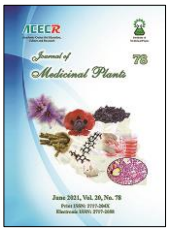
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Research Article

Regulation effects of *Trigonella foenum-graecum* seed extract on a mouse model of allergic asthma

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ABSTRACT

Background: *Trigonella foenum-graecum* L. (fenugreek) seeds have a long history of treating allergic asthma known as "Rabv" in Persian medicine. The protective effects of fenugreek on asthma have been noted in Persian medicine texts. **Objective**: The aim of the present study was to investigate the protective effects of aqueous fenugreek seed extract (AFSE) on the reduction of Th2 cytokines via decreasing the levels of mRNA expression of Th2 cytokines in bronchoalveolar lavage fluid (BALF). **Methods:** 28 female Balb/c mice were divided into four groups of seven animals. Negative and positive control groups received Phosphate-buffered saline and ovalbumin (OVA), respectively. The remaining two groups were firstly sensitized with OVA to induce asthma and then received Theophylline and AFSE. Thereafter, the mRNA expressions of Th2-type interleukins (IL-4, IL-5, and IL-13) and mucin5 as well as the concentrations of IL-5, IL-13, and IL-33 in BALF samples were measured and pathological alterations of lung tissues were analyzed. **Results**: AFSE treatment of Balb/c mice significantly decreased the number of eosinophils, the mRNA expressions of IL-4, IL-5, IL-13, and mucin5 and the concentrations of IL-5, IL-13, and IL-33 in their BALF specimens. It also considerably prevented peribronchial and perivascular inflammations, mucus hypersecretion, and goblet cell hyperplasia in lung tissues in comparison to OVA-sensitized mice. **Conclusion**: The present study demonstrated that the aqueous fenugreek seeds extract could be an alternative medication for the treatment of allergic asthma.

Abbreviations: ASFE, Aqueous Fenugreek Seed Extract; BALF, Bronchoalveolar Lavage Fluid; OVA, Ovalbumin

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1. Introduction

Allergic asthma is a type of lung disease that is characterized by airway inflammation, remodeling, and infiltration of leukocytes which results from a complex interplay between genetic and environmental factors [1]. Asthma is a serious public health problem. The morbidity and mortality of allergic asthma are increasing [2]. The cytokines IL-4, IL-5, IL-13, and IL33 are important role-players in asthma pathogenesis [3]. Corticosteroids and β-Agonists reduce asthma symptoms but they cannot cure the disease and long-term use of steroidal anti-inflammatory drugs will result in complications especially in children such as growth failure and tachyphylaxis [4]; therefore, the development of new therapeutic agents focusing on complementary and traditional medicine is immediately needed. These products are beneficial, accessible, and have low cost without or with low side effects.

Many previous studies have shown that *Trigonella foenum-graecum* L. (fenugreek) has beneficial effects on a variety of diseases such as lung diseases in which immunomodulatory defects are major issues [5]. Fenugreek seeds extract has shown anti-allergic effects on allergic skin inflammation [6]. It has also shown hepatoprotective [7], anti-lipidemia, anti-oxidant, anti-inflammatory, and anti-asthmatic effects in both experimental animals as well as humans in clinical trials [8-11]. A study by Emtiazy and colleagues showed that the aqueous extract of the seeds of fenugreek significantly improved the Quality of Life and the lung function tests of the patients with mild asthma compared to the placebo- and honey syrup-treated groups [12]. Purification and characterization of the chemical components of fenugreek seed oil revealed the presence of a number of phytochemicals with different pharmacological activities, including anti-asthmatic agents [13].

In Persian medicine (PM), fenugreek has been used for the treatment of respiratory and allergic diseases such as chronic coughs and asthma, hoarseness, pneumonia, and chronic bronchitis [14], [15]. This plant has several phytochemical components with a variety of biological activities. Trigonelline, for example, is a natural alkaloid isolated from fenugreek with protective effects on the heart and liver and treatment of hyperglycemia, hypercholesterolemia, nervous and hormonal disorders, and cancers [16]. Galactomannan, as an active polysaccharide isolated from fenugreek seeds, has shown radical scavenging activity and can be considered an efficient anti-oxidant [17].

