Teucrium polium Extract Alleviates Pathological Features of Asthma via IL-12 and IFN-γ Modulation in Murine OVA-induced Allergic Asthma

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ABSTRACT

Asthma is a chronic disorder characterized by airway overreaction and remodeling, eosinophilia, and neutrophilic inflammation, accompanied by thickening of the airways and breathlessness. Teucrium polium (TP) is a plant with anti-inflammatory properties and is considered for the treatment of allergic disorders.

In this study, we examined the effects of TP extract on ovalbumin (OVA)-induced asthma. Thirty female mice (5–6 weeks old) were divided into 5 groups of 6 each, including a control group and 4 groups treated with OVA, OVA + TP extract (50 mg/kg), OVA + TP extract (150 mg/kg), OVA + TP extract (300 mg/kg). Twenty-four hours after the last treatment, lung, serum, and spleen samples were collected and used for the evaluation of leukocyte infiltration, serum cytokine Interferon-γ (IFN-γ) levels, and the expression of the Interleukin-12A (IL12A) gene, respectively.

Hematoxylin-eosin staining was used to evaluate pathological changes in the lung tissue sections.

Treatment with TP extract reduced inflammatory cells such as eosinophils and neutrophils in the airways. Furthermore, it increased serum levels of IFN-γ and IL-12A at a dose of 50, 150, and 300 mg/kg compared to the OVA group.

This study showed that the administration of TP extract could improve pathological features, such as airway inflammation, and reduce systemic inflammation.

Keywords: Asthma; Interferon-gamma; Interleukin 12a; Inflammation; Teucrium polium

INTRODUCTION

Asthma is a chronic disorder characterized by excessive secretion of mucus, airway hyperresponsiveness (AHR) to allergens and the infiltration of inflammatory cells such as T lymphocytes, eosinophils, mast cells, and neutrophils into the airway tissue.1 AHR and increased mucus secretion from goblet

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cells lead to changes in the amount of mucus secretion, narrowing of the airways, and ultimately, shortness of breath and wheezing in asthma.\(^2\)

CD4\(^+\) T cells can be divided into T helper type 1 (Th1), Th2, Th17, and regulatory T (Treg) cells based on their function. Th2 cells are crucial to the induction of asthma.\(^3\)

Interleukin-12 (IL-12) induces the differentiation of naive T cells into Th1 cells and inhibits the differentiation into Th2 cells.\(^4\) It is a heterodimer cytokine consisting of two subunits, p35 (IL-12A) and p40 (IL-12B).\(^5\) This cytokine is necessary to stimulate interferon-gamma (IFN-\(\gamma\)) production by Th1 cells.\(^6\) A deficiency in IL-12 can lead to Th2 cell differentiation. Also, the expression of this cytokine is reduced in the bronchial biopsy of asthma patients.\(^7\) Recent studies have shown that the decrease in the expression of these subunits affects the development of asthma and allergies.\(^8,9\)

Th1 ameliorates the pathogenesis of asthma by producing IFN-\(\gamma\), which prevents the differentiation of naive T cells into Th2,\(^10\) and suppresses immunoglobulin (Ig)E isotype-switching in B cells.\(^11\) There are reduced levels of IFN-\(\gamma\) and increased production of Th2 cytokines in asthma.\(^12\) Therefore, herbal plants that increase the levels of IL-12 and IFN-\(\gamma\) can be considered targets for herbal asthma treatment.

*Teucrium polium* (TP) is a wild plant belonging to the Labiate family, found in Southwest Asia, Europe, and North Africa.\(^13\) It includes more than 340 species worldwide, and 12 species have been discovered in Iran.\(^14\) The biological activities of this plant have been reported as having anti-inflammatory, antioxidant, antimicrobial, and antidiabetic properties.\(^15\) Also, in recent studies on animals, the anti-inflammatory and pain-relieving effects of this plant have been examined, and positive effects have been reported.\(^16,17\)

One study has shown that TP extract can reduce the severity of neutrophilic and eosinophilic airway inflammation and the serum levels of IgE and IL-4 in allergic animal samples.\(^18\) In another study, the effect of this extract on induced allergy was investigated in a mouse model, and the results showed that TP improves the disease by reducing the serum level of IgE and the number of blood eosinophils.\(^19\) These features are summarized in Figure 1.

