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Original article

Anti-Inflammatory Effect of *Pimpinella anisum* Extract in a Mouse Model of Allergic Asthma

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Abstract

Background and Objectives: Allergic asthma is a chronic inflammatory disease of the airways which has become prevalent globally. There are reports about the immunomodulatory and antioxidant effects of *Pimpinella anisum* L. seeds; so, in this study, we explored the suppressive effects of aqueous *P. anisum* L. seeds extract on ovalbumin-induced asthma in a mouse model. **Methods:** The seeds were extracted with water and the extract was dried by freeze-drying method. Twenty-eight BALB/c male mice weighing 15–20 g were divided into four groups of seven animals. Ovalbumin was used to trigger allergic asthma in these animals. Negative and positive control mice received phosphate-buffered saline and ovalbumin, respectively. The remaining two groups were challenged with ovalbumin and then received budesonide and the seed extract, respectively. Thereafter, the eosinophils count and expression of IL-5, -13, and -33 were measured in bronchoalveolar lavage fluid of mice. Histopathological changes of the lung tissues were also analyzed. **Results:** Aqueous extract of *P. anisum* seeds hindered ovalbumin -stimulated asthmatic complications by declining eosinophils number and expression of IL-5, -13, and -33 in bronchoalveolar lavage fluid of mice. It also inhibited the hyperplasia of goblet cells, hypersecretion of mucus, and inflammation in peribronchial and perivascular spaces, which were consequences of ovalbumin exposure. The activity of the extract in suppressing inflammatory responses of asthma in our murine model was comparable to budesonide. **Conclusion:** Our data underscored the effect of aqueous *P. anisum* seeds on the suppression of inflammatory responses of allergic asthma, proposing a promising suggestion for the treatment of the disease.

Keywords: asthma; budesonide; eosinophil; ovalbumin; *Pimpinella anisum*

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Introduction

Allergic asthma is a chronic disease that affects respiratory system and is usually presented by several recurring symptoms such as coughing, wheezing, airway remodeling, and shortness of breath [1,2]. It is characterized by extreme airway mucus production, chronic pulmonary

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eosinophilia, and elevated serum immunoglobulin E (IgE) [1]. Asthma mainly occurs in childhood and a variety of genetic and environmental factors are associated with its development [3]. According to recent reports, the prevalence and economic burden of clinically diagnosed asthma is increasing throughout the world [4]. Currently, three types of medications are prescribed for the treatment of asthma patients, which include inhaled short and long acting β_2 agonists, inhaled and oral corticosteroids, and leukotriene antagonists; these treatments are accompanied by multiple adverse side effects [5]. Therefore, it is necessary to develop novel therapies that target allergic asthma with few or no side effects.

Pimpinella anisum L., also called anise, is a medicinal herb that grows in different parts of the world, including the Middle East West Asia, Egypt, Eastern Mediterranean Region, Mexico, and Spain [6].

In Persian medicine, "Rabv" symptoms are similar to signs of asthma in conventional medicine. Many medications have been reported for the treatment of "Rabv", including anise seeds. Several traditional Persian medicine books such as "Makhzan ul-Adwia" by "Aghili Khorasani", "Tuhfat al-Mu'minin" by "Hakim Momen", and "Gharabadin Kabir" have recommended *Polypodium vulgar* with liquorice root and anise for the treatment of cough, shortness of breath, and asthma [7-9]. Anise has also been recommended by Rhazes, in "Al Hawi Fi Al Tibb"as an effective traditional herbal remedy for the alleviation of asthma symptoms [10]. Anise seeds are not only used for the treatment of gastrointestinal diseases, but also for the diseases of the upper respiratory tract, fever, and inflammation of the mouth and throat [11]. Anise has aromatic seeds with an expectorant-like effects on the lungs and airways [12]. Some beneficial and therapeutic effects of anise on digestive disorders, gynecologic disease, dyspnea, and its use as an anticonvulsant and anti-asthma agent have been reported in Persian medical books [13,14]. The seeds of this herb have a dry and warm temperament with a variety of medical uses such as the stimulation of milk production, regulating menstruation, urine excretion, and sweat secretion. This medicinal plant has been traditionally consumed to treat seizure, epilepsy melancholy, and nightmare [15]. Anise has also a variety of pharmacological effects such as antibacterial, antifungal, insecticidal, antiviral, muscle relaxant of the tracheal chain, anticonvulsant, analgesic, antiulcer, palliative of nausea, laxative, antidiabetic, hypolipidemic, antioxidant, alleviative of menopausal hot flashes and the pain in dysmenorrhea properties [6].

