



Teucrium polium Extract Attenuates Inflammation in Asthma by Reducing ROR γ t Transcription and Increasing IL-10 Secretion in an Ovalbumin-induced Murine Asthma Model

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ABSTRACT

Background: One of the inflammatory diseases of the respiratory system is asthma. Teucrium polium (TP) has anti-inflammatory and anti-allergic properties and its anti-asthmatic effects have not been investigated yet. ROR γ t is an inflammatory transcription factor for Th17 differentiation. By secreting IL-17, Th17 leads to neutrophilic inflammation in the lungs. As an anti-inflammatory cytokine, IL-10 reduces the dissemination of inflammatory elements in the airways.

Objective: To evaluate the effect of TP extract in asthma treatment.

Methods: Thirty female Balb/c mice were distributed into 5 groups (n=6) including the control, treated with ovalbumin (OVA), and OVA+ various doses of TP (50, 150, and 300 mg/kg). All groups except the control group were sensitized to OVA solution on days 0, 7, and 14 by subcutaneous injection. The challenge was performed on days 18 to 21 by the inhalation of 1% OVA and the treatment was done with TP extract in the treatment groups, half an hour before the challenge. On day 22, the serum and spleen samples were collected to determine IL-10 serum levels and ROR γ t gene expression, respectively.

Results: In the treatment groups, the expression of ROR γ t significantly decreased when using OVA+ Tp extract (150 mg/kg and 300 mg/kg), and IL-10 serum levels significantly increased when using OVA+ TP extract (150 mg/kg) compared with the OVA group.

Conclusion: It is possible that TP extract can be effective in improving asthma by reducing inflammation.

Keywords: Asthma; Extract; IL-10; Inflammation; ROR γ t; Teucrium polium

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INTRODUCTION

Asthma is a chronic disease of the airways characterized by airway over-reactivity to a wide range of stimuli. This overreaction leads to inflammation and airway obstruction. Symptoms of asthma are coughing, wheezing, shortness of breath, and chest tightness (1). This disease affects about 300 million people all over the world, and it is assessed that the number of people suffering from this disease will reach 400 million by 2025 (2).

T cells subtypes including Th1, Th2, Th17, and regulatory T cells play a role in asthma by producing cytokines (3).

Th17 cells are associated with autoimmune and inflammatory diseases, and the cytokines essential for their differentiation are IL-6, TGF- β , IL-23, and IL-1 β (4). The above cytokines activate two main transcription factors, retinoic acid receptor-related orphan receptor- γ t (ROR γ t) and signal transducer and activator of transcription 3 (STAT3), in Th17 cells. High ROR γ t expression is critical for the Th17 differentiation (5). Th17 cells secrete IL-17A, an inflammatory cytokine (6). IL17A production increases in the lungs and sputum of patients with asthma, leading to excessive mucus secretion (5). IL-17A stimulates the inflammation caused by the recruitment of neutrophils into the airways and ultimately causes neutrophilic asthma (7). Therefore, the upregulation of ROR γ t can be considered an exacerbation factor of asthma. In addition, IL-10 is an anti-inflammatory cytokine that balances the immune system and regulates the function of immune cells (8). It is mainly produced by regulatory T cells (Treg) and can inhibit IgE production (9, 10), leading to the reduction of eosinophil penetration in the airways and the control of eosinophilic asthma (11). The decrease in the production of IL-13, IL-5, and IL-4 cytokines, which are necessary for allergic reactions, is done by this cytokine by inhibiting the activation of Th2 cells (12).

Teucrium polium (TP) belongs to the Lamiaceae family and has a white and cottony

appearance. Its branches are round and hairy and the length of the leaves is about 3 cm (13).

This plant has many medicinal properties, including antioxidant, diuretic, antifungal, anti-rheumatism, anti-cancer, antibacterial, and anti-inflammatory (14). Recent studies have shown that this plant, with its anti-allergic properties, is able to improve allergies in animal models (15, 16). Therefore, this project was conducted to investigate the effects of this plant on the expression of ROR γ t and IL-10 levels in serum in the mouse model of acute asthma.