In this study, we investigate the possible effects of TP on asthma through its effects on Th1 cells and the treatment of a murine model of ovalbumin (OVA)-induced acute allergic asthma (Figure 2).

![Figure 1. Features of *Teucrium polium*](image)
Teucrium polium Improved Allergic Asthma in an Animal Model

Figure 2. Visual representation of asthma induction, Teucrium polium (TP) extract treatment, and its therapeutic effects on the immune system of mice. 
Asthma-induced mice were divided into 5 groups (n=6 each): Control group (A); OVA group (B); OVA + TP extract (50 mg/kg) group (C); OVA + TP extract (150 mg/kg) group (D); OVA + TP extract (300 mg/kg) group (E). The pathological examination of lung tissues (Groups A to E) revealed a significant reduction in inflammation in the treatment groups (C to E) compared to the OVA group. Reduction in inflammation has been indicated: 0: no inflammation, 1: mild inflammation, 2: moderate inflammation, 3: severe inflammation (F). TP extract increased the levels of IFN-γ (G) and IL-12A (H). (hematoxylin-eosin staining, magnification ×40). *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

MATERIALS AND METHODS

Teucrium polium Extract Preparation
TP leaves were purchased from an herbal shop in Rafsanjan, Kerman Province, Iran, and were approved by botanists at the Vali-e-Asr University of Rafsanjan. Aerial parts of the TP were separated for the present study.

To prepare the extract, 200 grams of ground powder were mixed with 250 mL of 70% ethanol. The mixture was left at room temperature for 72 hours and mixed continuously, then filtered with a Whatman No. 1 filter paper (125 mm), transferred to a flat dish, and left at 30°C for 48 hours in an incubator to evaporate and dry the alcohol. After drying, to prepare the studied concentrations, the extract was dissolved in normal saline and stored at 4°C until use.

Experimental Animals
Thirty female Balb/c mice (5–6 weeks old, weighing 18–22 g) were obtained from the Kerman University of Medical Sciences and kept in the animal room at Rafsanjan University of Medical Sciences to adapt to the conditions (20±2°C and 12-hour light-dark cycles). The mice were supplied with a sterilized pellet diet and water.

OVA Sensitization and Inhalation
The animals were randomly divided into 5 groups (6 mice in each group) including the Control group (received saline only), OVA group (induced asthma model), OVA treated with 50 mg/kg TP extract, OVA treated with 150 mg/kg TP extract, and OVA treated with 300 mg/kg TP extract.

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The induction of acute asthma has been described in a previous study.\textsuperscript{20} Briefly, 20 mg of OVA (Sigma-Aldrich, St. Louis, MO, USA) mixed with 80 mg of aluminum hydroxide adjuvant (Alum; Sigma-Aldrich) dissolved in 4 mL of saline solution and 100 μL of the solution were injected subcutaneously into each mouse on days 0, 7, and 14 (sensitization step). The control group was injected with only saline (100 μL).

Then, during the challenge phase, from day 18 to day 21, the mice received OVA via inhalation, where they were exposed to 1% OVA daily for 30 minutes in a glass container using a compressor nebulizer. The control group was treated like the other groups but received saline by inhalation. Three doses (50, 150, and 300 mg/kg) of T. p extract were used to treat animal models during a period of 4 days (days 18 to 21), once a day by intraperitoneal injection of 100 μL doses of 50, 150, and 300 mg/kg TP extract half an hour before OVA challenge. No injections were given in the control and OVA groups during the treatment step (Figure 3).

