



Sceptridium ternatum extract exerts antiasthmatic effects by regulating Th1/Th2 balance and the expression levels of leukotriene receptors in a mouse asthma model

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ABSTRACT

Aim of the study: *Sceptridium ternatum* Lyon (ST), a common Chinese herb, has been used in treatment of allergic asthma and whooping cough. In the present study, we investigated the Th1/Th2 ratio of peripheral blood and mRNA levels of leukotriene receptors after the treatment of ST in allergic asthma mouse model.

Materials and methods: Mouse asthma model was developed by ovalbumin (OVA) sensitization followed by the inhalation of aerosol allergen. Montelukast (10 mg/kg), as a positive control drug, and ST were administrated six days before the OVA sensitization for ten days. Airway responsiveness was evaluated by the Medlab 12.0 biological signal processing system. The ratio of Th1/Th2 cells was determined by flow cytometry. The expression level of *Cyslt1* was measured by PCR. Pathological changes of lung tissues were examined by H&E staining.

Results: ST significantly reduced the airway responsiveness, elevated the ratio of Th1/Th2, and decreased *Cyslt1* mRNA level in a dose-dependent manner. High-dose ST distinctly prevented the pathological changes of lung tissues.

Conclusion: High-dose ST had the same efficacy as Montelukast in a mouse asthma model, and ST could be a potential anti-asthmatic agent.

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1. Introduction

Atopic asthma is a chronic inflammatory disorder in the respiratory tract and has characteristics of airway hyper-responsiveness (AHR) and infiltration of a variety of inflammatory cells induced by cytokines and other mediators. It has been reported that atopic asthma is tightly associated with the imbalance of Th1/Th2 cells and their distinct cytokine profiles (Racke and Bonomo, 1999; Simon et al., 1999; Elewaut et al., 2002; Kanik et al., 2002). Th2 cells secrete Th2 specific cytokines, such as interleukin (IL)-4, 5 and 13, which mediate humoral immune responses and IgE production and recruit eosinophils to airways, thus further inducing the secretion of histamine and leukotriene. In contrast to Th2 cells, Th1 cells play the opposite role. Th1 cytokines, such as IFN- γ and IL-12, are involved in the mediation of cellular immune reaction and the antagonism of Th2 immune responses and IgE synthesis to restrain the development of asthma. In physiological condition, the ratio of Th1/Th2 cells achieves dynamic

balance. Whenever this balance is disturbed, diseases will occur. Therefore, one effective strategy for curing asthma is to stimulate Th1 cells and simultaneously inhibit Th2 immune responses to restore the ratio of Th1/Th2.

As the principle inflammatory mediators, leukotrienes (LTs) play pivotal roles in allergic asthma, not only promoting the accumulation of inflammatory cells in airways and enhancing vascular permeability and mucus secretion, but also inducing the contraction of airway smooth muscles and the proliferation of epithelial cells of respiratory tract. Since LTs gain the function through their specific receptors, LT receptor antagonists have been widely used in clinics with well proved therapeutic effectiveness.

Sceptridium ternatum (Thunb.) Lyon (ST), also named Herba *Sceptridium*, is a common herb in Zhejiang Province, China. It has been considered to have anti-toxic effects in the body. ST has been prescribed to treat asthma and whooping cough. Although a number of studies has found that ST has anti-inflammatory and expectorant effects (Wang et al., 2001), the intrinsic mechanism of its anti-asthma activity is unclear. In the present study, we investigated the effects of ST on Th1/Th2 balance, mRNA levels of several LT receptors and the pathophysiological changes of lung tissues in an allergic asthma mouse model.

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2. Materials and methods

2.1. Preparation of ST extract

The Herba *Sceptridium* was collected from Lishui, Zhejiang province, in May 2012. Voucher specimens were identified by Dr. Fangzhi Kong, professor of Zhejiang Academy of TCM and preserved at the Institute of basic clinical research. Dried and pulverized Herba *Sceptridium* (80 g) was extracted by 10 times volume of 70% (v/v) aqueous ethanol for two hours and repeated for 3 times. The filtrates were combined and concentrated into crude product by vacuum. The ethanol extract was then suspended in water to reach concentration of 500 mg/mL.

2.2. Animals

Eight-week-old female Balb/c mice (20–22 g) were purchased from Shanghai B&K Universal Group Limited and raised in specific pathogen free (SPF) conditions at the Experimental Animal Center, Zhejiang Academy of TCM. Animal studies were conducted strictly under international guidelines for laboratory animal use and care as found in the U.S. National Institutes of Health guidelines.