MATERIALS AND METHODS

Teucrium polium Extract Preparation

The *Teucrium polium* plant was obtained from a local shop in Rafsanjan, Kerman. It was used after the approval of the herbarium expert of the Vali-e-Asr University of Rafsanjan, Iran, and aerial parts and leaves of this plant were used to prepare the extract. To prepare the extract, 200 grams of TP powder were weighed and mixed with 250 ml of 70% ethanol in a container. The container containing a solution was moved at room temperature in darkness for 72 h. Then the extract was filtered and poured into flat glass containers and placed at 30 °C for 48 h in an incubator until dry. After complete drying, our extract was prepared. To inject the extract into the animals of the treatment groups, different amounts of the extract were weighed and after dissolving in normal saline, they were injected into the animals.

Mice

Thirty female Balb/c mice (5 to 6 weeks old) with an approximate weight of about 18-22 g were bought from the Kerman University of Medical Sciences (KMU).

They were kept in the animal room at Rafsanjan University of Medical Sciences at a temperature of 21-23 °C, a natural light-dark cycle (12 h a day/12 h a night), and standard conditions in terms of adequate water and

food for one week to adapt to environmental conditions. After this period, the animals were included in the study. All protocols on mice were approved by the Animal Ethics Committee of Rafsanjan University of Medical Sciences (approval No. IR.RUMS.REC.1399.161).

OVA Sensitization and Inhalation

The mice were randomly divided into 5 groups (n=6), including the followings: 1. The control group: receiving only normal saline. 2. The asthma group: in this group, the asthma was induced by OVA, but no treatment protocol was performed on them. 3. OVA+ treated with 50 mg/kg TP extract, 4. OVA+ treated with 150 mg/kg TP extract, 5. OVA+ treated with 300 mg/kg TP extract (17, 18).

For acute allergic asthma induction, according to the last studies (19), asthma induction was performed in two stages, the sensitization and challenge stages.

In the sensitization phase, 80 mg of aluminum hydroxide adjuvant (Alum; Sigma-Aldrich) was mixed with 1 mL of OVA (Sigma-Aldrich, St. Louis, MO, USA) in 3 ml of normal saline, and 100 µl of suspension was injected subcutaneously (SC) into each of the mice on days 0,7,14 (sensitization step). The control group received only 100 µl of normal saline SC.

In the challenge stage, the mice were

placed in an asthma induction device (nebulizer) for 30 minutes. At this stage, the mice were exposed to 1% OVA and the asthma was induced by the inhalation of OVA in mice. This stage was done on days 18 to 21. In the control group, normal saline was used instead of a 1% OVA solution (Figure 1). In the treatment phase, in each group, 100 µl of the extract was injected intraperitoneally (IP) into each mouse (20). There was no treatment in OVA and the control groups (Figure 1).

Cytokines Analysis

On the 22nd day, the mice were bled by a retro-orbital method, to separate the serum from the mice's blood, the samples were centrifuged for 5 min at speed of 1200 rpm. The serum for each mouse was separated from the blood and collected and stored at -80 °C for IL-10 level measurement. Briefly, to evaluate the levels of IL-10 in the serum samples a 96-well plate coated with anti-IL-10 -monoclonal capture antibodies was obtained from the company (Karmania Pars Gene, Kerman, IRAN). According to the instructions of the kit, the ELISA procedures were performed. Finally, the absorption of each sample was measured at 540 nm wavelength by an ELISA reader.

