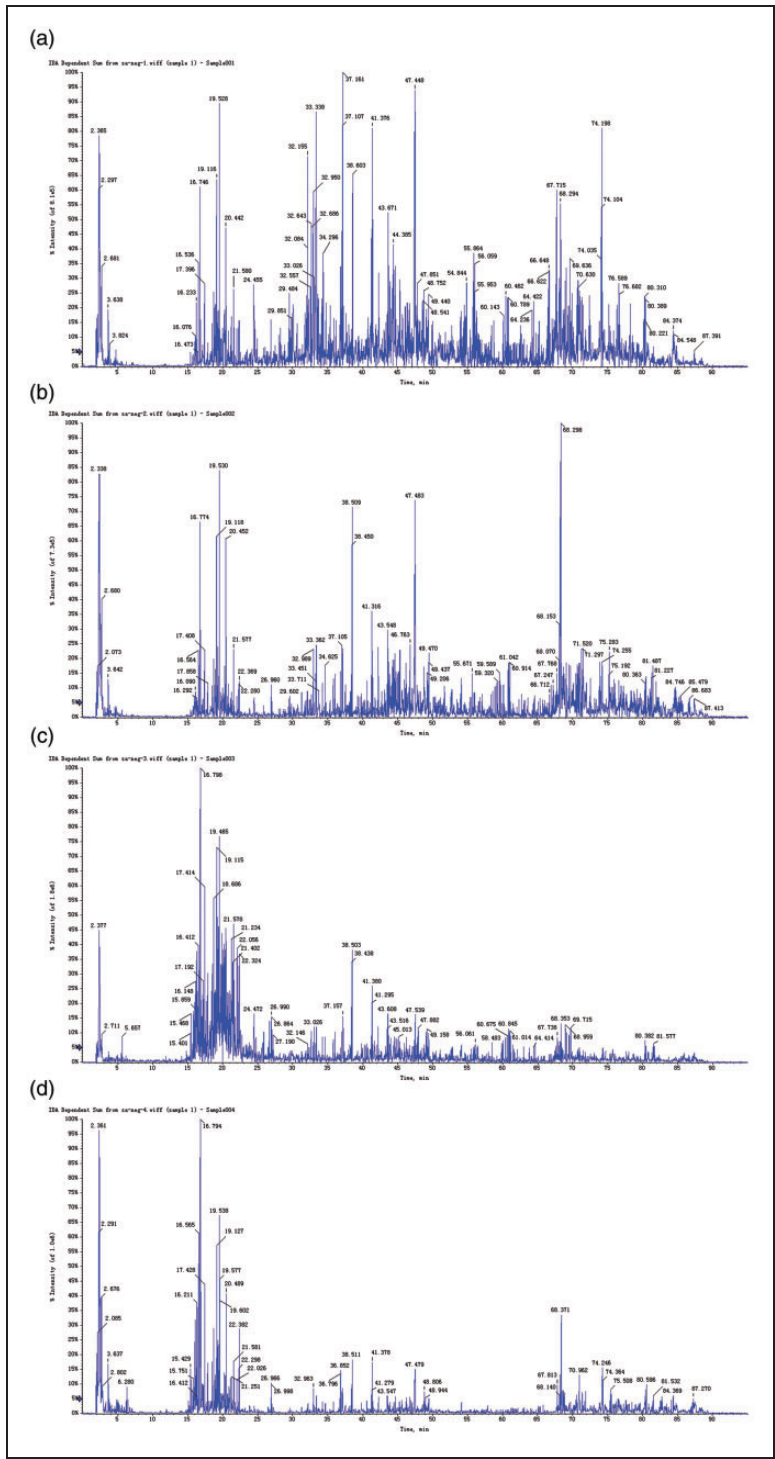


Figure 3. Total iron chromatogram (TIC) graphs of *Sceptridium ternatum* herb extracts. (a) chloroform extracts; (b) petroleum ether extracts; (c) n-butanol extracts; (d) ethyl acetate extracts.

(2) *Building of bronchial asthma model*¹³

All groups except the control group were sensitized by intraperitoneal injection of 0.2 ml liquid aluminum adjuvant containing 0.8 mg/ml OVA gel. Then the mice were placed in a closed container in which 1% OVA gel was atomized once daily for 20 minutes each time. Normal saline was atomized for the blank control group. The mice were sacrificed on day 28 of the experiment.

(3) *Drug administration*

The petroleum ether layer, chloroform layer, ethyl acetate layer and n-butanol layer doses were 30 mg/kg⁻¹, 12 mg/kg⁻¹, 10 mg/kg⁻¹ and 40 mg/kg⁻¹, respectively. The montelukast group dose was 10 mg/kg⁻¹. Starting in the first 18 days, doses were administered once daily by continuous gavage for 10 days. Doses were administered daily to the normal saline (control) group.