In the present study, we have investigated the protective effects of aqueous fenugreek seeds extract (AFSE) on ovalbumin (OVA)-induced allergic asthma complications in a murine model of the disease.

2. Materials and Methods

2.1. Reagents

Ovalbumin and aluminum hydroxide (Sigma-Aldrich, USA), urethane (Sigma-Aldrich, USA), Mouse interleukins 5 (Cat. No. M5000), 13 (Cat. No. CSB-E04602m) and 33 (Cat. No. M3300) enzyme-linked immunosorbent assay (ELISA) kits (Abcam, USA), TRIzol (Invitrogen, USA), cDNA synthesis kit (Thermo Scientific, USA), Rotor-Gene Q thermal cycler (Qiagen, Germany) and theophylline (Ramopharmin, Iran) were used in the present study.

2.2. Preparation of AFSE

Fenugreek seeds were provided from the pharmacy of the School of Persian Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The scientific name was confirmed

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at the Herbarium of Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran and a sample was kept at TMRC Herbarium for future reference (HMS-534). Fenugreek analysis: Fenugreek seed powder was analyzed according to British Pharmacopoeia (BP) and the swelling index, total ash and loss on drying were evaluated [18]. The powdered seeds were boiled in water until one third of the volume evaporated. The mixture was filtered and after cooling, the extract was freeze dried and kept in refrigerator until further analyses.

2.3. Animals

Eight-week-old female Balb/c mice (approximately 20-22 g) were purchased from Pasture Institute of Iran. The mice were acclimatized under standard laboratory conditions (24 ± 2 °C, 50 ± 5% humidity and 12 h light-dark cycle and allergen-pathogen free conditions) for one week. They were allowed free access to food and tap water. All animals were handled in accordance with National Institutes of Health (NIH) guideline for the care and use of Laboratory animals (Publication 1985, Edition 85-23). This study was approved by ethical committee of Shahid Beheshti University of medical sciences (No.: IR.SBMU.RETECH. REC.1396.153).

2.4. Experimental protocol

Twenty eight Balb/c mice were randomly divided into four groups of seven animals. The mice in positive (OVA group) and negative control groups were challenged with OVA and phosphate-buffered saline (PBS). The remaining two groups were first challenged with OVA and then received 1 % theophylline (drug control group) and AFSE (2 mg/kg) (AFES group).

2.5. Asthma induction and drug treatments

The induction of allergic asthma was accomplished during a month. Briefly, the mice were sensitized via intraperitoneal (*i.p*) injection of 20 μg OVA and 50 μL aluminum hydroxide in 50 μL PBS (pH 7.4) on days one and fourteen. The mice were challenged by inhalation of 1% OVA solution aerosolized by an ultrasonic nebulizer (NB- 500, Rossmax, Switzerland), for 30 min per day on days 24, 26, 28 and 30 (Fig. 1) [19]. Then, drug treatment groups received AFSE theophylline for eight consecutive days (days 23-30). On day 31 of study, all mice were killed and then separated into two groups; one group of four animals for lung histopathology analysis and the other group of three mice for Bronchoalveolar lavage fluid (BALF) sampling [20].

2.6. Collection of BALF and measurement of interleukins

BALF sampling is an experimental procedure that is generally used to assess the cellular and acellular content of the lung lumen. This fluid is elicited via inserting a catheter into the trachea of a terminally anesthetized animal, through which a saline solution is instilled into the bronchioles [21]. After the mice were anesthetized by urethane, BALFs were collected via tracheal cannulation and the samples were stored at -70 °C for cytokines measurement [19]. The levels of IL-5, IL-13 and IL-33 in the BALF were determined by using ELISA method, according to the manufacturer's instructions.