RNA Isolation and cDNA Synthesis

We extracted total RNA from spleen samples (21). Briefly, 40 mg of spleen tissue

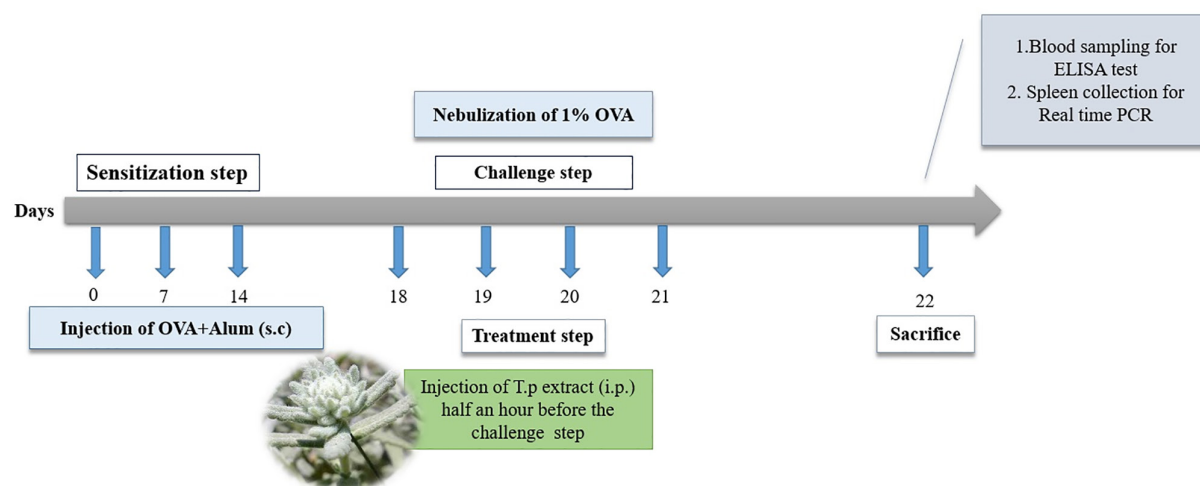


Figure 1. Schematic figure of asthma induction by OVA in a mouse model and time of treatment; OVA: ovalbumin; IP: Intraperitoneal; SC: subcutaneous.

was powdered and homogenized after mixing with 750 µl of Trizol solution. The extraction of RNA was done according to the manufacturer's guidelines (Karmania Pars Gene Company, Kerman, IRAN). NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific, Inc.) was used to determine the purity and evaluate the quality of RNA. For the synthesis of cDNA by Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, Inc.), 5ng of pure RNA was used according to the protocol of the kit (Pars Tous, Iran).

Real-time PCR

The real-time PCR technique was used to measure the changes in RORγt gene expression. Real-time quantitative PCR technique, primers, and Master Mix SYBR Green PCR kit (Biosystem, China) were used to measure RORγt gene expression. β-actin housekeeping gene was used as the internal control.

Primer sequences for RORγt and β-actin transcripts are shown in Table 1. For duplication, we used SYBR Green PCR Master Mix containing (12.5 µl total volume): 1 µl cDNA sample, 0.5 µl primers, 5 µl SYBR Green Dye, and 6 µl diethylpyrocarbonate (DEPC)-treated water. PCR thermocycling was done according to the kit protocol (SYBR Green PCR Master Mix, Biosystem, China). A melting-curve analysis was used for the amplification specificity. The level of RORγt gene expression was normalized to β-actin. Relative fold changes of RORγt and β-actin gene expression were shown by $2^{-\Delta\Delta CT}$.

Statistical Analysis

All the data obtained in this study were analyzed and checked by SPSS software

(version 20). ANOVA test was used for normal data distribution and Tukey's multiple comparison tests were used to obtain the data values. The graphs were plotted with Excel software. A value of $P \leq 0.05$ is considered statistically significant. The amount of RORγt gene expression level in all groups was calculated using $2^{-\Delta\Delta CT}$ (Livak method).

RESULTS

The Teucrium polium Extract Increased the Level of the Anti-inflammatory Cytokine, IL-10, in the Treated Groups

IL-10 serum level was evaluated in different groups by the ELISA method. The production of IL-10 decreased by OVA treatment. The levels of IL-10 in the serum of the OVA+ 150mg/kg TP extract group increased significantly compared with the OVA-treated group ($P \leq 0.001$).