(4) *Determination of airway responsiveness*¹⁴

Penh value and tidal volume (TV) were measured. At 48 h after the last atomization, airway resistance was measured using a non-invasive respiratory and lung function measurement system and Penh values and TV were calculated. The peak inspiratory pressure (PIP), peak expiratory pressure (PEP), relaxation time (Tr) and expiration time (Te) were measured (Figure 4). Penh value was calculated by the formula

$$\text{Penh} = \text{PEP}/\text{PIP} \times \text{Pause},$$

$$\text{Pause} = (\text{Te} - \text{Tr})/\text{Tr}$$

First, mice were placed in the body plethysmograph system (each mouse in one plethysmograph box) and four measurements were taken. According to the setting,

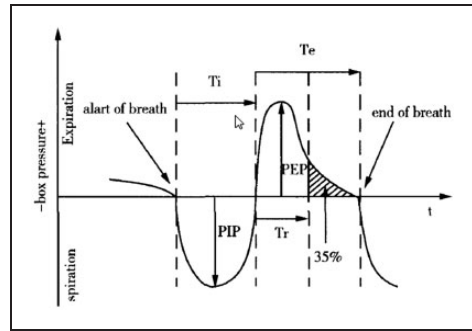


Figure 4. Enhanced pause (Penh) measurements.

the first baseline airway resistance was recorded, followed by determination of 30 μ L of normal saline and atomization of 30 μ L of double concentration Mch to stimulate airway constriction. Airway resistance, Penh values and TV were measured. The atomization excitation was carried out for 1 minute and observations were made for 3 minutes. Mch excitation gradient concentrations were 3.12, 6.25, 12.5 and 25 mg/ml. The Penh and TV values of physiological saline were used as standard values. The difference between the Mch Penh and TV values and the standard values was used as a statistical measure of airway reactivity.

(5) *Determination of IL-4 and IFN- γ expressions*

After measurement of airway responsiveness, a 0.7 ml sample of blood was drawn from the eye and added to the anticoagulant heparin. The blood samples were allowed to stand for 2 h and then centrifuged at 626 g for 15 minutes. The plasma was separated and preserved at -20°C . The expressions of IL-4 and IFN- γ were measured using the ELISA kit according to the manufacturer's instruction. The IL-4/IFN- γ expression ratio was calculated.

(6) *Histopathological observation of the left lung*

Table 2. Leukotriene receptor reverse primer design.

| Gene | Primer | Sequences (5'-3') | Amplified fragment size |
|--------------------|----------------|-------------------------|-------------------------|
| CysLT ₁ | Forward primer | CCAAGGCACCAAGCAGACATT | 157 bp |
| | Reverse primer | GCCAAAGAAACCCACAACAGA | |
| CysLT ₂ | Forward primer | TGGGAAAGGAAGAGTGAAGAATA | 241 bp |
| | Reverse primer | GTTGACAGAGGCGAGGACTAA | |
| GAPDH | Forward primer | AGGTTGTCTCTGCGACTTCA | 187 bp |
| | Reverse primer | GGGTGGTCCAGGGTTTCTTAC | |

CysLT: cysteinyl leukotrienes; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

The left lung was harvested and fixed in 4% paraformaldehyde. The tissues were embedded in paraffin and sliced into pieces 5 μ m thick. Hematoxylin and eosin staining was used to observe histopathological changes in the left lung. First the paraffin-embedded specimens were dewaxed twice in xylene for 5–10 minutes each time. Gradient elution was performed. Hematoxylin and eosin staining was carried out at room temperature for 5–10 minutes and the color was washed off with acid water and ammonia. The specimens were gently washed with running water three times to remove the staining solution and then once with distilled water. The specimens were dehydrated in gradient ethanol and transparentized in xylene, and the cover slip was sealed with neutral balsam. Five high-power visual fields were randomly selected under a light microscope and photographed. The images were treated and analyzed using ImageJ software (US National Institutes of Health, Bethesda, MD, USA). The experimental groups were compared with the control group in terms of bronchial inflammatory cell infiltration.

Effect of ST extracts on mRNA expression of CysLT receptors

The right lung tissues were removed from the fridge at -80°C and 50–100 mg of tissue was weighed for each group. Total RNA was

isolated from homogenized right lung tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Absorbance measurements at A260 and A280 and A260/A280 were obtained and computed using the nucleic acid protein analyzer (ND-2000, Thermo Fisher Scientific Inc., Rockford, IL, USA) to determine the concentration and purity of total mRNA. The extracted mRNA was preserved at -80°C . Two micrograms of RNA were then reverse transcribed to cDNA using the PrimeScript RT reagent kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) according to the manufacturer's instructions. Oligonucleotide sequences of PCR primers are shown in Table 2. The resulting cDNA product was added to SYBR Green fluorescent dye, in accordance with the FastStart Universal SYBR Green Master operation for PCR amplification, increased conditions: 95°C 10 minutes, 95°C 10 s for 40 cycles and 60°C 30 s for 40 cycles.

Agarose gel electrophoresis was carried out to determine the quantitative PCR specificity. We used $0.5 \times \text{TAE}$ 1.5% agarose gel buffer preparation, adding GoldView stock solution (containing 0.5% GoldView), 20 ml of PCR product and 4.0 ml of $6 \times \text{DNA}$ loading buffer. After mixing, 4 μ l mixed solution was gently added to a dispersion-like hole. Electrophoresis was carried out for 30

minutes at 80 V. A DAN Ladder was used as a control to determine the specific products.

DNA melting curves were determined. The 5 μ l PCR product was added to 5 μ l SYBR Green I (1:5000) and 10 μ l of water was detected using the quantitative PCR instrument. Analysis of PCR product melting curve was performed in the range 70°C to 98°C linear temperature per second. For preparation of the standard curve, real-time quantitative PCR (RT-qPCR) gene amplification of a 10-fold dilution of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA template CysLT₁ was used, five concentrations each, three wells per concentration. Δ cycle threshold (Δ Ct) values were calculated for each concentration of CysLT₁ and GAPDH gene cDNA and Δ Ct values were plotted against logarithmic concentration values. If the slope is close to zero, $2^{-\Delta\Delta Ct}$ can be used to represent the relative expression of the target gene.