**Blood Sampling**

Mice were anesthetized on day 22 with an intraperitoneal injection of 1.5 mL ketamine hydrochloride and 0.5 mL xylazine in 10 mL saline to collect blood samples for further investigations. Blood samples were collected from the inner corner of the mice’s eyes. The blood was centrifuged at 4°C (300g) for 4 minutes, and serum was collected and stored at 80°C to measure IFN-γ cytokine.

Figure 3. Treatment process briefly: Experimental protocol of asthma induction with OVA and treatment with *Teucrium polium* extract in the murine model; OVA: ovalbumin; i.p.: intraperitoneal; s.c.: subcutaneous; PCR: polymerase chain reaction; Alum: Aluminum hydroxide; T.p: *Teucrium polium*. 

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Histological Evaluation

The lung tissues of the mice were collected and fixed in 10% formalin. The tissues were dehydrated with ethanol and embedded in paraffin. The thickness of the tissue sections was 4 μm, and then hematoxylin-eosin (H&E) staining was performed according to the protocol to evaluate the degree of inflammatory cell infiltration into the airways. Sections were analyzed for leukocyte infiltration into lung tissues, especially neutrophils and eosinophils. After the examination of the tissue sections by the pathologist, the degree of inflammation and morphological changes in the tissues were described descriptively.

The degree of inflammation was determined by the following scales:

0: no inflammation, 1: mild inflammation, 2: moderate inflammation, and 3: severe inflammation.

Total RNA Isolation and Real-time Quantitative PCR of Spleen Tissue

Real-time PCR analysis was performed to evaluate changes in the gene expression of IL12A (encoding IL-12p35).

Total RNA was extracted from the spleen using an RNA extraction kit (Karmania Pars Gene Company, Kerman, Iran) according to the kit instructions.

After extraction, the quality of the RNA was checked by a NanoDrop instrument (Thermo Fisher Scientific, Inc.). Then, 700 nanograms of total RNA were applied for the cDNA synthesis by a cDNA synthesis kit (Pars Tous, Iran) according to the kit instructions. The relative expression of the gene was measured by quantitative real-time PCR technique using specific primers (Supplementary table) and SYBR green PCR Master Mix Kit (Biosystem, China).

The relative expression of the gene was normalized using a housekeeping gene, β-actin, and evaluated by the following formula: normalized relative ratio = 2^{-ΔΔCt}.

Cytokines Analysis

According to the manufacturer’s protocol (Karmania Pars Gene Company, Kerman, Iran), the enzyme-linked immunosorbent assay (ELISA) method was used to check the serum level of IFN-γ in the serum samples of the mice.

The sensitivity of the kit was 3 pg/mL. Briefly, to measure cytokine concentrations in serum samples, a 96-well plate kit was prepared.

Adding 50 μL of the studied sera to each well was followed by performing the remaining steps according to the kit protocol. Finally, the absorbance of the samples was measured using an ELISA reader at a wavelength of 455 nm.

Statistical Analysis

Data were processed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. All results are expressed as Mean±SD. The diagrams were drawn using Prism version 9.0 (GraphPad Software, San Diego, CA, USA). A p value≤0.05 was considered statistically significant.

RESULTS

TP Extract Treatment Significantly Reduced the Inflammation in OVA-induced Asthma Mice

Excessive mucus secretion and the infiltration of inflammatory cells into the lungs are important features of asthma pathology. The lung sections were stained with H&E. Staining was used to evaluate the infiltration of inflammatory cells (eosinophils and neutrophils) and the thickness of the bronchial epithelium in the lungs. The OVA group had high inflammation and mucus secretion (Figure 4B). However, the treatment groups showed significantly decreased inflammation, mucus secretion, and airway thickness (Figures 4C-E).