2.3. Mouse asthma model and drug treatment

Mouse model of allergic asthma was developed by ovalbumin (OVA, Shanghai kayon Biological Technology co., Ltd, China) sensitization and inhalation. Balb/c mice were randomly divided into six groups ($n=10/\text{group}$): untreated control group (normal), OVA control group, OVA+ST 19.35 g/kg group, OVA+ST 9.675 g/kg, OVA+ST 1.935 g/kg, and OVA+montelukast (Merck Pharmaceutical Co., Ltd.) 10 mg/kg group as a positive control. Except the normal, all groups were sensitized on day 1 and 14 by intraperitoneal (i.p.) injection with 0.1 mL of 0.08% OVA mixed with equal volume of Inject Alum (Al (OH)₃/Mg(OH)₂, Pierce, USA) as adjuvant. From day 24, the mice were exposed for 20 min to aerosolized OVA (1% OVA, 10 mL) once per day for 3 days. From day 18, ST or montelukast was orally administered daily for 10 days.

2.4. Determination of AHR

AHR was measured by the Medlab12.0 biological signal processing system (Nanjing Medease Science and Technology Co., Ltd, China). After final inhalation of OVA, mice were placed in a closed container (plethysmograph box), which is connected with the baroreceptor. The changed pressure caused by breathing was recorded by Sensor (TRD5700). After processing by the amplifier (MAX1420, MAX2270, MAX4371), the signal transmitted to the system for enhanced pause (Penh%) calculation. The Penh% was calculated as follows: $\text{Penh}\% = \text{Pause} \times (\text{PEF}/\text{PIF}) \times 100\%$, while the $\text{Pause} = (\text{Te} - \text{Tr})/\text{Tr}$ (PIF, peak inspiratory flow; PEF, peak expiratory flow; Te, expiratory time; Tr, relaxation time).

2.5. Histological analysis

Mice were euthanized by cervical dislocation and the lung samples were fixed in 4% paraformaldehyde solution. After fixation, the paraffin sections were stained with haematoxylin and eosin (H&E) to evaluate the inflammatory cell infiltration. Tissue morphology was observed under microscope (Eclipse 80i, Nikon).

2.6. Flow cytometry analysis

A total of 100 μL of peripheral blood sample from each mouse was added to 100 μL of RPMI 1640 in cell culture tubes, then incubated with monensin mixture (Bender MultiSciences, Austria)

in the presence of PMA (50 ng/mL), Monensin (100 $\mu\text{g}/\text{mL}$), and Ionomycin (1 $\mu\text{g}/\text{mL}$) for 6 h in a 5% CO₂ humidified incubator. One hundred microliters of Peripheral blood were then labeled with anti-mouse CD3 and anti-mouse CD8 antibody (eBioscience, USA) at room temperature for 20 minutes. Erythrocytes were lysed for 15 min at room temperature with 20 μL hemolytic agent and centrifuged. Blood cells were permeabilized with 200 μL of Permeabilizing Solution for 10 min at room temperature. After centrifugation, cells were divided equally, followed by incubating with 5 μL of anti-IL-4 antibody and anti-IFN- γ antibody (eBioscience, USA) for 20 min in the dark, at room temperature. After washing in PBS, the cells were detected by flow cytometer (Beckman Colter, USA). The same processing of control tube was performed as experimental group, except stimulating by PMA and Ionomycin.

2.7. Agarose gel electrophoresis analysis and quantitative real-time PCR

Total RNA was isolated from homogenized lung tissues using the Trizol reagent (Invitrogen, Carlsbad, CA) as described in the instruction. The RNA concentration was quantitative measured by UV spectrophotometer, and then stored in the refrigerator at -70°C . Reverse transcribed to cDNA was conducted according to the instruction of reverse transcription kit (Roche, USA). Oligonucleotide sequences of PCR primers for *Cyslt1*, *Cyslt2* and *GAPDH* were listed in Table 1. For amplification on the qPCR (Stepone Plus, ABI, USA), a LightCycler DNA Master SYBR Green I reagent set (Roche, USA) was used. Before amplification, 10 μL of 1 $\times$ LightCycler DNA Master SYBR Green I was mixed with Forward primer and Reverse primer. PCR was performed in 18 μL reactions containing 10 μL of 1 $\times$ LightCycler DNA Master SYBR Green I, and 300 nM (0.2 μL) of each primer. PCR was initiated by 1 cycle of 95°C for 10 min and 40 cycles of 10 s at 95°C for denaturation and 30 s at 60°C for annealing. The fluorescence of SYBR Green I was measured once per cycle to monitor template amplification.