2.7. RNA extraction and quantitative real time PCR

To evaluate the effects of *Trigonella foenum-graecum* water extract on the expression of interleukins 4, 5, 13, as well as Muc5 genes in

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BALF samples, the quantitative Real time-PCR was used. Briefly, the total RNA was extracted from BALF cells via TRIzol solution (Invitrogen life technologies, NY, USA). Then, the cDNAs were synthesized by using a cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). The changes in the expressions of the mentioned interleukins, Muc5, and GAPDH (as housekeeping gene) were measured by quantitative reverse transcriptase PCR (qRT-PCR) in a rotor gene (Qiagen, Hilden, Germany) detection system SYBR GREEN® (nonspecific DNA-binding factors). The primer sequences used in the present study are shown in Table 1.

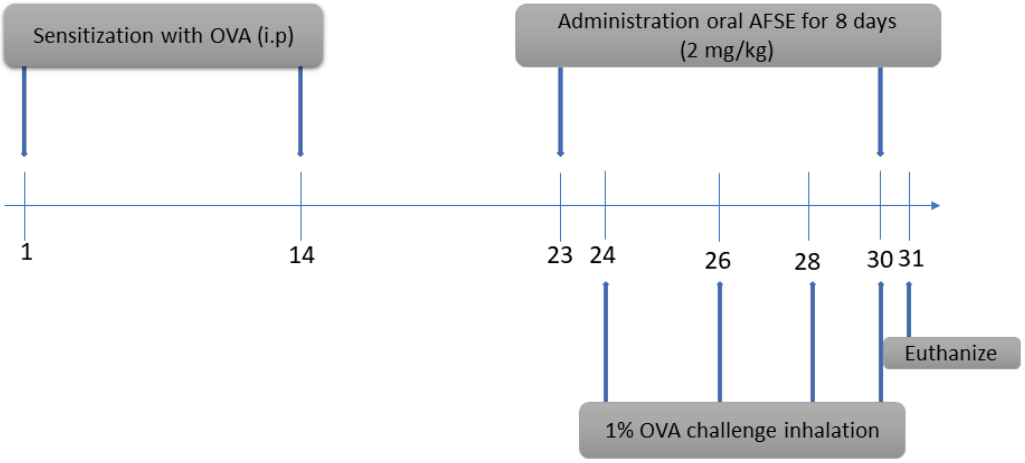


Fig. 1. The mice were sensitized via intraperitoneal (i.p) injection of OVA on days 1 and 14. Then, they were challenged by inhalation of 1 % OVA solution aerosolized by an ultrasonic nebulizer for 30 min per day on days 24, 26, 28 and 30. The days between 14th and 23th days were allocated as a re-sensitization period. Two of the three sensitized and challenged groups were treated with AFSE and theophylline for eight consecutive days (days 23-30).

Table 1. Primer sequences used in this study

Gene name	Forward primer	Reverse primer
IL−4	5'-TTTGGCACATCCATCTCCG-3′	5'-AGATCATCGGCA TITTGAACG-3′
IL−5	5'- ATCCAGGAACTGCCTCGTC -3′	5'- ACATTGACCGCCAAAAAGAG -3′
IL−13	5'- AATAAGATCAAGAAGAAATGTGCTCAA -3′	5'- GGTCCACACAGGGCAACT -3′
Muc5	5'-AAGGCTCGTACCACAGGGA-3′	5'-CAGGACTCTCTGAAATCGTACCA-3′
GAPDH	5'-GGTCCTCAGTGTAGCCCAAG-3	5'-TGTTCCTACCCCCAATGTGT-3

2.8. Histological evaluation

On day 31, three mice in each group were sacrificed, their lungs were isolated. Then, the isolated lungs were immersed in 10 % neutral buffered formalin and embedded in paraffin. These paraffin-embedded tissues were subsequently cross-sectioned and the tissue sections were stained with hematoxylin and eosin (H&E). Finally, the sections were observed by an expert pathologist.

2.9. Statistical analysis

Data were expressed as mean ± SD and analyzed by parametric one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test for multiple comparisons. SPSS software version 17.0 was used for analysis of the present data. The results were accepted statistically significant when P-value was < 0.05.