Statistical methods

All results were means \pm standard deviations ($x \pm s$). SPSS version 20.0 software

(SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Analysis of variance (ANOVA) and t-tests were used to compare groups. Values of $P < 0.05$ were considered significant and values of $P < 0.01$ considered very significant. Airway reactivity was compared using repeated-measures ANOVA and there was a significant between-group difference. Single-factor ANOVA was used to compare dose groups. Values of $P < 0.05$ were considered significant and values of $P < 0.01$ considered very significant.

Results

Screening ST for ingredients with antitussive action

The antitussive effect of different ST extracts was compared (Figure 5). The latency to the first cough induced by ammonia atomization was similar in mice from different experimental groups compared with the control group. The number of coughs in mice treated by petroleum ether extract and n-butanol was lower than in the control group, but not significantly. Mice treated

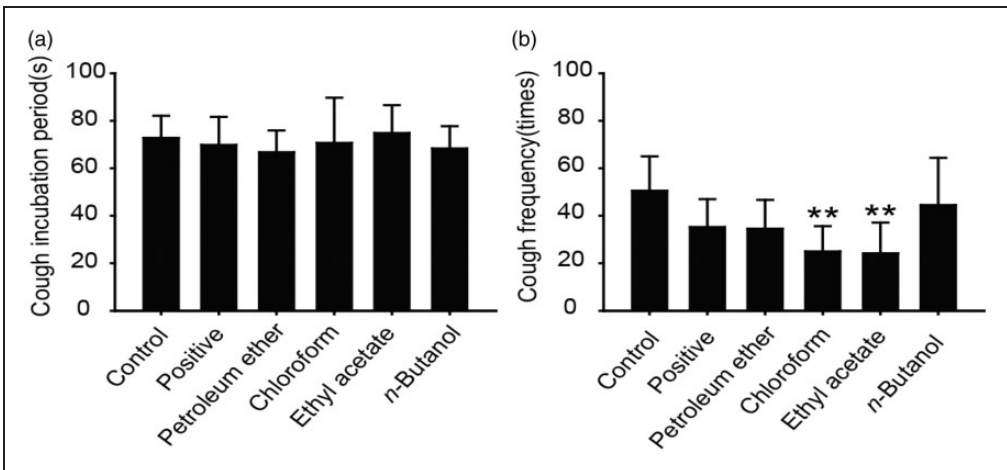


Figure 5. Effect of different *Sceptridium ternatum* extracts on cough latency and the number of coughs induced by ammonia in mice ((a) cough incubation period; (b) cough times). Note: compared with the control group, ** $P < 0.01$.

with chloroform extract and ethyl acetate extract showed a significantly lower number of coughs within 3 minutes than control group mice (25 ± 10.6 and 24.2 ± 12.9 vs. 50.5 ± 14.5 ; $P < 0.01$). The number of coughs within 3 minutes in mice receiving the two extracts was even smaller than that in mice treated by montelukast (35.2 ± 11.8).

The results indicated that different extracts of ST and montelukast did not affect latency to the first ammonia-induced cough. The chloroform extract and the ethyl acetate extract greatly reduced the number of coughs within 3 minutes in mice. Thus, these two ST extracts were effective in reducing cough.

Screening ST for ingredients with antiasthmatic action

To observe the antiasthmatic effect of different ST extracts, we used an ovalbumin-sensitized and late asthmatic mouse model (including atomization) and examined each ST extract for asthmatic airway reactivity, alveolar lavage inflammatory cells and the percentage of IL-4/IFN- γ ratio to screen for antiasthmatic effects.

(1) Effect of different ST extracts on suppressed airway hyperresponsiveness in a mouse asthma model

Airway hyperresponsiveness, which is a measure of airway resistance, was used as the main indication of asthma. We observed significant reduction in airway hyperresponsiveness in all treatment groups; mice in the model group had significantly greater ($P < 0.01$) airway responsiveness with each concentration of Mch compared with the control group. This indicated a dose-dependent inhibition of airway hyperresponsiveness. The petroleum ether layer, chloroform layer and n-butanol layer groups showed significantly less airway hyperresponsiveness ($P < 0.01$) than the model group at each Mch concentration, as measured by Penh values (Figure 6(a)). Different ST extracts significantly reduced airway hyperresponsiveness, as significantly higher TV was detected in both the chloroform and ethyl acetate layers ($P < 0.01$). Ventilation in asthmatic mice was increased (Figure 6(b)). The data indicate that different ST extracts have an inhibitory effect on airway hyperresponsiveness.