In order to determine the specificity of the qPCR, agarose gel electrophoresis was performed. 1.5% agarose gel with 0.5 $\times$ TAE buffer was prepared with addition of the GoldViewer stock solution (containing goldviewer 0.5%). 20 μL of each PCR product was mixed with 6 $\times$ DNA loading buffer, and then 4 μL of mixture was loaded on the gel. DNA Maker I (Genemed Synthesis, USA) was used as a control.

DNA melting curves were acquired on the qPCR by measuring the fluorescence of SYBR Green I during a linear temperature transition from 60°C to 98°C at the rate of $0.3^\circ\text{C}/\text{s}$. Fluorescence data were converted into melting peaks to plot the negative derivative of fluorescence over temperature versus temperature ($-dF/dT$ vs. T). PCR reagents were carefully mixed according to the directions. Melting curve analysis was performed immediately after the amplification. The method of $2^{-\Delta\Delta\text{Ct}}$ was employed to analyze the relative expression level of target gene. Calculation formulas were as follows: amount of target = $2^{-\Delta\Delta\text{Ct}}$, while $\Delta\Delta\text{Ct} = [\text{Ct}(\text{sample}) - \text{Ct}(\text{internal reference})] - [\text{Ct}(\text{blank}) - \text{Ct}(\text{internal reference})]$. Finally, the relative transcript levels of the target gene were used as a parameter for statistical analysis. The method for validation of $2^{-\Delta\Delta\text{Ct}}$ was as follows: The

Table 1
Primer sequences for quantitative real-time PCR.

Gene	Primer	Sequences (5'–3')	Amplified fragment size
<i>Cyslt1</i>	Forward primer	CCAAGGCACCAAGCAGACATT	157 bp
	Reverse primer	GCCAAAGAAACCCACAACAGA	
<i>Cyslt2</i>	Forward primer	TGGGAAAGGAAGAGTGAAGAATA	241 bp
	Reverse primer	GTTGACAGAGGCGAGGACTAA	
<i>GAPDH</i>	Forward primer	AGGTTGTCTCTCGCACTTCA	187 bp
	Reverse primer	GGGTGGTCCAGGGTTCTTATC	

blank group was diluted 10, 100, 1000 and 10,000 times, and then measured the ΔC_t concentration and value. A plot of the log cDNA dilution versus ΔC_t was made. If the absolute value of the slope was less than 0.1, it meant that the efficiencies of the target and reference genes were similar, and the $\Delta\Delta C_t$ which represents the relative quantification of target could be adopted for further analysis.

2.8. Statistical analysis

All values are expressed as means $\pm$ S.D. SPSS 12.0 was used for statistical analysis. Differences between groups were tested using a one-way analysis of variance followed a post-hoc Tukey's test to identify the difference between the two groups.

3. Results

3.1. ST suppressed airway AHR in a mouse asthma model

AHR is the critical indication of asthma, which happens in response to the airway inflammation. In this experiment, asthma mouse was used to study the effect of ST on AHR. To establish mouse asthma model, we first used OVA to stimulate normal mice

then quantitatively measured the extent of enhanced pause (indicated as Penh (%)). OVA could drastically induce AHR (OVA control) compared to that in normal mice ($p < 0.05$) (Table 2). We treated OVA control mice with either Montelukast or three different doses of ST. Significant reduction on AHR was observed in all treatment groups ($p < 0.05$); ST at the high dose (OVA+ST-H) had the same potency as montelukast (OVA+Montelukast). ST showed a dose-dependent inhibition of AHR.

3.2. ST inhibited inflammation in asthma mouse model

Airway inflammation and consequential airway remodeling are the two main pathological courses during bronchial asthma (Pierre et al., 2011). In the present study, we investigated the effects of ST on the morphological changes of lung tissues in asthma mice. The typical allergic inflammation reaction was observed under microscope in lung tissues of OVA control group compared with that of normal mice, such as the accumulation and infiltration of lymphocytes and plasma cells, bronchial dilatation and cavity secretion (Fig. 1A and B). Montelukast treatment significantly attenuated the symptoms (Fig. 1C). Similar effects were also observed when treating with high dose of ST (Fig. 1D). The lower

Table 2

Effect of ST on enhanced pause in asthma model mice.