At a dose of 300 mg/kg, TP extract significantly reduced the thickening of the bronchial epithelium, inflammation, and mucus production, and the inflammation reached its minimum (Figure 4E). The infiltration rate of eosinophils and neutrophils in this group was reduced to 1 (Figure 4E). The degree of inflammatory cell infiltration for the OVA-treated group was 3 (Figure 4B), while the scores of the OVA groups treated with 50 and 150 mg/kg TP extract were significantly reduced to 2 (Figures 4C and 4D). In the control group, inflammation was not observed (Figure 4A). The intensity of inflammation in the studied groups for a better understanding of the effect of TP extract on asthma is shown in the diagram (Figure 4F).
Figure 4. Effect of *Teucrium polium* (TP) extract on morphological changes of lung tissues in OVA-induced asthma in a murine model (n=6). Lung tissues from all 30 mice were processed for histological evaluation. TP extract modulated the infiltration of inflammatory cells in the bronchus.

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; (hematoxylin-eosin staining, magnification ×40)

F: Diagram related to lung pathology and the level of inflammation in the control and treated groups.

The degree of inflammation: 0: no inflammation, 1: mild inflammation, 2: moderate inflammation, 3: severe inflammation. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

*Teucrium polium* Extract Increased the Production of Th1-specific Cytokines and Genes

Th1-related cytokines, such as IFN-γ and IL-12, were decreased in the OVA group. OVA reduced IFN-γ production by Th1 cells. Production and secretion of IFN-γ were upregulated by TP extract treatment. Serum levels of IFN-γ in the Control group and OVA + 150 mg/kg TP extracts were significantly higher than in the OVA group (*p<0.0001, Figure 5A*). IFN-γ levels increased in OVA + 50 mg/kg and OVA + 300 mg/kg extract groups compared to the OVA group, but there were no statistical differences (Figure 5A).

OVA downregulated *IL12A* gene expression. *IL12A* gene expression was upregulated by TP extract in the treatment groups and was significantly increased in the OVA + 300 mg/kg TP extract group compared to the OVA group (*p<0.0001). The expression of the *IL12A* was increased in the OVA + 50 mg/kg and OVA + 150 mg/kg (*p≤0.05) extract groups compared to the OVA group (Figure 5B). The results showed that the expression of *IL12A* was dose-dependent, and the highest rate was observed in the group treated with the dose of 300 mg/kg.
Figure 5. Effects of TP extract on serum levels of IFN-γ and gene expression of IL12A in OVA-sensitized asthmatic mice and treated mice (n=6 per group). Data are presented as mean±SD. TP extract increased the levels of IFN-γ (A) and IL12A gene expression, as confirmed by real-time polymerase chain reaction (B). *p<0.05, ****p<0.0001.

**DISCUSSION**

Asthma is a chronic inflammatory and noncommunicable disease that is characterized by inflammation, excessive response to allergens, and deformity in airway epithelial cells.21 In this study, OVA was used to induce acute asthma in a mouse model. OVA probably causes asthma by disrupting the Th1 to Th2 balance in the immune system. Differentiation of Th2 cells increases the cytokines secreted by these cells, such as IL-13, IL-5, and IL-4. These cytokines increase mucus secretion, eosinophil infiltration, and IgE production in the airways, respectively.22 Studies have shown that the compounds such as flavonoids and terpenoids in TP have antioxidant and anti-inflammatory properties.23 Thymol and carvacrol present in TP extract are among the terpenoids known as anti-inflammatory and anti-allergic agents.24 The protective role of carvacrol and thymol in some body systems, including the respiratory, digestive, and endocrine systems, has also been reported.25 Flavonoids are polyphenols with broad biological activities, including antiviral, anti-allergic, anti-inflammatory, antineoplastic, and antioxidant properties.26 According to phytochemical studies, compounds such as β-caryophyllene, caryophyllene oxide, and α-humulene have anti-inflammatory properties.27 Studies have been conducted on the anti-allergic effect of TP, and the results of our study confirmed this property in animal models. The anti-inflammatory effects of this plant can suppress allergic inflammation and improve the disease.28 In asthma, eosinophilic inflammation in the airways leads to airway damage and eosinophilic asthma.29 Neutrophils also infiltrate and damage airways. Reducing the infiltration of these cells prevents AHR and damage to the pulmonary epithelial cells, thus preventing inflammation.30 Evaluation of lung tissue in this study showed increased infiltration of neutrophils and eosinophils and mucus secretion in the air bubbles of OVA-induced asthmatic mice (Figure 4B). The injection of TP extract into mice with OVA-induced asthma could reduce the infiltration of eosinophils and neutrophils into lung tissues (Figures 4C, 4D, and 4E). In other words, the anti-inflammatory compounds found in TP extract may have led to the reduction of cytokines involved in the differentiation of neutrophils and eosinophils (i.e., IL-17 and IL-5) and the reduction of lung inflammation in this
study. Unfortunately, it was not possible to evaluate these two cytokines.