The airway hyperresponsiveness of ovalbumin-sensitized/challenged BALB/c mice treated for 10 days with different extracts of ST was examined. Mice were subject to an aerosolized OVA (25 mg/mL) inhalation challenge. Penh (A) and TV (B) values were determined. Values were expressed as means \pm SEMs ($n = 10$ /group). Statistical

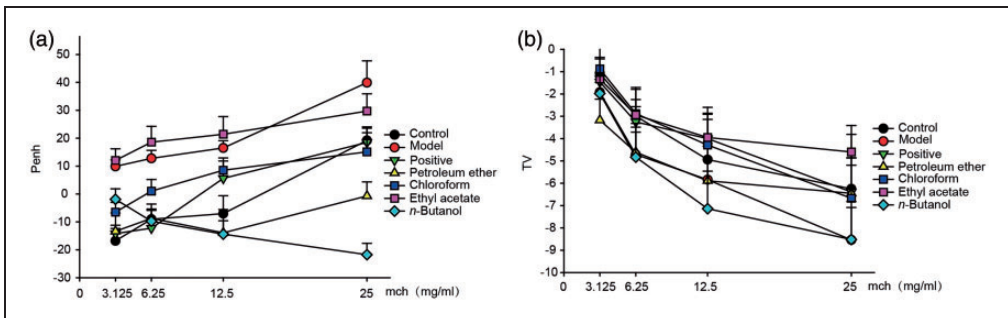


Figure 6. Effect of different *Sceptridium ternatum* extracts on Penh (a) and tidal volume (b) values in mice Mch: methacholine chloride; TV: tidal volume.

analysis was performed using Student's t-test ($P < 0.05$, $P < 0.01$).

(2) Effect of different *ST* extracts on IL-4/IFN- γ ratio in an asthma mouse model

The concentration of IL-4 and the ratio of IL-4/IFN- γ in the blood increased greatly in the model group compared with the

control group (Table 3 and Figure 7). An IL-4 level increase was significantly inhibited in the petroleum ether and ethyl acetate layer ($P < 0.05$) groups compared with the model group. Although the Th1 cytokine IFN- γ was not significantly affected, the Th1/Th2 ratio was significantly reduced (Table 3 and Figure 7(b)). These data suggest that the petroleum ether and ethyl

Table 3. Effect of different extracts of *Sceptridium ternatum* on mouse plasma levels of IL-4 and IFN- γ ($\bar{x} \pm s$, $n = 10$).

| Group | Dosage | IL-4 (ng/L ⁻¹) | IFN- γ (ng/L ⁻¹) | IL-4/IFN- γ |
|-----------------|------------------------|----------------------------|-------------------------------------|--------------------|
| Control | – | 132.4 \pm 24.2 | 693.5 \pm 112.0 | 0.20 \pm 0.05 |
| Model | – | 193.6 \pm 15.5# | 705.3 \pm 116.9 | 0.26 \pm 0.13# |
| Positive | 10 mg/kg ⁻¹ | 143.4 \pm 17.4 | 710.0 \pm 104.6 | 0.20 \pm 0.04* |
| Petroleum ether | 30 mg/kg ⁻¹ | 133.6 \pm 25.3* | 693.5 \pm 167.2 | 0.19 \pm 0.06* |
| Chloroform | 12 mg/kg ⁻¹ | 158.8 \pm 26.6 | 675.1 \pm 95.8 | 0.24 \pm 0.05 |
| Ethyl acetate | 10 mg/kg ⁻¹ | 156.4 \pm 25.1* | 734.7 \pm 114.7 | 0.21 \pm 0.05* |
| n-butanol | 40 mg/kg ⁻¹ | 157.2 \pm 17.3 | 700.7 \pm 132.1 | 0.22 \pm 0.04 |

Compared with control group, # $P < 0.05$; compared with model group, * $P < 0.05$. IL-4: interleukin-4; IFN- γ : interferon gamma.

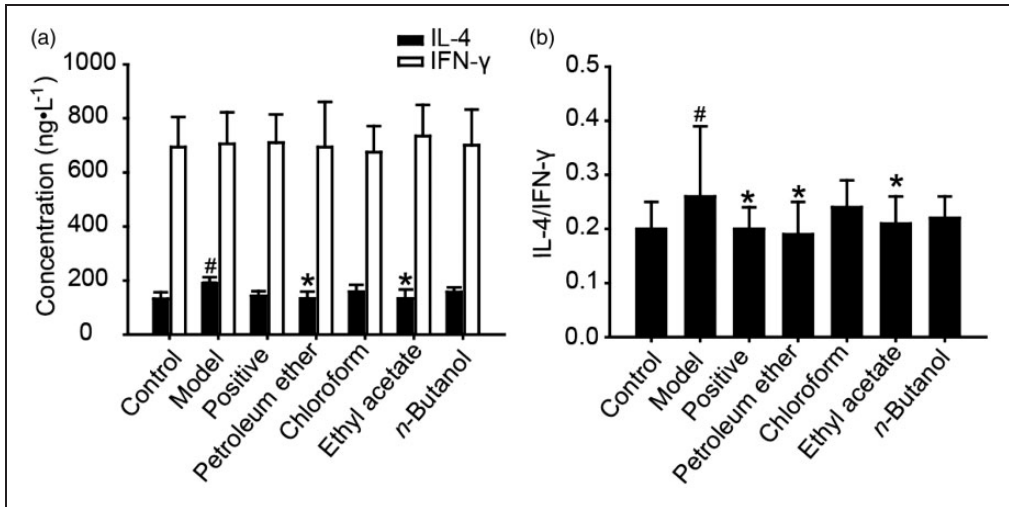


Figure 7. Effect of different *Sceptridium ternatum* extracts on mice plasma levels of interleukin (IL)-4 and interferon gamma (IFN- γ). The cytokines in supernatant were measured using an ELISA kit. Values are expressed as means \pm SEMs ($n = 10$ /group). Compared with control group, # $P < 0.05$; compared with model group, * $P < 0.05$.

acetate layers can inhibit allergen-specific stimulated Th2 cell activity and reduce the Th1/Th2 ratio.