In the current investigation, we explored the anti-allergic impacts of aqueous extract of *Pimpinella anisum* seeds on the OVA-induced allergic asthma in a murine model.

Materials and Methods

Ethical consideration

The experimental design was approved by Ethical Committee of Shahid Beheshti University of Medical Sciences with the code of IR.SBMU.RETECH.REC.1397.504. The animals were handled according to the National Institutes of Health (NIH) guideline for the care and use of Laboratory animals.

Chemicals

Aluminum hydroxide, ovalbumin (OVA) powder, and urethane were purchased from Sigma-Aldrich, USA. Mouse IL-5, IL-13, and IL-33 ELISA kits (Abcam, USA), TRIzol (Invitrogen life technologies, USA), cDNA synthesis kit (Thermo Scientific, USA), and budesonide (Ramopharmin, Iran) were used in the present study.

Plant material

Anise (100 g) was purchased from the medicinal herbal market of Tehran, Iran (2019). The plant seeds were gently powdered by an electrical grinder, mixed with 1000 mL water, and boiled for 30 min. Then, the extract was filtered twice and lyophilized by the freeze-drying method to preserve perishable materials and for convenient transportation.

Animals and experimental groups

Twenty-eight 6–8 weeks old male BALB/c mice weighing 15–20 g were purchased from Pasteur Institute of Iran (Tehran, Iran). The mice were maintained under controlled standard conditions (12-h-light/dark cycle, humidity 45-65%, temperature 22-24 °C) with free access to food and water. The animals were randomly divided into 4 groups (n=7 for each group): negative control group (sensitized and challenged with PBS only);

positive control group (sensitized and challenged with OVA); treatment group, which was first sensitized and challenged with OVA and then received anise extract orally; and drug control group, which was treated with OVA and then with budesonide by gavage [16].

Induction of allergic asthma

This experiment was carried out during a month. To induce allergic asthma in the experimental mice model, the animals were sensitized with OVA (20 μg/mouse) via intraperitoneal injection of this compound on days 0 and 14. Then, the sensitized mice inhaled 1% (w/v) OVA aerosol by using a nebulizer once daily for 20 min on days 24, 26, 28, and 30. As mentioned before, the negative control mice were injected and challenged with PBS. *Pimpinella anisum* aqueous seeds extract (at the dose of 0.16 mg/kg, based on pilot tests) was administrated to animals by oral gavage once daily 15 min after the OVA challenge on days 24-30. Budesonide 1 % was administrated to animals according to the same protocol as the anise-treated group [16].

Bronchoalveolar lavage fluid collection

On day 31, the mice were euthanized using inhaled halothane for the collection of blood and bronchoalveolar lavage fluid (BALF) as well as the dissection of their lung tissues. Four mice of each group were used for BALF collection and the remaining three undergone surgery to dissect their lungs for histopathological examinations. BALF sampling was carried out via tracheostomy. Briefly, the mice were anesthetized then they underwent lung lavage twice with cold PBS. The number of eosinophils was counted by centrifuging BALF samples using a cytospin and attaching pellets to a slide glass [17].

Quantification of cytokine expressions at mRNA and protein levels

Enzyme-linked immunosorbent assay (ELISA) method was used to measure the protein concentration of interleukins 5, 13, and 33 in BALF specimens by ELISA kits according to protocols of manufacturers. The sensitivity for IL-5, IL-13, and IL-33 kits were 1 pg/mL, 1.2 pg/mL, and 5.3 pg/mL, respectively. Intra-assay precision for IL-5, IL-13, and IL-33 kits were < 10%, 2.2%, and 5.9%, respectively. Inter-assay precision for IL-5, IL-13, and IL-33 kits were < 12%, 1.4%, and 12.4%, respectively.

