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Original article

Effect of nettle seed on immune response in a murine model of allergic asthma

Effet de la graine d'ortie sur la réponse immunitaire dans un modèle murin d'asthme allergique

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ABSTRACT

Asthma is regarded as an inflammatory disease of the lung characterized by inflammatory mediator release, eosinophil recruitment, mucus hypersecretion and bronchoconstriction. The aim of the present study was to evaluate the protective effect of *Urtica dioica* seeds on allergic asthma. Mice were sensitized via intraperitoneal injection of ovalbumin (OVA) containing aluminum hydroxide on days 1 and 14. On days 24, 26, 28 and 30, the mice underwent challenge through inhalation of 1% OVA aerosolized using an ultrasonic nebulizer. Animals sensitized and challenged with OVA were treated with theophylline 1% while the other asthmatic group received 3.3 g.kg⁻¹ nettle seed extract by gavage 15 min after each challenge for eight consecutive days (days 23–30). At the end of this period, the mice were euthanized on day 31 for analysis of lung tissue, bronchoalveolar lavage fluid (BALF) and blood. The results showed that nettle seed significantly ($P < 0.05$) decreased the following in lung tissue: percentage number of eosinophils in blood ($1.4 \pm 0.3\%$) and BALF ($22 \pm 5\%$); Th2 cytokines including IL-5, IL-13, and IL-33 (43.12 ± 3.5 , 63.76 ± 3.3 pg/ml, 10.40 ± 0.81 ng/ml respectively); mRNA expression of IL-4 (1.56 ± 0.3), IL-5 (1.42 ± 0.1), IL-13 (1.58 ± 0.04) and Muc5 (1.83 ± 0.2); inflammatory cell infiltration (perivascular inflammation: 1 ± 0.3 ; peribronchial inflammation: 1.5 ± 0.2); goblet-cell hyperplasia (1.5 ± 0.2); and mucus hypersecretion ($25 \pm 10\%$). The findings demonstrated a protective effect of nettle seed on OVA-induced allergic asthma in mice.

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1. Introduction

Asthma is a chronic disease characterized by recurrent attacks of breathlessness and wheezing.

The symptoms occur as a result of an inflammatory process in the airways that induces bronchospasm and bronchoconstriction

[1]. Management of allergic asthma is a key problem in the industrial world. As major controllers of the immune system, cytokines play an important role in allergic asthma. Symptoms include eosinophilic inflammation, airway obstruction, mucus hypersecretion, markedly increased IgE production, airway hyperresponsiveness and smooth muscle spasm [2].

Some 235 million people are currently affected by asthma and it is common among children. According to the latest WHO estimates, released in December 2016, there were 383 000 deaths due to asthma in 2015 [3]. While inhaled corticosteroids are effective in the treatment of chronic asthma, adverse effects can result from long-term use and high-dose therapy [4]. Efforts must therefore be continued to find new anti-asthmatic treatments; medicinal plants represent potent therapeutic resources and could prove useful in the search for natural products having few side effects.

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Urtica dioica L., or nettle, is a medicinal herb belonging to the *Urticaceae* family. It has been used in Iranian traditional medicine to treat respiratory diseases [5,6]. This plant is used to treat cough and rheumatic pain [7], stomach ache [8], and liver insufficiency [9]. Nettle leaves are recommended against rheumatism, gout and asthma [10]. Some studies have demonstrated an effect of nettle leaves on allergic rhinitis [11], joint pain [12], arthritis and rheumatism [13]. In addition, immunomodulatory [14], anti-inflammatory [15], anticancer [16,17] and antidiabetic [18] properties of this plant have been reported. Further, Zemmouri et al. have shown that nettle leaves may confer protective effects against airway inflammation [19]. Alcoholic extract of nettle seeds has demonstrated a protective effect against fluoride-induced toxicity, with strengthening of antioxidative defenses [20]. A number of chemical components in nettle have been identified, including phenolic acids, scopoletin, carotenoids, anthocyanidins, lignan glucosides and flavanol glycosides [21].

However, while the effects of nettle leaves on airway inflammation have been investigated and the seeds have a stronger effect than leaves according to sources on Iranian traditional medicine [5], there have been no studies to date of the effects of nettle seeds against allergic asthma. Thus, the aim of the present study was to evaluate the anti-asthmatic effects of an aqueous extract of nettle seeds on ovalbumin (OVA)-induced allergic asthma in mice.

2. Materials and Methods

2.1. Reagents

Aluminum hydroxide (Sigma-Aldrich, USA), OVA (Sigma-Aldrich, USA), urethane (Sigma-Aldrich, USA), Mouse IL-5, IL-13 and IL-33 ELISA kits (Abcam, USA), TRIzol (Invitrogen Life Technologies, NY, USA), cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA) and theophylline (Ramopharm, Iran) were used in the present study.