(3) *Effect of different ST extracts on ovalbumin-induced airway inflammatory cells in broncho-alveolar lavage fluid (BALF)*

To evaluate the pulmonary inflammatory response to different ST extracts, BALF cells were recovered and the total and differential leukocyte counts were compared (Figure 8). The ratio of eosinophils, lymphocytes and neutrophils increased significantly ($P < 0.01$) in the model group. The chloroform and ethyl acetate layer groups showed a significantly decreased ($P < 0.01$) ratio of eosinophils, lymphocytes and neutrophils compared with the model group. These

data suggest that ST inhibited the infiltration of inflammatory cells to BALF in asthmatic mice, especially the chloroform and ethyl acetate layers.

(4) *Effect of different ST extracts on ovalbumin-induced inflammation in lung tissue*

Airway remodeling and inflammation are the two main pathological courses of bronchial asthma. We investigated the effects of different ST extracts on morphological changes in lung tissues in asthmatic mice. The control group showed normal histological structures and no or minimal inflammation, whereas the model group showed an increase in inflammatory cell infiltration in the airway lumen. Compared with the model group, all treatment groups showed significantly reduced inflammatory cell infiltration and mucus production. Chloroform and ethyl acetate layers can effectively reduce airway inflammatory cell infiltration and airway remodeling (Figure 9).

Effects of different ST extracts on the mRNA expression of CysLT receptors in mice

(1) *Agarose gel electrophoresis and melting curve*

The ST products were analyzed using gel electrophoresis after amplification. The CysLT₁ showed a sharp band with clean background, whereas the CysLT₂ mRNA had one non-specific band that could be quantified effectively (Figure 10). The melting curve for the CysLT₁ ($T_m = 82.07^\circ\text{C}$) exhibited a single melting peak, whereas the CysLT₂ generated two peaks, indicating two different PCR products: the one with a T_m of 76.35°C is the right product. The primer dimers resulting from CysLT₂ PCR indicate non-specific amplification, which

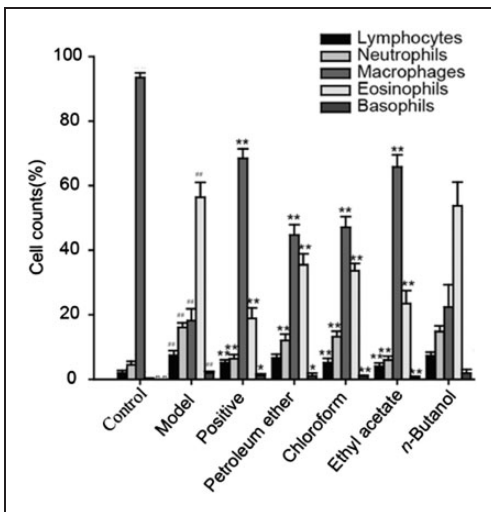


Figure 8. Effect of different *Sceptridium ternatum* extracts on the percentage of inflammatory cells in broncho-alveolar lavage fluid of asthmatic mice. Values are expressed as means \pm SEMs ($n = 10$ /group). Statistical analysis was performed using Student's t-test. Compared with control group, # $P < 0.05$; compared with model group, * $P < 0.05$, ## $P < 0.01$; compared with model group, ** $P < 0.01$.

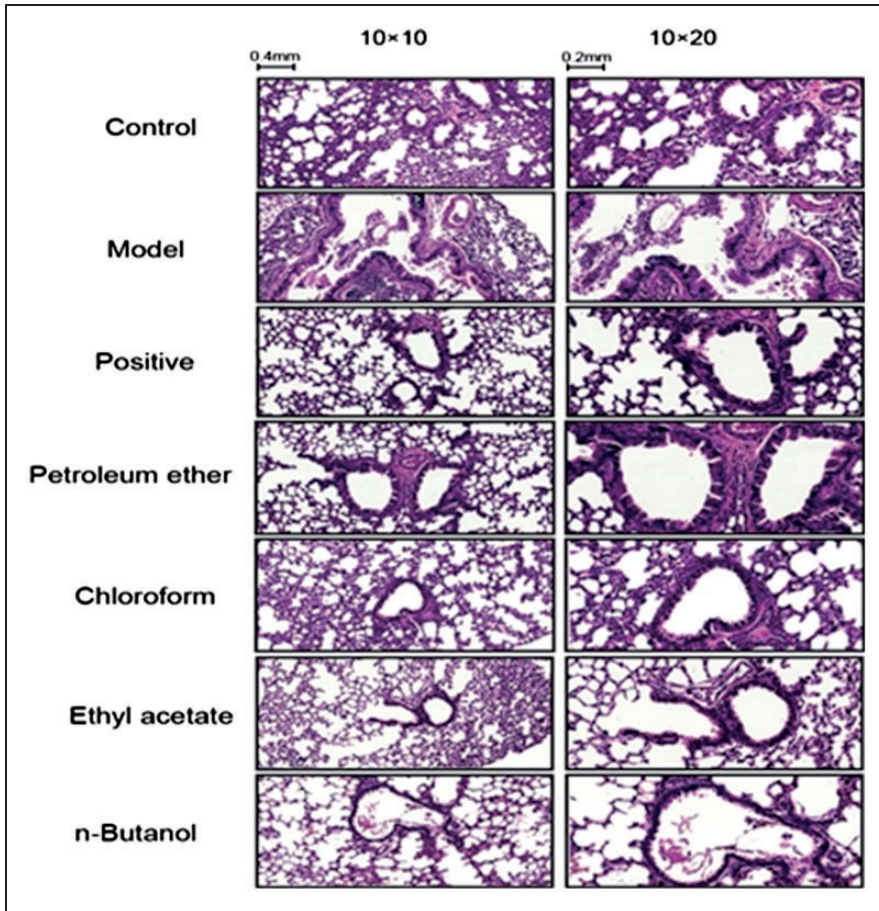


Figure 9. Biopsies showing effect of different *Sceptridium ternatum* extracts on lung tissue.