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3. Results

The swelling index, total ash and loss on drying of fenugreek seeds were measured according to BP Pharmacopeia. The results are shown in Table 2.

3.1. AFSE inhibited inflammatory cells infiltration in BALF

This study indicated that the AFSE had an inhibitory effect on the infiltration of inflammatory cells and eosinophils into lung tissue. The results showed that the number of eosinophils in BALF increased significantly in OVA-group mice (69.18 ± 3.85 %) compared with the negative control group (1.23 ± 1.09 %). However, treatment of the AFSE group (28.11 ± 2.88 %) reduced eosinophils infiltration in BALF of the OVA-induced groups (Fig. 2). In addition, OVA-induced inflammatory cell infiltration into BALF was significantly reduced due to theophylline treatment of animals.

Table 2. Analysis of fenugreek seeds according to BP Pharmacopeia

Tests	Fenugreek sample	Limit in BP Pharmacopeia
Swelling index	7.2	≥ 6
Total ash	2.95%	≤ 5%
Loss on drying	7.12	≤ 12 %

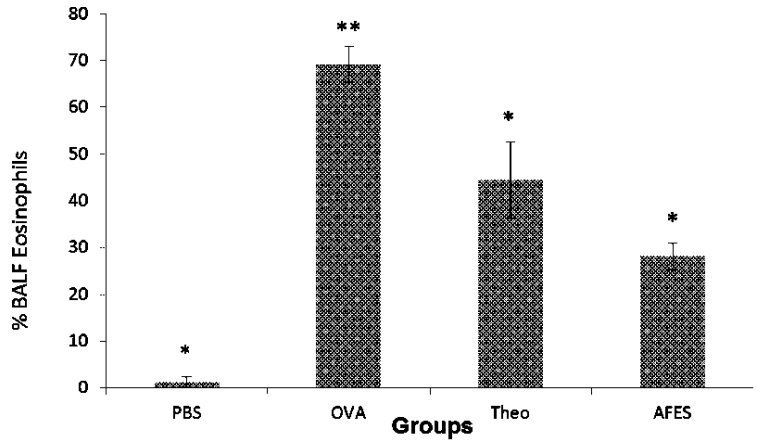


Fig. 2. Treatment with AFSE exhibited a remarkable reduction in the level of eosinophil in BALF compared with those of the OVA group. An AFSE effect on the reduction of eosinophil counts is more significant in comparison to theophylline. Results are expressed as the mean ± SD, $P < 0.05$.

3.2. AFSE reduced Th2 cytokines levels in BALF

OVA-challenged mice showed increased levels of IL-5 (100.18 ± 4.89 $Pg.ml^{-1}$), IL-13 (153.10 ± 4.61 $Pg.ml^{-1}$), and IL-33 (25.16 ± 0.99 $ng.ml^{-1}$) in BALF. In the present study, in AFSE group, IL-5 (49.16 ± 7.92 $Pg.ml^{-1}$), IL-13 (80.98 ± 4.36 $Pg.ml^{-1}$), and IL33 (8.96 ± 0.93 $ng.ml^{-1}$) levels of BALF samples significantly decreased compared to positive control group ($P < 0.05$) (Fig. 3).

3.3. Effects of AFSE on lung histopathology

Treatment of mice with AFSE and theophylline considerably hampered mucus hypersecretion, goblet cell hyperplasia, and peribronchial and perivascular inflammation (Fig. 4 and Table 3).