Groups	Treatment reagents	Mouse number	Treatment dose	Penh(%)
Normal control	Physiological saline	10	0.5 ml d ⁻¹	18.27 $\pm$ 3.54
OVA model control	Physiological saline	10	0.5 ml d ⁻¹	112.37 $\pm$ 15.99*
OVA+Montelukast	Montelukast	10	10 mg ⁻¹ kg d ⁻¹	30.27 $\pm$ 5.191; [△]
OVA+ST-L	<i>Sceptridium ternatum</i>	10	1.935 g ⁻¹ kg d ⁻¹	57.01 $\pm$ 8.711; [△]
OVA+ST-M	<i>Sceptridium ternatum</i>	10	9.675 g ⁻¹ kg d ⁻¹	40.87 $\pm$ 4.371; [△]
OVA+ST-H	<i>Sceptridium ternatum</i>	10	19.35 g ⁻¹ kg d ⁻¹	32.64 $\pm$ 3.821; ^{△,2;°}

All data were expressed as means $\pm$ SD.

Penh:enhanced pause.

* $p < 0.05$ vs. normal control.

[△] $p < 0.05$ vs. model control.

[△] $p > 0.05$ vs. OVA+Montelukast.

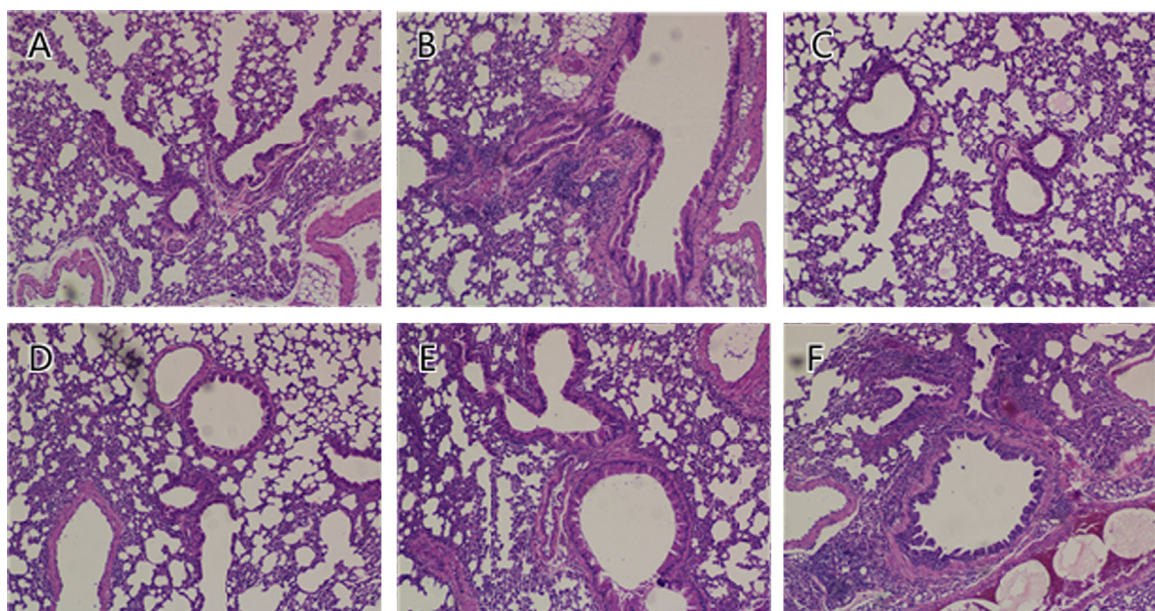


Fig. 1. Effects of ST on histological changes in a mouse asthma model. Lung tissue slices were fixed and stained using hematoxylin and eosin (H&E). (A) untreated control group (normal control) (B) OVA model control group (C) OVA+Montelukast (D) OVA+ST 19.35 g/kg group (E) OVA+ST 9.675 g/kg group (F) OVA+ST 1.935 g/kg group.

doses of ST also had beneficial effects but less remarkable than high dose (Fig. 1E and F).