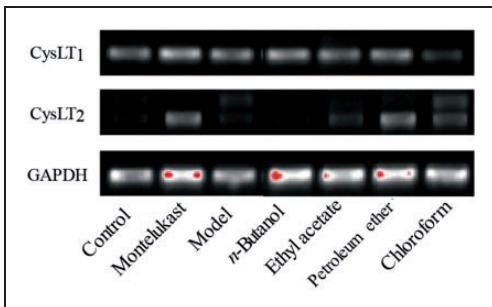


Figure 10. Agarose gel electrophoresis of CysLT₁ and CysLT₂. CysLT: cysteinyl leukotrienes; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

inevitably affects the accuracy of the experiment. This affects accurate quantification. Therefore, the results indicate that CysLT₁ showed better amplification and could be relatively quantified, but that CysLT₂ was not effectively amplified and could not be relatively quantified.

(2) Investigation of CysLT₁ standard curve

At a 10-fold dilution of the cDNA template, CysLT₁ and GAPDH genes were amplified and five sequential concentrations were selected for qRT-PCR. All qRT-PCR

Table 4. Δ Ct values for five concentrations of CysLT₁ and GAPDH.

| | | | | | |
|---------------|--------|-------|------|------|------|
| Concentration | 0.0001 | 0.001 | 0.01 | 0.1 | 1 |
| Δ Ct | 8.43 | 8.44 | 8.41 | 8.39 | 8.38 |

Δ Ct: Δ cycle threshold; CysLT: cysteinyl leukotrienes; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

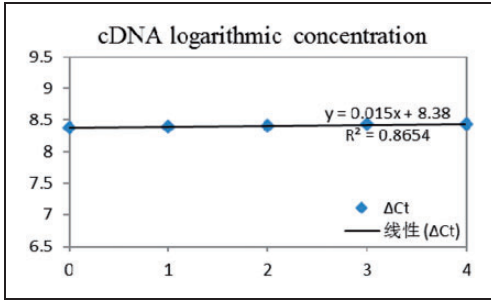


Figure 11. The curve of the relation between Δ Ct and cDNA logarithmic concentration for CysLT₁ and GAPDH. CysLT: cysteinyl leukotriene; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Δ Ct: Δ cycle threshold.

reactions were run in triplicate to minimize error. The Δ Ct value (Ct CysLT₁–Ct GAPDH) of CysLT₁ and GAPDH was calculated using the dilutions against cDNA concentration (Table 4). We generated a linear graph with a correlation coefficient of 0.015, indicating the similarity of amplification efficiency (Figure 11). The $2^{-\Delta\Delta$ Ct method can be used to analyze the relative expression of CysLT₁ mRNA receptors.¹²

(3) *Measurement of CysLT₁ expression*

The CysLT₁ expression level in mouse lung tissues was subjected to different treatment layers using the Ct value ($2^{-\Delta\Delta$ Ct) method. Compared with the control group, the CysLT₁ mRNA expression level was significantly greater in the model group ($P < 0.05$). Compared with the model group, the CysLT₁ mRNA expression level in the treatment group was significantly lower, especially in the chloroform and

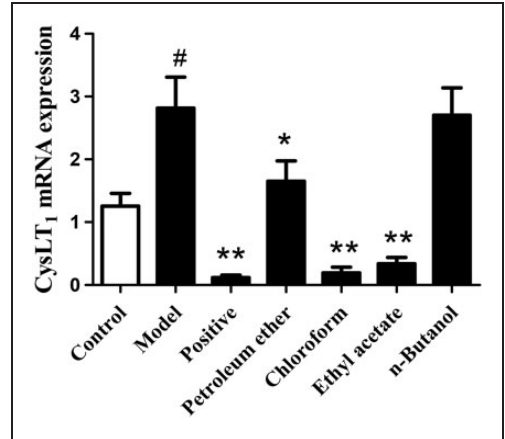


Figure 12. Effect of different *Sceptribidium ternatum* extracts on the expression of mRNA in lung tissue of asthmatic mice. Values are expressed as means \pm SEMs ($n = 10$ /group). Compared with

ethyl acetate layers ($P < 0.01$). The petroleum ether layer was also statistically different from the model group ($P < 0.05$) (Figure 12).

Discussion

The ingredients of ST showed antitussive and antiasthmatic actions. We found that different ST extracts relieved the symptoms of cough induced by exposure to ammonia in mice. The chloroform extract and the ethyl acetate extract showed strong antitussive and antiasthmatic effects compared with the control group ($P < 0.01$). A delayed asthma model was generated using ovalbumin as the sensitizer and mice were treated with different extracts of ST. The petroleum ether extract, the chloroform extract and the

n-butanol extract all reduced Penh values in asthmatic mice. The chloroform extract and the ethyl acetate extract increased TV and decreased the number of inflammatory cells in the BALF, thus reducing neutrophil infiltration in the airway. Thus, the chloroform extract and the ethyl acetate extract contained effective ingredients.