Real time-PCR technique was used to quantify the mRNA expression levels of interleukins 5 and 13 in BALF. Briefly, TRIzol solution was applied to extract the total RNA from the cellular content of the BALF. Thereafter, the extracted RNA samples were exposed to cDNAs synthesis process using a cDNA Synthesis Kit. Gene alterations of interleukins 5 and 13 and GAPDH (as housekeeping gene) were measured by quantitative reverse transcriptase PCR (qRT-PCR) using a rotor gene (Qiagen, Hilden, Germany) detection system SYBR GREEN® (nonspecific DNA-binding factors). The sequences of the primers used for the amplification and quantification of the mentioned genes are shown in Table 1 [18].

Histopathological study

The lung tissues of three mice of each group were isolated to be analyzed for histopathological changes. These tissues were first immersed in 10% neutral buffered formalin and then embedded in paraffin. The paraffin-embedded tissues were cross-sectioned at a thickness of 3-5 μm and the sections were stained. Three sections were prepared for each tissue. The stained tissues were evaluated by a pathologist to observe and report any inflammatory responses and pathological changes of lung tissues. Hematoxylin and eosin (H&E) staining method was applied to report both perivascular and peri-bronchial inflammations. The number of goblet cells was counted using periodic acid–Schiff (PAS) staining of the tissue sections. Evaluation of mucus production and mucosal cell proliferation was performed by PAS staining of the tissue mucin molecules and counting them in 10 high-power fields (HPF) [19].

Table 1. Primer sequences used in this study

Gene name	Forward primer	Reverse primer	Accession number	Amplicon size
IL−5	5'- ATCCAGGAACTGCCTCGTC -3′	5'-ACATTGACCGCCAAAAAGAG -3′	NM_010558.1	62 bp
IL−13	5'- AATAAGATCAAGAAGAAATGTGCTCAA -3′	5'- GGTCCACACAGGGCAACT -3′	NM_008355.3	60 bp
GAPDH	5'-GGTCCTCAGTGTAGCCCAAG-3	5'-TGTTCCTACCCCCAATGTGT-3	XM_030244453.2	137

IL: interleukin

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare the data between the treated and control groups. We used the Shapiro-Wilk test to affirm that our data were normally distributed. All values presented in this study are expressed as mean ± SD. GraphPad Prism software version 7 was used for the analysis of the present data. P-values <0.001 and <0.0001 were considered statistically significant.

Results and Discussion

Figure 1 shows the effects of OVA sensitization and drug treatment on the number of eosinophils. OVA challenge significantly increased eosinophil count (69.18±3.85) in BALF of the positive control group in comparison to PBS-administered negative controls (1.23±1.09) ($p<0.0001$). However, the extract treatment remarkably reduced the number of eosinophils (31±7) in BALF samples of the asthmatic group compared with the OVA-challenged mice (69.18±3.85) ($p< 0.0001$). Also, the budesonide-treated group showed a significantly decreased number of BALF eosinophils (44.41±8.12) as compared with OVA-treated group ($p<0.001$).

As illustrated in Figure 2, OVA-challenged mice showed significantly elevated mRNA expression of IL-5 and IL-13 in BALF specimens compared with PBS group ($p<0.0001$).