IL-10 levels increased in other treated groups compared with the OVA group and were not significant. Among the groups treated with TP extract, the highest amount of IL-10 was seen in the OVA group+ 150mg/kg TP extract. Table 2 shows the numerical values of this cytokine in different groups.

RORγt Gene Expression as an Inflammatory Gene Decreased in the Treatment Groups

Real-time PCR was used to evaluate RORγt transcription factor levels in spleen cells. OVA treatment increased the expression of the RORγt gene. The expression of this gene significantly reduced in the control, OVA+ 150 mg/kg and OVA+ 300 mg/kg TP extract compared with the OVA group ($P < 0.05$); in the other group treated with the

Table 1. Primer sequences for real-time PCR. Primers were designed using Beacon designer software and checked by the BLAST program.

Gene	Primer sequence (5' to 3')
RORγt forward	5-TGCGACTGGAGGACCTTCTAC-3
RORγt reverse	5-TCACCTCCTCCCCTGAAAAG-3
β-actin forward	5-CGATGCCCTGAGGCTCTTT-3
β-actin reverse	5-TGGATGCCACAGGATTCCA-3

Table 2. Comparison of serum IL-10 levels (pg/mL) between OVA and other studied groups.

Groups	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	P value
A	6	30.5480	2.82927	1.26529	25.65	32.50	0.060
B	6	23.3167	3.84326	1.56901	19.00	29.35	-
C	6	29.0000	7.34847	3.67423	23.00	38.00	0.242
D	6	54.4800	3.99400	1.78617	50.00	58.00	0.000***
E	6	25.1680	1.24945	0.55877	24.00	26.84	0.943

Group definitions: A: Control group, B: OVA group, C: OVA+ TP extract (50 mg/kg) group, D: OVA+ TP extract (150 mg/kg) group, E: OVA+ TP extract (300 mg/kg) group. *P≤0.05, **P≤0.01, ***P≤0.001

Table 3. Real-time quantitative PCR of RORγt gene expression level between OVA and other studied groups.

Groups	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum	P value
A	6	0.6930	0.24381	0.10903	0.33	0.95	0.001***
B	6	1.3833	0.23166	0.09458	1.00	1.60	-
C	6	1.1750	0.20616	0.10308	1.00	1.40	0.667
D	6	0.9100	0.12450	0.05568	0.75	1.00	0.029*
E	6	0.7830	0.33716	0.15078	0.20	1.01	0.004**

Group definitions: A: The control group, B: OVA group, C: OVA+ T.p extract (50 mg/kg) group, D: OVA+ T.p extract (150 mg/kg) group, E: OVA+ T.p extract (300 mg/kg) group. *P≤0.05, **P≤0.01, ***P≤0.001

extract, the expression of this gene decreased compared with the OVA group, but it was not significant. In Table 3, the numerical values of RORγt gene expression are given in more detail.

DISCUSSION

Allergic asthma is an inflammatory disease of the respiratory system characterized by increased infiltration of leukocytes and inflammatory cells in the lung tissue and respiratory dysfunction. This inflammation leads to bronchial obstruction, increased sensitivity of the airways to allergens, and excessive production of mucus (22, 23).

Inflammation is one of the main indicators of asthma, related to the permeation of inflammatory cells such as neutrophils, eosinophils, T cells, and other cells into the airways. The inflammation leads to the swelling of the airways and makes it difficult for air to pass through the airways, moreover, complications such as shortness of breath and wheezing occur in patients with asthma (24).

According to previous studies, it was

determined that the compounds in TP extract such as triterpenoids, flavonoids, and alkaloids have anti-inflammatory properties (25). Flavonoids and alkaloids mainly regulate inflammatory cytokine production, therefore, it can be declared that they may improve asthma by reducing the inflammatory elements (26).