3.3. ST restored Th1/Th2 ratio of peripheral blood in asthma mouse model

Th1/Th2 ratio is the key index of bronchial asthma. Imbalanced Th1/Th2 ratio exists during the course of the disease. A series of allergic reaction could occur when either Th1 cell activity was reduced or Th2 cell activity was enhanced (Lee et al., 2001). The numbers of Th1 and Th2 cells of the asthma mice showed a trend of increase in lung tissues, but Th1/Th2 ratio was significantly reduced ($P < 0.05$). ST showed dose-dependent recovery of Th1/Th2 ratio, with the high dose group most effective (Table 3).

3.4. ST reduced *Cyslt1* expression level in asthma mouse model

Given that leukotriene family proteins play key roles in asthma, we amplified both *Cyslt1* and *Cyslt2* mRNAs from mouse tissues by quantitative real-time PCR (qPCR). The melting curve for *Cyslt1* exhibited a single melting peak (Fig. 3A) which indicating optimal PCR condition and specific amplification, while the *Cyslt2* PCR generated two peaks (Fig. 3B), implying two distinct PCR products, probably owing to substantially low mRNA level of *Cyslt2*. Consistent with the readout of *Cyslt1* melting curve, correct products were validated upon the analysis of agarose gel electrophoresis: *Cyslt1* showed sharp band with clean background (Fig. 2A).

Table 3
Influence of ST on Th1/Th2 cell ratio of peripheral blood in asthma model mice.

Groups	Mouse number	Th1/Th(%)	Th2/Th(%)	Th1/Th2
Normal control	10	4.16 ± 0.27	0.80 ± 0.13	6.53 ± 1.06
OVA model control	10	6.41 ± 1.19	2.16 ± 0.47	$3.53 \pm 0.50^*$
OVA+Montelukast	10	6.11 ± 0.99	1.84 ± 0.27	4.15 ± 0.84
OVA+ST-L	10	6.10 ± 0.56	1.28 ± 0.17	$5.35 \pm 0.501; ^\Delta$
OVA+ST-M	10	5.53 ± 0.53	1.07 ± 0.04	$5.48 \pm 0.621; ^\Delta$
OVA+ST-H	10	5.97 ± 0.54	1.92 ± 0.08	$6.10 \pm 0.581; ^\Delta$

All data were expressed as means $\pm$ SE.

* $p < 0.05$ vs. normal control.

$^\Delta$ $p < 0.05$ vs. model control.

Whereas *Cyslt2* had one non-specific band in addition to the desired one: the one that T_m is 76.35°C with relatively lower abundance is the right product (Fig. 2B). As the main product for *Cyslt2* PCR was the non-specific amplification which will inevitably affect the accuracy of further measurement, we did not include its quantitative analysis in this paper.

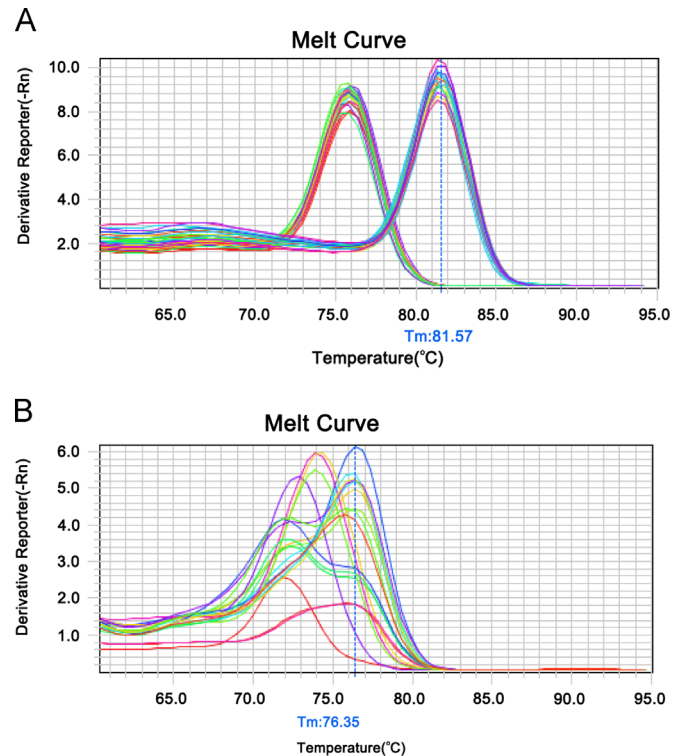


Fig. 3. Influence of ST on *Cyslt1* and *Cyslt2* mRNA expression in mouse lung tissue. Melting-curve analysis of *Cyslt1*, *Cyslt2* and *GAPDH* during the real-time PCR. The rate of changes (first derivative) of the relative fluorescence units with time on the Y-axis versus the temperature on the X-axis, which peaks at the melting temperature. Similar peaks indicate no contamination, mispriming, primer-dimer artifact, etc. (A) Melting-curve of *Cyslt1* and *GAPDH*. (B) Melting-curve of *Cyslt2* and *GAPDH*. The T_m value of 76.35°C represents *GAPDH*.