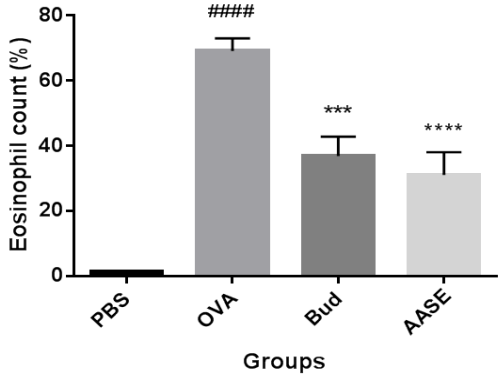

Figure 1. The effect of *Pimpinella anisum* aqueous seeds extract (AASE) on the number of eosinophils in BALF samples. The positive and negative groups received phosphate-buffered saline (PBS) and ovalbumin (OVA), respectively. Drug treatment groups were administered anise extract and budesonide (Bud) after sensitization with OVA. The eosinophils counts are shown as mean ± SD. ####p<0.0001 (compared to PBS); ****p<0.0001 (compared to OVA); ***p<0.001 (compared to OVA)

The treatment of mice with budesonide and the extract significantly diminished the expression levels of both IL-5 and IL-13 in comparison to OVA-treated asthmatic group. Treating animals with anise extract or budesonide significantly reduced the expressions of both IL-5 and IL-13 in BALFs in comparison to positive control mice ($p<0.0001$ and $p<0.001$).

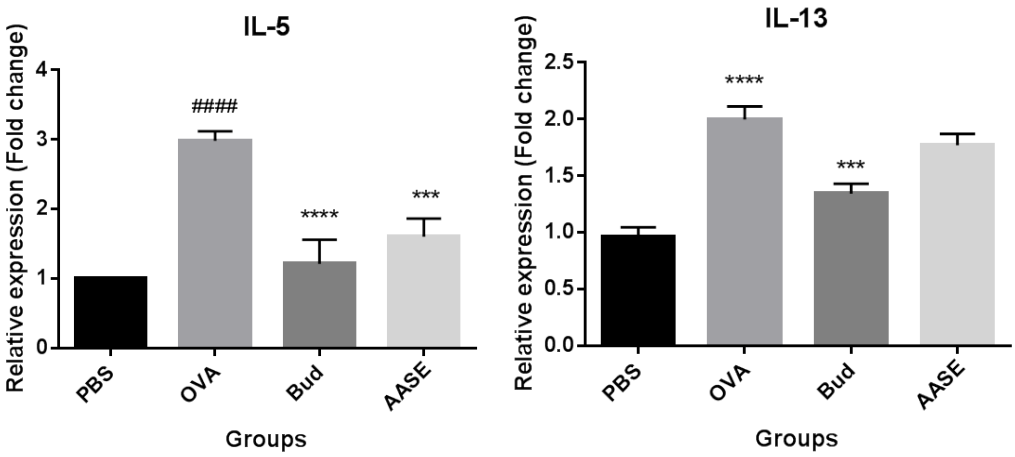

Figure 2. Effects of asthma induction and drug treatments on the expressions of IL-5 and 1L-13 in BALF samples. Ovalbumin (OVA) treatment significantly elevated gene expressions of these two interleukins. *Pimpinella anisum* aqueous seeds extract (AASE) and budesonide downregulated mRNA expressions of both IL-5 and 1L-13 in BALFs. Values are expressed as mean ± SD. ####p< 0.0001 (compared to PBS); ****p<0.0001 (compared to OVA); ***p< 0.001 (compared to OVA)