The serum level of IFN-γ was also measured in our study, which showed that the serum levels of the cytokine were decreased in the OVA group compared to the healthy control group and showed a significant increase in the dose of 150 mg/kg. Th1 cells inhibit the proliferation and differentiation of eosinophils and Th2 cells by secreting IFN-γ. Recent studies have shown that in people with asthma, the serum levels of IFN-γ are decreased, leading to airway eosinophilia, increased mucus and sputum in the airways, and ultimately shortness of breath.

Previous studies have shown the positive effect of α-humulene (one of the elements found in TP extract) in the improvement of allergy by increasing the production of IFN-γ and reducing airway eosinophilia, leading to the improvement of allergy in treated allergic mice. It can be claimed, according to the results of our study, that the extract, by inducing the secretion of this cytokine, leads to an increase in the differentiation of Th1 cells and a decrease in the differentiation and number of Th2 cells, consequently interfering with the development of asthma and allergies.

IL-12 stimulates the differentiation of Th1 cells and the production of IFN-γ by these cells. Reduced IL-12 levels have been shown in patients with asthma, leading to increased production of IgE and IL-4 in serum and increased eosinophil differentiation in bronchial lavage fluid (BALF).

Recent studies have shown that IL-12α serum levels are decreased in patients with asthma and have a direct relationship with asthma severity. In our study, IL12A gene expression was significantly decreased in the OVA group compared to the healthy control group. As a result, airway inflammation and the narrowing of airway epithelial cells increased in the OVA group. In treatment groups, TP extract increased the expression of the IL12A gene and significantly inhibited the pathological complications of asthma. According to the role of this cytokine in the differentiation of Th1 cells, it can be concluded that the increase in IL12A gene expression in the treated groups is directly related to the reduction of Th2 cell differentiation and the improvement of asthma symptoms. Considering the role of Th2 cells in the induction of mucus secretion and the differentiation of eosinophils in the respiratory tracts of asthma and allergy patients, this extract has probably suppressed the differentiation of these cells to a certain extent by inducing genes related to Th1, leading to disease improvement.

The limitations of this study include the following: the effects of the extract on Th1 cells were investigated in the whole body of animals with asthma. In future studies, it will be better to measure the effects of this extract on Th1 cells in the animal’s BALF. Since Th1 cells play an important role in inhibiting the differentiation of Th2 cells and thus improving asthma, in this study, we investigated the role of TP extract on Th1 cells. We measured the expression of IL12A in the spleen of animals, which is a crucial gene in Th1 differentiation. The authors suggest that in future studies, the researchers measure the effect of this extract on the levels of IL-4, IL-5, IL-13, and IL-9 in the BALF and evaluate the Th1 and Th2 responses.

We concluded for the first time that TP effectively alleviates OVA-induced asthma complications by modulating Th1-related cytokines. Briefly, IFN-γ and IL-12 productions were upregulated. TP prevented lung morphological changes, mucus secretion, and the infiltration of inflammatory cells such as eosinophils and neutrophils near the bronchi.

STATEMENT OF ETHICS

In this study, all the provisions related to the ethical standards of working on laboratory animals were carried out with great care according to the common protocols in the world to avoid the least harm and inconvenience to the animals. In addition, the methods were approved by the Ethics Committee of Rafsanjan University of Medical Sciences (Approval No. IR.RUMS.REC.1399.161).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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