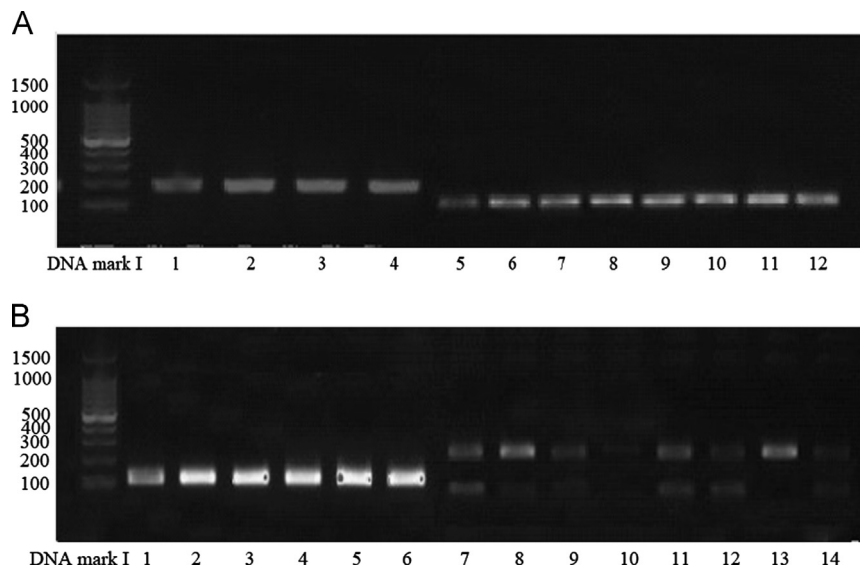


Fig. 2. Influence of ST on *Cyslt1* and *Cyslt2* mRNA expression in mouse lung tissue. Agarose gel electrophoresis analysis method was employed to determine mRNA expression of *Cyslt1* and *Cyslt2* in the lung tissues. (A) From 1 to 4 represent *GAPDH*, from 5 to 12 represent *Cyslt1*. (B) From 1 to 6 represent *GAPDH*, from 7 to 14 represent *Cyslt2*.

Table 4
Cyslt1 mRNA expression in mouse lung tissue.

Groups	Mouse number	ΔCt	2 ^{−ΔΔCt} of <i>cyslt1</i> mRNA
Normal control	10	8.24 ± 1.37	1
OVA model control	10	7.21 ± 0.97*	2.05
OVA+Montelukast	10	11.72 ± 1.791; [○]	0.09
OVA+ST-L	10	8.66 ± 1.18	0.75
OVA+ST-M	10	9.34 ± 0.74	0.48
OVA+ST-H	10	10.34 ± 1.582; [△]	0.23

* $p < 0.05$ vs. normal control.

[○] $p < 0.05$ vs. model control.

[△] $p < 0.05$ vs. model control.

Table 5
ΔCt values of 5 concentrations of *Cyslt1* and *GAPDH*.

Concentration	0.0001	0.001	0.01	0.1	1
ΔCt	8.45	8.46	8.43	8.41	8.39

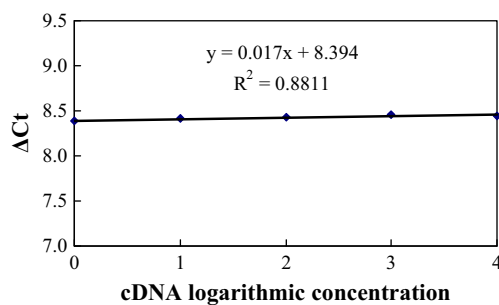


Fig. 4. The curve of the relation between ΔCt and cDNA logarithmic concentration for *Cyslt1* and *GAPDH*.