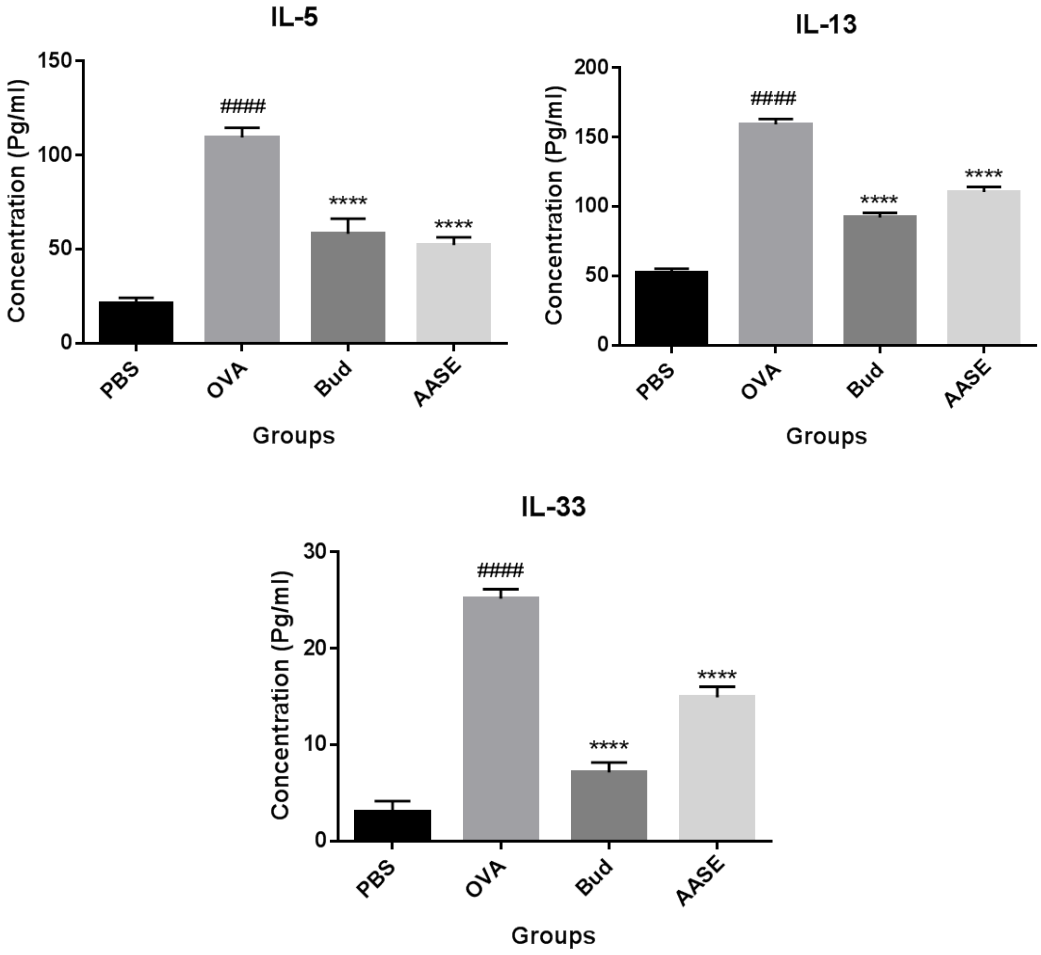

Figure 3. Effects of *Pimpinella anisum* aqueous seeds extract (AASE) on the concentrations of IL-5 (A), IL-13 (B), and IL-33 (C) in BALF; ####P<0.0001compared to PBS; ****p<0.0001compared to Ovalbumin (OVA); all data are expressed as mean±SD.

Table 2. Histological analysis of lung tissues

	Goblet cells	Mucus (%)	Peribronchial inflammation	Perivascular inflammation
PBS	1±0.2	25±5	0.5±0.1	0.5±0.1
OVA	4±0.1#	100±5#	4±0.2#	4±0.2#
Bud	1.3±0.7*	35±1*	1.1±0.2*	1.2±0.2*
AASE	1±0.2*	25±1*	3±0.4*	1±0.1*

PBS: phosphate-buffered saline; OVA: ovalbumin; Bud: budesonide; AASE: *Pimpinella anisum* aqueous seeds extract; the results are expressed as mean ± SD; *p<0.05 was considered statistically significant compared to OVA group; #p< 0.05 was considered statistically significant compared to PBS group

Using ELISA technique, the protein levels of IL-5, IL-13, and IL-33 in BALFs of the mice were quantified. Figure 3 demonstrates that OVA treatment of positive control mice significantly enhanced the concentrations of these three interleukins of their BALFs when compared with PBS-treated controls (p<0.0001). However, extract administration of asthmatic mice diminished the concentrations of all three mentioned interleukins in their BALF in comparison to OVA-challenged animals (p<0.0001). Additionally, treating the animals with the standard drug, budesonide, also significantly declined the levels of these three cytokines in BALF as compared to positive control group.