The Th1/Th2 ratio is important in the recruitment and activation of these cells at sites of inflammation. Allergic asthma is a clinical syndrome well characterized by Th1/Th2 imbalance¹⁵. Th1/Th2 imbalance may be an immunologic cause of bronchial asthma. IL-2 and IFN- γ are secreted by Th1 cells and are mediators of cellular defense mechanisms. Th2 cells generate cytokines such as IL-4, IL-5, IL-6, IL-9 and IL-13, which mediate allergic inflammation. The IL-4/IFN- γ ratio can reflect the Th1/Th2 balance. During an allergic response, such as in bronchial asthma, the function of Th1 cells will decline, whereas Th2 cells may be highly activated. This response is known as Th2 dominance. Thus, Th1/Th2 imbalance may initiate or maintain asthma and may promote airway inflammation and hyperresponsiveness. In this study, the IL-4 level noticeably increased in the model group, the IFN- γ level remained essentially constant and the IL-4/IFN- γ ratio notably increased compared with the control group. This indicated a shift to Th2 dominance, as described above. The petroleum ether extract and the ethyl acetate extract significantly reduced the IL-4 level and the IL-4/IFN- γ ratio in asthmatic mice ($P < 0.05$). This suggests that treatment with ST may alleviate airway inflammation by regulating the Th1/Th2 balance in asthmatic mice.

CysLTs can cause airway smooth muscle contraction, promote the accumulation of inflammatory cells and the release of cytokines or even induce changes in constituent cells in the airway. Thus, CysLTs play an important role in bronchial asthma. The expression of CysLT receptors is closely

related to the occurrence and progression of asthma. The inhibition of CysLT receptor expression can effectively relieve asthma.

The CysLT receptor regulator works by antagonizing leukotriene receptors or inhibiting leukotriene synthesis. CysLTs have two receptors:¹⁶ the CysLT₁ receptor can be blocked by a specific receptor antagonist, whereas the CysLT₂ receptor cannot. CysLT₁ receptor expression can enhance the accumulation of eosinophilic granulocytes and the release of adhesion factors and thus can aggravate inflammation.^{17,18} CysLT₂ receptor expression can promote the release of CysLTs and nucleotides and induce the release of IL-8 by damaged tissue, which leads to acute asthma characterized by neutrophil infiltration.¹⁹

We attempted to reveal the mechanism of the antiasthmatic effects of ST by detecting the mRNA expression of CysLT receptors in mice. The CysLT₁ mouse receptor comprises 339 amino acids and the CysLT₁ receptor comprises 309 amino acids; these receptors exhibit high similarity with the hCysLT₂ receptor in humans.^{20,21} The mRNA of the CysLT₁ receptor is widely expressed in C57BL/6 mice, including in the skin, lung and small intestine. CysLT₂ receptor mRNA has even wider expression in mice and is present in the spleen, lung and small intestine. In strain 129 mice, CysLT₂ receptor mRNA expression is very low in the spleen, lung and small intestine, which indicates the variation of CysLT₂ expression in different strains of mice.^{22,23} In the present study, CysLT₂ expression was very low. Thus, CysLT₂ receptor mRNA in BALB/c mice could not be quantitatively analyzed.

We detected the expression of CysLT₁ and CysLT₂ receptor mRNA. After one round of cDNA amplification, the amount of amplified product did not reach the RT-PCR detection threshold. Therefore, PCR amplifications were performed twice under the same conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 s and

40 cycles of 60°C for 30 s. We did not perform 80 cycles at once because the reliability of repeating 40 cycles twice was greater than that of performing all 80 cycles at once.

After several preliminary experiments with CysLT₂, we obtained satisfactory amplification results. The first PCR was carried out on CysLT₂ and GAPDH genes using 2 µl of the amplified product for 20 cycles. Then, 2 µl of the amplified fragments was diluted 10 times and subjected to 40 cycles of quantitative PCR. A satisfactory amplification diagram was obtained, but the amplified fragments were not specific. Two peaks were observed and the peak that corresponded to a T_m of 75.6°C was the target product (Figure 10). As indicated by agarose gel electrophoresis, one band (250 bp) was equivalent to the target product (241 bp) and this band was the target product (Figure 8). The target band was obtained using amplification for 20 cycles. However, the expression of CysLT₂ receptor mRNA was low, and there were a large number of primer dimers. This finding is in accord with previous research. The CysLT₂ receptor mRNA expression was therefore not studied quantitatively.

In summary, we identified effective antitussive and antiasthmatic ingredients in ST, and revealed that the mechanism of the antitussive and antiasthmatic actions was possibly related to the inhibition of the mRNA expression of CysLT₁ receptors in asthmatic mice.

Acknowledgment

The authors wish to acknowledge the Zhejiang Province Traditional Chinese Medicine key scientific research fund project (2016ZZ009); Zhejiang Province Traditional Chinese Medicine scientific research fund project (2017ZB022); Zhejiang Province Traditional Chinese Medicine youth scientific research fund project (2011ZQ001); Zhejiang Province health innovation personnel

training program (Huang Ping); and the second level training program of Zhejiang Province's 151 Talents Project (Huang Ping).