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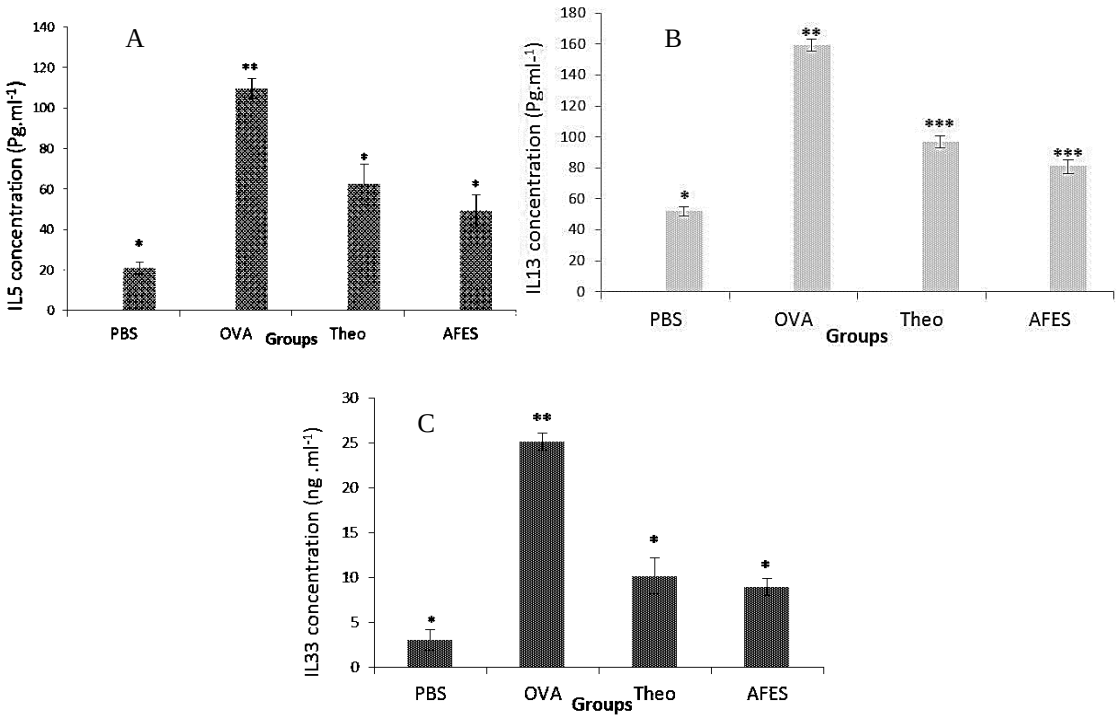


Fig. 3. Cytokine levels in BALF. Treatment with AFSE exhibited a remarkable reduction in the levels of IL-5 (A), IL-13 (B), and IL33 (C) in BALF compared with those of the OVA group. Results are expressed as the mean ± SD ($P < 0.05$).

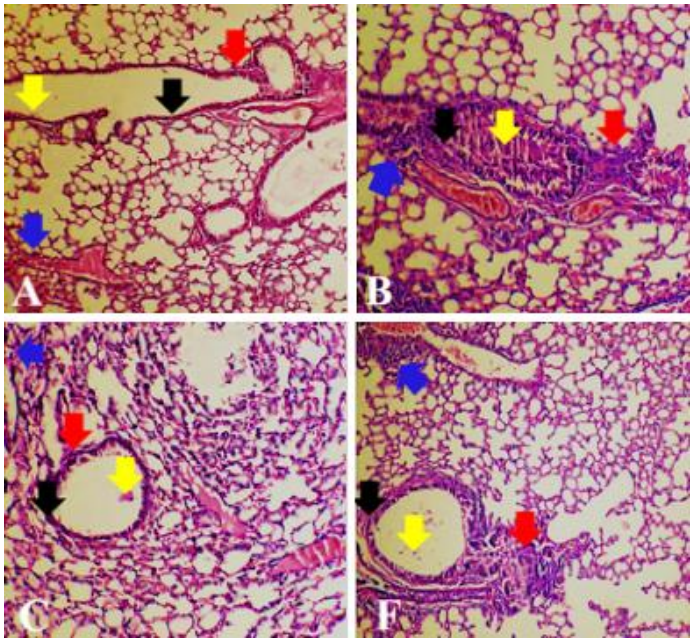


Fig. 4. Effect of AFSE on allergic airway inflammation in lung tissues (H&E staining). (A) Normal; (B) OVA; (C) AFSE, (F) theophylline. Goblet cell (black arrows); mucus (yellow arrows); peribronchial (red arrows); perivascular (blue arrows). OVA treatment showed increased inflammatory cells throughout the peribronchial, perivascular regions causing additional mucus secretion from hyperplastic goblet cells and significantly increased goblet cell hyperplasia in the airway. While the above factors were significantly reduced in the AFSE group similar to theophylline receiving group.