Analysis of histopathological changes of lung tissues (Figure 4 and Table 2) of the OVA-challenged mice exhibited a considerable entrance of inflammatory cells into peribronchial and

perivascular spaces of this tissue (Figure 4B). However, these infiltrates were not present in the lung tissue sections from PBS- treated animals, as negative controls, (Figure 4A). Figures 4C and D show that budesonide and extract treatments inhibited both perivascular and peribronchial inflammations. We found that hyperplasia of goblet cells, secretion of mucus, and peribronchial and perivascular inflammations were significantly augmented in the OVA-challenged group compared to PBS-challenged mice ($p<0.05$), as shown in Table 2. We also observed that the budesonide and extract treated mice had exposed a significantly reduced hyperplasia of goblet cells, hypersecretion of mucus, and inflammations of peribronchial and perivascular spaces compared to OVA-challenged mice ($p<0.05$). Interestingly, the perivascular inflammation in the extract-administered group was equal to that of the budesonide group, but the peribronchial inflammation in the extract-treated group was significantly higher than that of the budesonide group ($p<0.05$).

Allergic asthma is a chronic inflammatory airway disease characterized by recurrent episodes of airway obstruction, wheezing, and mucus overproduction. This disease afflicts approximately 300 million people worldwide [20,21]. In the present study, we evaluated the therapeutic effects of *P. anisum* aqueous seeds extract on allergic inflammatory responses in a murine model of OVA-induced asthma. Previous works have suggested the need for further investigation of the *P. anisum* on airway inflammatory responses triggered by asthma [22]. OVA-induced asthma is an appropriate model for investigating the mechanisms causing asthma and the therapeutic effects of medicines. OVA sensitization of animals causes asthma-related symptoms similar to chronic asthmatic patients [23]. In this study, the gene expressions and protein levels of inflammatory cytokines including IL-5, IL-13, and IL-33 in the BALF significantly increased due to OVA challenge of mice. However, anise extract treatment significantly inhibited the elevation of these interleukins in comparison to OVA-challenged group. We also found that the extract could hamper the eosinophils trafficking, which was augmented by the sensitization of mice lungs with OVA. Anise extract also reversed the goblet cell hyperplasia, mucus hypersecretion, and migration of inflammatory cells into peribronchial and perivascular spaces in comparison to ovalbumin sensitized group. Our observed positive impacts of the anise extract on asthma-associated adverse side effects were comparable with those of budesonide, which is a standard medication for allergic asthma therapy. Eosinophilic trafficking is considered a crucial aspect of allergic asthma and seems to be associated with the elevation of Th2 cytokines [24]. These responses activate T cells, B cells, smooth muscle cells, and goblet cells [25]. An augmented expression and function of Th2 cytokines such as IL-5, IL-13, and IL-33 triggers the progression of immune signal transduction and induces the promotion of eosinophilia [26,27]. Increased expression of IL-5, IL-13, and IL-33 in the lungs plays a key role in aggravating the severity of asthma. IL-5 has been indicated to activate eosinophils and stimulate their migration into bronchi. IL-13 accumulation in the airways is a trigger for mucus hypersecretion and airway hyper-responsiveness (AHR) [28].