It was shown that β-caryophyllene in the TP extract improved obesity-associated airway hyperresponsiveness (AHR) in the mice by reducing transcription of IL-1β and TNF-α inflammatory cytokine genes and infiltration of inflammatory leukocytes into the airways (27). The results of the above studies are consistent with the present study and indicate the anti-inflammatory properties of TP extract. IL-10 is continuously produced in the airways of healthy individuals, but its production is reduced in the sputum and serum of patients with asthma and allergies (28).

In previous studies, the use of monoclonal antibodies against the IL-10 receptor in asthmatic mice increased the level of inflammatory cytokines such as TNF-α, IL-1β and IL-17 in the mice's serum and caused neutrophilic and eosinophilic inflammation in their lung tissues (29). In 2012, the effect

of TP extract on intestinal inflammation in dogs with inflammatory bowel disease (IBD) was investigated, and was concluded that the administration of this extract diminished inflammatory cytokines and enhanced the number of anti-inflammatory cytokines in the treated groups (29).

The data analysis of this study showed that the IL-10 serum level decreased in the asthma control group. On the other hand, in the treated groups, the amount of this cytokine at a dose of 150 mg/kg of TP extract was significantly higher than in the asthma control group, thus, it can be argued that the extract in this therapeutic dose was able to stimulate the main IL-10 producing cells to produce this cytokine and reduce pulmonary inflammation.

In the dose of 300 mg/kg, the secretion rate of this cytokine was lower than the dose of 150 mg/kg. It can be said that at a dose of 300 mg/kg, the extract may have negative effects on the survival or proliferation of Tregs and decrease the production of this cytokine at higher doses.

Flavonoids and alkaloids interfere with nitric oxide (NO) synthase and IL-17A production (26). IL-17A is an inflammatory cytokine secreted by Th17 cells (30). ROR γ t is a major transcription factor in the differentiation of Th17 cells. Activated Th17 produces IL-17A, an inflammatory cytokine, which leads to the accumulation of inflammatory cells such as neutrophils in the inflammatory areas (31). The possible role of IL-17A in the pathogenesis of allergy and asthma has been shown. Recent studies have shown that the increase in IL-17A serum level in patients with asthma has increased the infiltration of neutrophils into the lung tissue and, finally neutrophilic asthma (32). Recently, ROR γ t gene expression has been measured in the peripheral blood and sputum of asthma patients and healthy individuals, and a significant increase in the expression of this gene has been observed in the blood and sputum of patients (33). In 2015, Latifnia used TP extract as an adjuvant in the *Leishmania* parasite vaccine, and the results showed that

this extract prevented the increase of serum IL-17A levels in patients and improved the disease (34).

The results of this study showed that the expression of the ROR γ t gene decreased in the groups treated with TP extract compared with the OVA control group. In the highest therapeutic dose of TP extract, this gene had the lowest expression compared with the other doses (300 mg/kg dose). It can be stated that TP extract, by inducing its anti-inflammatory properties, is probably able to reduce the expression of this inflammatory gene and reduce the differentiation of Th17 cells, and ultimately lead to the reduction of IL-17A production from these cells and the reduction of neutrophil infiltration into lung tissues.

According to the above description, IL-10 has an essential role in inhibiting inflammation and reducing IL-17, which means that IL-10 inhibits neutrophilic inflammation by inhibiting Th-17 differentiation. In this study, by increasing the production of IL-10, the extract decreased the expression of the ROR γ t gene, which has a special role in the production of IL-17.

It is shown that by regulating IL-10 secretion and ROR γ t expression, involved in the development and pathogenesis of asthma, TP extract can be useful in improving asthma; however, more experiments are needed to affirm our findings.

CONCLUSION

In summary, in the groups treated with TP extract, IL-10 secretion increased, while ROR γ t expression decreased. Our results show that TP by having anti-inflammatory compounds can be effective in the treatment of asthma and can be used as low-complication herbal medicine in the treatment of asthma.

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Conflict of Interest: None declared.

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