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Table 3. Goblet cell hyperplasia, peribronchial and perivascular inflammation and mucus hyper-secretion changing were showed in studied groups.

Groups	Goblet cell	Perivascular inflammation	Peribronchial inflammation	Mucus (%)
PBS	1 ± 0.2*	0.5 ± 0.1*	0.5 ± 0.1*	25 ± 5*
OVA	4 ± 0.4**	4 ± 0.2**	4 ± 0.3**	100 ± 0**
Theo	1.5 ± 0.1*	1.5 ± 0.2*	1.5 ± 0.3*	30 ± 5*
AFSE	1 ± 0.2*	0.9 ± 0.1*	0.7 ± 0.2*	25 ± 5*

PBS: Phosphate-buffered saline; OVA: Ovalbumin; Theo: Theophylline; AFSE: Aqueous fenugreek seed extract. All data are expressed as mean ± SD.* $P < 0.05$, ** $P < 0.01$

3.4. Effects of AFSE on gene expression of cytokines and mucin

In the OVA group, the mRNA expression of IL-4 (8.0000), IL-5 (2.9801), IL-13 (2.0000) and mucin (12.0141), were elevated compared to PBS group (1.00 ± 0.14, 1.00 ± 0.11, 1.00 ± 0.13 and 1.00 ± 0.09, respectively) ($P < 0.05$). In the AFSE group, there was a markedly attenuation in IL-4 (1.6245), IL-5 (1.2311), IL-13 (1.0718) and mucin (1.4870) mRNA expression compared to OVA group ($P < 0.05$) (Fig. 5). The results showed that AFSE-treated group exhibited remarkable reduction in the mRNA levels of all cytokines tested compared with those of the OVA group ($P < 0.05$).