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This research received specific grants from the Zhejiang Province Traditional Chinese Medicine key scientific research fund project (2016ZZ009); Zhejiang Province Traditional Chinese Medicine scientific research fund project (2017ZB022); Zhejiang Province Traditional Chinese Medicine youth scientific research fund project (2011ZQ001); Zhejiang Province health innovation personnel training program (Huang Ping); and the second level training program of Zhejiang Province's 151 Talents Project (Huang Ping).

References

1. Chu KH, Lee CC, Hsin SC, et al. Arsenic trioxide alleviates airway hyperresponsiveness and eosinophilia in a murine model of asthma. *Cell Mol Immunol* 2010; 7: 375–380.
2. Galli SJ, Tsai M, Piliponsky AM, et al. The development of allergic inflammation. *Nature* 2008; 454: 445–454.
3. Hiroshima Y, Garthwaite L, Hsu K, et al. ISU201 enhances the resolution of airway inflammation in a mouse model of an acute exacerbation of asthma. *Mediators Inflamm* 2015; 2015: 405629.
4. Kudo M, Ishigatsubo Y and Aoki I. Pathology of asthma. *Front Microbiol* 2013; 4: 263.
5. Wang HY, Dai Y, Wang JL, et al. Anti-CD69 monoclonal antibody treatment inhibits airway inflammation in a mouse model of asthma. *J Zhejiang Univ Sci B* 2015; 16: 622–631.
6. Jeon WY, Shin IS, Shin HK, et al. Samsocum water extract attenuates allergic airway inflammation via modulation of Th1/Th2

- cytokines and decrease of iNOS expression in asthmatic mice. *BMC Complement Altern Med* 2015; 15: 47.
7. Tang F, Wang F, An L, et al. Upregulation of Tim-3 on CD4(+) T cells is associated with Th1/Th2 imbalance in patients with allergic asthma. *Int J Clin Exp Med* 2015; 8: 3809–3816.
 8. Dahlen SE, Hansson G, Hedqvist P, et al. Allergen challenge of lung tissue from asthmatics elicits bronchial contraction that correlates with the release of leukotrienes C4, D4, and E4. *Proc Natl Acad Sci U S A* 1983; 80: 1712–1716.
 9. Hay DW, Torphy TJ and Udem BJ. Cysteinyl leukotrienes in asthma: old mediators up to new tricks. *Trends Pharmacol Sci* 1995; 16: 304–309.
 10. Murphy RC, Hammarstrom S and Samuelsson B. Leukotriene C: a slow-reacting substance from murine mastocytoma cells. *Proc Natl Acad Sci U S A* 1979; 76: 4275–4279.
 11. Yuan Y, Yang B, Ye Z, et al. Sceptridium ternatum extract exerts antiasthmatic effects by regulating Th1/Th2 balance and the expression levels of leukotriene receptors in a mouse asthma model. *J Ethnopharmacol* 2013; 149: 701–706.
 12. Chen ML, Wu CH, Hung LS, et al. Ethanol extract of perilla frutescens suppresses allergen-specific Th2 responses and alleviates airway inflammation and Hyperreactivity in ovalbumin-sensitized murine model of asthma. *Evid Based Complement Alternat Med* 2015; 2015: 324265.
 13. Wang SM, Ruan JS, Zhuang J, et al. Effects of Sceptridium ternatum oral liquid on the pathological morphology and expectorant effect of chronic bronchitis model mice. *Fujian Journal of TCM* 2001; 32(3): 18–19. DOI 10.13260/j.cnki.jfjtc.007834.
 14. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. *Methods* 2001; 25: 402–408.
 15. Shi YH, Shi GC, Wan HY, et al. Coexistence of Th1/Th2 and Th17/Treg imbalances in patients with allergic asthma. *Chin Med J (Engl)* 2011; 124: 1951–1956.
 16. Chen T, Xiao L, Zhu L, et al. Anti-asthmatic effects of Ginsenoside Rb1 in a mouse model of allergic asthma through relegating Th1/Th2. *Inflammation* 2015; 38: 1814–1822.
 17. Robinson DS. The role of regulatory T lymphocytes in asthma pathogenesis. *Curr Allergy Asthma Rep* 2005; 5: 136–141.
 18. Zhang X, Zhong W, Meng Q, et al. Ambient PM2.5 exposure exacerbates severity of allergic asthma in previously sensitized mice. *J Asthma* 2015; 52: 785–794.
 19. Xu WH. Repetitive measurements of enhanced pause (Penh). *Respir Physiol Neurobiol* 2015; 206: 41–44.
 20. Brannan JD and Loughheed MD. Airway hyperresponsiveness in asthma: mechanisms, clinical significance, and treatment. *Front Physiol* 2012; 3: 460.
 21. Wei Y, Luo QL, Sun J, et al. Bu-Shen-Yi-Qi formulae suppress chronic airway inflammation and regulate Th17/Treg imbalance in the murine ovalbumin asthma model. *J Ethnopharmacol* 2015; 164: 368–377.
 22. Garcia-Lafuente A, Guillamon E, Villares A, et al. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* 2009; 58: 537–552.
 23. Warashina T, Umehara K and Miyase T. Flavonoid glycosides from Botrychium ternatum. *Chem Pharm Bull (Tokyo)* 2012; 60: 1561–1573.