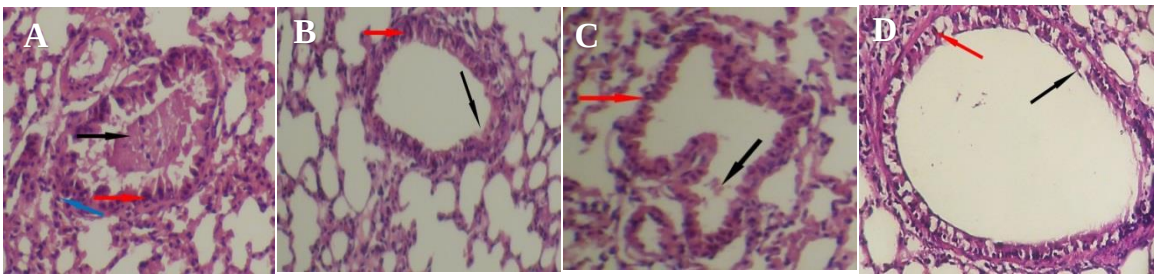

Fig 4. Histopathological analysis of lung tissues stained with Hematoxylin-eosin. A) The positive control asthmatic group which was challenged with ovalbumin; B) The negative control group which was treated with phosphate-buffered saline; C) The asthmatic group that received budesonide; D) The tissue section from asthmatic mice treated with *Pimpinella anisum* aqueous seeds extract. Black, red, and blue arrows are representatives of mucus, goblet cell, and peribronchial and perivascular infiltrates, respectively.

During an eosinophilic attack, these cells undergo a degranulation process and tend to secret some toxic proteins which cause damage to bronchioles [29]. Uncontrolled production of IL-33 can activate of Th2 cells, dendritic cells, eosinophils, mast cells, and basophils. Therefore, IL-33 is considered a promising candidate for therapeutic intervention. Activation of these cells ultimately leads to increased expression of inflammatory mediators that promote the disease symptoms [30]. Our results indicated that the anti-asthmatic effects of anise extract may be associated with reduced production of Th2 cytokines. Th2 cytokines are closely involved in AHR and mucus production [31]. Th2 cytokines activate mast cells and eosinophils, which produce autacoids such as histamine and leukotrienes [32]. These mediators lead to the contraction of airway smooth muscle, resulting in AHR [33-35]. Additionally, Th2 cytokines elevate the production of mucus by goblet cells via activation of inflammatory signaling pathways [36]. Yang et al. treated ovalbumin-induced asthmatic mice with a Chinese medicinal herbal formula. They found that this herbal remedy reversed histopathological changes of lung tissues of asthmatic mice by hindering the influx of inflammatory cells into the lung and trachea of the animals [37]. Another study on the effects of *Involucrum castanea* extract on ovalbumin-induced asthma in guinea pigs showed that the reduction of eosinophilic trafficking in BALF and expression of IL-5 in lungs are possible mechanisms for anti-inflammatory effects of this plant extract on asthma [38]. The results of these two studies show that inflammatory cells attack the lungs and airways, eosinophilia, and IL-5 amplification can be major mechanisms involved in allergic asthma complications. Therefore, targeting them could be an effective way to tackle this disease. Our study also affirmed that the anti-asthmatic activities of anise extract were mainly exerted by targeting the mentioned mechanisms.

Conclusion

Anise extract suppressed the increase in allergic lung inflammatory responses in an OVA-induced allergic asthma model. Thus, the possible mechanism of action of the extract may involve inhibition of eosinophilic trafficking and the blockade of Th2 cytokines responses, leading to the prevention of the inflammatory cell infiltration, the trigger of tissue inflammations, and mucus secretion. Therefore, anise has an important role in reducing inflammation of the airways. Our results suggest that anise extract can be suggested as a promising natural remedy for the treatment of allergic asthma.

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Author contributions

Rasool Choopani and Reza Ilkhani had a major role in developing ideas and supervising the study; Azadeh Ghiaee and Roya Arbabtafti contributed to collecting data and preparing the draft for this paper. Shirin Fahimi and Seyyed Shamsadin Athari contributed to data collection, writing and editing the manuscript. Fatemeh Jafari, Hanieh Kashafroodi and Tahereh Dargahi performed the experiments. All authors have read and agreed to the final version of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

AASE: *Pimpinella anisum* aqueous seeds extract; OVA: ovalbumin; Bud: budesonide; PBS: phosphate-buffered saline; BALF: bronchoalveolar lavage fluid; IgE: immunoglobulin E; qRT-PCR: quantitative reverse transcriptase PCR; H&E: Hematoxylin and eosin; PAS: periodic acid–Schiff; ELISA: Enzyme-linked immunosorbent assay; HPF: high-power fields; ANOVA: one-way analysis of variance; AHR: airway hyper-responsiveness