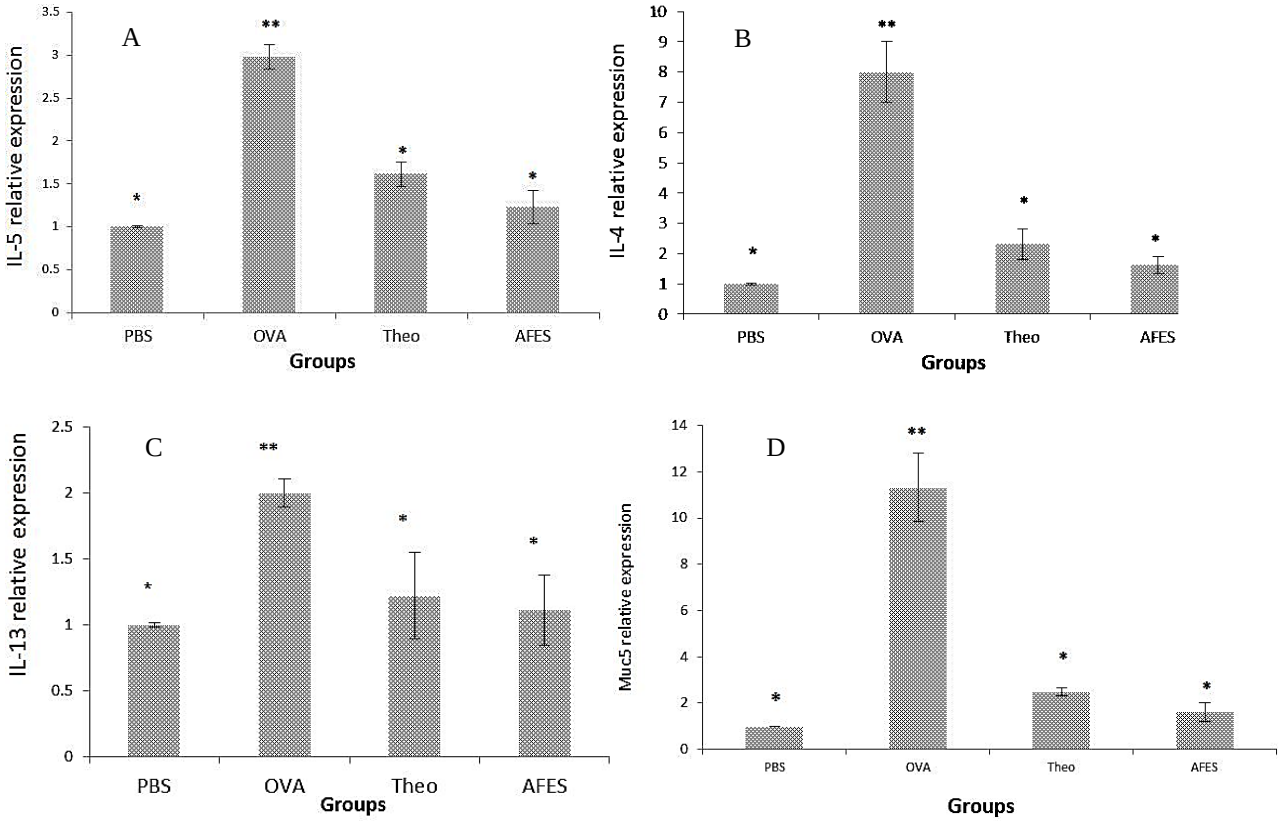


Fig. 5. Treatment with AFSE exhibited remarkable reduction in the mRNA expression of IL-4 (A), 5 (B), 13 (C) and mucin (D) compared with those of the OVA group ($P < 0.05$). AFSE had more significant effects on the reduction of the expression levels of the mentioned cytokines in comparison to theophylline. All data are expressed as mean ± SD and $P < 0.05$ was considered statistically significant level.

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4. Discussion

According to the results of the present study, AFSE decreased the concentrations of Th2 cytokines such as IL-5, IL-13, and IL33 as well as the expression levels of IL-4, IL-5, IL-13, and mucin genes in the treated groups. Also, it was shown that AFSE had anti-inflammatory effects on the lung tissues of mice with OVA-induced asthma. In addition, the efficacy of AFSE was comparable with the theophylline.

Allergic asthma is a serious inflammatory disease that results from elevated Th2-type cytokines and infiltration of inflammatory cells such as eosinophils into the lung tissue [3]. Chemical anti-asthma drugs have adverse effects and do not completely cure the disease [4]. Thus, medicinal herbs could be promising alternatives and complementary therapies that have partly fewer adverse effects to treat asthma [22]. Fenugreek seeds and leaves are used as the spices and also have been recommended for a wide variety of therapeutic purposes in traditional medicine [6].

Oral administration of *Trigonella foenum-graecum* to OVA-induced mice model of asthma decreased the amount of Th2 cytokines in their airway fluids [23]. Saponins are the basic chemical constituents of fenugreek, [24] and specifically, are characterized as immunostimulating factors [25]. It is believed that the allergic asthma is related to the imbalance of Th1/Th2 cells [26]. Cytokines IL-4, IL-5, IL-13, and IL-33 are closely related to asthma symptoms [3].

In the present study, the results showed that AFSE significantly decreased Th2 cytokines (such as IL- 5, IL-13, and IL33), inflammatory cell infiltration, and mucus hyper-secretion in lung tissues. In addition, the results showed that AFSE significantly decreased the transcription of Th2 cytokines and mucin at the mRNA levels in BALF cells. Indeed, lung histological analysis showed decreased inflammatory cells and inhibited mucus hyper-secretion. Also, mucosal thickening was frequently observed in the control group, but in the AFSE group, mucosal thickening was significantly decreased.

Previous studies have shown that *Trigonella foenum-graecum* inhibited Th2 cytokines and inflammatory cells especially eosinophils in BALF and lung homogenates and increased the Th1 cytokines, including INF-g. These findings effectively proved that *Trigonella foenum-graecum* regulated the balance of Th1/Th2 cells [23] and also fenugreek is effective in the treatment of mild asthmatic symptoms [12].