Next, we determined *Cyslt1* expression level in mouse lung tissues subjected to different conditions of treatment by using relatively CT value ($2^{-\Delta\Delta Ct}$) method (Table 4, Kenneth et al., 2001). The expression level of *Cyslt1* in normal mice was arbitrarily set as 1 and had almost two fold increase, reaching to 2.05 in OVA control group. After feeding with Montelukast, *Cyslt1* expression was significantly declined ($P < 0.05$). ST reduced expression of *Cyslt1* mRNA level in a dose-dependent manner ($P < 0.05$). In that the necessary condition for $2^{-\Delta\Delta Ct}$ method exists when target gene has the same amplification efficiency with internal control DNA, cDNA templates of *Cyslt1* and control gene *GAPDH* was subject to 10-fold serial dilutions and 5 sequential concentrations for *Cyslt1* and *GAPDH* were selected for qPCR. All qPCR reactions were run in triplicate to minimize error. At each dilution, the Ct value for *Cyslt1* and *GAPDH* was calculated and by plotting the ΔCt values (Ct *Cyslt1* – Ct *GAPDH*) for the dilutions against cDNA concentration, we drew a linear graph with the correlation coefficient of 0.8811, indicating the similarity of amplification efficiency (Table 5 and Fig. 4).

4. Discussion

Bronchial asthma is a allergic disease that recruits various inflammatory cells, cytokines and inflammatory mediators. AHR is known to be the characteristic symptom of bronchial asthma (O'Byrne and Inman, 2003). In the present study, we unraveled the therapeutic activity of ST in asthma mouse in the aspects of pathology and pathophysiology, unveiling the mechanism of ST.

Indeed, similar to montelukast, high dose ST could effectively reduce Th1/Th2 ratio, leukotriene family protein *Cyslt1* expression, and meanwhile inhibit inflammation.

With the understanding of asthma inflammation and its immune mechanism, Th1/Th2 cells imbalance is considered to be the immunological cause of asthma, led by the dominant up-regulation of Th2 cells in immune response (Romagnani, 2000; Crosby et al., 2002; Shen et al., 2002). However under normal circumstances, T lymphocyte (Th0) differentiates to Th1 and Th2 cells proportionally, meanwhile keeps their amount in a relative balanced state through the manner of antagonizing and feedback promotion, forming a complicated but well-ordered cytokine network to maintain body's normal immune response (Murphy and Reiner, 2002; Kharmate et al., 2007). In allergic diseases such as bronchial asthma, the activity of Th1 cells is reduced, while Th2 cell activity is activated, resulting in Th2-dominated immune responses. The alternation of Th1/Th2 ratio is proved to be an initial factor for asthma that gives rise to airway inflammation and hyper-responsiveness (Carneiro et al., 2010). Therefore, it will be of the greatest significance for asthma therapy if the imbalanced status of Th1/Th2 cells can be reversed. ST improved the asthmatic conditions mainly on Th2 cells. Remarkably, high dose ST could restore the Th1/Th2 ratio to close to the normal level. Even lower doses of ST had more beneficial effects than montelukast, indicating ST would be more effective in this regard.

Leukotrienes are the essential mediators of inflammation in pathogenesis of asthma. Their receptors are widely distributed in the human airway. They can stimulate the accumulation of a bunch of inflammatory cells, causing the contraction of airway smooth muscle and increase of airway resistance, mucus secretion and vascular permeability and ultimately will bring about airway remodeling and AHR. Thus, arresting leukotriene synthesis or blocking leukotriene receptors has emerged as a major direction in the treatment of bronchial asthma. In the present paper, we quantitatively assayed the expression levels of *Cyslt1* and *Cyslt2* in lung tissues of asthma mice. After different drug treatment, the expression of *Cyslt1* was greatly reduced, and montelukast and high dose ST reduced *Cyslt1* mRNA levels by 23 fold and 9 fold, respectively. *Cyslt2* however after multiple attempts to optimize PCR conditions and primer sets, still had a very faint yield and dual peaks of melting curves, indicating that *Cyslt2* was very lower in the lung tissues. Therefore, we only quantitatively measured the level of *Cyslt1* not *Cyslt2* to evaluate the function of ST.

In summary, by mouse asthma model, we revealed the therapeutic mechanism of herbal medicine ST for asthma treatment. ST can restore Th1/Th2 ratio, reduce the expression of *Cyslt1*, attenuate inflammation reaction, and hence relieve asthma symptoms. Our study lays the foundation for the further exploration on how ST regulates expression of leukotriene receptors in the future.

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