Trigonella foenum-graecum extract suppressed the secretion of IL-4 and mRNA expression of IL-4 and transcription factor GATA- 3 [6]. Our study was consistent with these results. In brief, our results showed that AFSE inhibited airway inflammation in OVA-induced asthmatic mice. Also, AFSE decreased the Th2 cytokines by decreasing the mRNA expression of IL-4, IL-5, IL-13, and mucin, which have been elevated in asthmatic mice. Based on our results, AFSE could be an alternative medication for the treatment of allergic asthma; however, further studies are necessary for evaluating the safety and effectiveness of the plant in humans.

5. Conclusion

According to our observations, AFSE could effectively suppress major inflammatory responses triggered by OVA in our established murine model of allergic asthma. AFSE exerted these modulating effects on asthma-related complications at molecular, cellular, and tissue levels, which showed by the reduction of Th2 cytokines, eosinophils trafficking, and histopathological alterations of the lungs due to

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inflammatory responses of allergic asthma induced by OVA. More interestingly, AFSE alleviating effects on allergic asthma-stimulated inflammation were non-significantly higher than theophylline. Overall, the present data suggest that AFSE may have potential beneficial activities against processes of asthma.

Author contributions

The core idea of this study came from S.S.A, M.KH, and R.CH; M.K and S. A.A contributed to the development of the study design and data collection; S.S.A and R.CH analyzed data and prepared manuscript draft; M.HM edited the manuscript scientifically.

Conflict of Interest

The authors declare that there is no conflict of interest.

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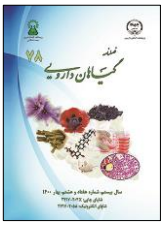
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مقاله تحقیقاتی

اثر تنظیمی عصاره بذر شنبلیله بر آسم آلرژیک در مدل موشی

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چکیده

مقدمه: در منابع طب ایرانی شنبلیله برای درمان ربو (آسم آلرژیک) و مشکلات تنفسی بسیار مورد توجه قرار گرفته است. در متون طب ایرانی به اثرات محافظتی شنبلیله در آسم اشاره گردیده است. **هدف:** هدف از این تحقیق، بررسی اثرات حفاظتی عصاره آبی بذرهای شنبلیله در کاهش سایتوکاین‌های Th2 از طریق کاهش سطح بیان mRNA آنها در مایع لاواژ برونکوآلوئولار (BALF) می‌باشد. **روش بررسی:** ۲۸ موش ماده نژاد Balb/c به چهار گروه یکسان تقسیم شدند. گروه کنترل منفی و مثبت به ترتیب بافر سالین فسفات (PBS) و اووالبومین (OVA) دریافت کردند. دو گروه باقیمانده ابتدا با OVA جهت القای آسم تیمار شدند و سپس تئوفیلین و AFSE دریافت کردند. سپس، بیان mRNA اینترلوکین‌های IL-4، IL-5 و IL-13 و موسین ۵ و نیز غلظت IL-5، IL-13 و IL-33 در نمونه های BALF اندازه گیری شد. تغییرات پاتولوژیک بافت‌های ریه موشها نیز مورد بررسی قرار گرفت. **نتایج:** نتایج تایید کرد که عصاره آبی بذر شنبلیله به طور قابل توجهی بیان ژنهای IL-4، IL-5 و IL-13 و غلظت پروتئین‌های IL-5، IL-13 و IL-33 در مایع BALF را کاهش داد. این عصاره گیاهی، نفوذ سلول‌های التهابی به بافت ریه و ترشح بالای موکوس در بافت‌های ریه را نیز کاهش داد. **نتیجه‌گیری:** عصاره آبی بذر شنبلیله می‌تواند به عنوان درمان مکمل برای آسم در تحقیقات کارآزمایی بالینی مورد استفاده قرار گیرد.

مخففها: AFSE، عصاره آبی بذرهای شنبلیله؛ BALF، مایع لاواژ برونکو آلوئولار؛ OVA، اووالبومین

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