2.2. Extract Preparation

Nettle seeds were purchased from an herbal supplier in Tehran and were powdered using an electrical grinder. 100 g of powdered seeds were boiled in 1000 ml of distilled water with occasional stirring until one-third of the original volume had evaporated. The mixture was filtered and lyophilized in a freeze dryer to obtain nettle seed extract (NSE).

2.3. Animals

Balb/c female mice were purchased from the Pasteur Institute of Iran. Mice aged 6 to 8 weeks and weighing 15–20 g were used. All animals were housed at $22 \pm 1^\circ\text{C}$ with a 12/12 h light/dark cycle, with relative humidity of $50 \pm 5\%$, under allergen- and pathogen-free conditions, for one week prior to the sensitization period. They also had free access to food and tap water ad libitum throughout the experimental period. All animals were handled in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

The experimental protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (No.: IR.SBMU.RETECH.REC.1396.527).

2.4. Experimental Groups

The animals were randomly divided into four groups ($n = 7$ per group) as follows:

- a group sensitized to and challenged with Phosphate Buffered Saline (PBS) (negative controls);
- a group sensitized to and challenged with ovalbumin (OVA) (positive controls);
- an OVA-challenged group treated orally with 1% theophylline (Theo) according to the experimental protocol;
- an OVA-challenged group treated orally with 3.3 g.kg⁻¹ nettle seed extract (NSE) according to the experimental protocol.

2.5. Induction of allergic asthma and treatment

The groups were sensitized by intraperitoneal (i.p.) injection of a solution containing 20 μg OVA and 50 μl aluminum hydroxide in 50 μl phosphate buffered saline (PBS) (pH 7.4) on days 1 and 14. On days 24, 26, 28 and 30, the mice were challenged for 30 min by inhalation of OVA (1% in sterile saline solution) aerosolized using an ultrasonic nebulizer (NB500, Rossmax, Switzerland). The negative control group received injections and was challenged with PBS only. Animals sensitized and challenged with OVA were treated with either 100 μl of Theo 1% or 3.3 g.kg⁻¹ NSE by gavage (p.o.) 15 min after each challenge on 8 consecutive days (days 23–30). Finally, the mice were euthanized on day 31 for analysis of lung tissue, bronchoalveolar lavage fluid (BALF) and blood (fig. 1).

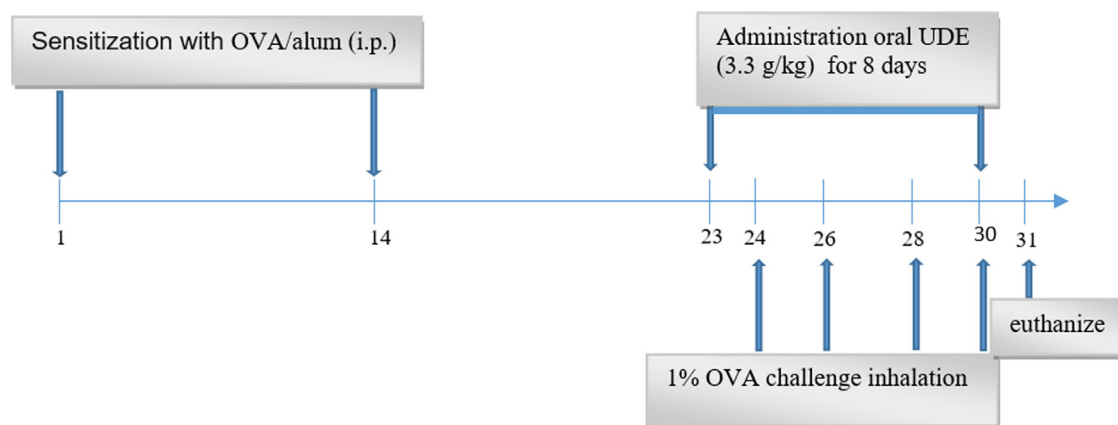


Fig. 1. The mice were sensitized via intraperitoneal injection of 20 μg OVA and 50 μl aluminum hydroxide in 50 μl PBS on days 1 and 14. They then underwent challenge by inhalation of 1% OVA solution, aerosolized in an ultrasonic nebulizer, for 30 min per day on days 24, 26, 28 and 30. The sensitized and challenged groups were treated with 1% Theo or 3.3 g.kg⁻¹ NSE on 8 consecutive days (days 23–30). Finally, the mice were euthanized on day 31 for analysis of lung tissue, BALF and blood.

2.6. Collection of BALF

Following anesthesia of the mice with urethane, BALF was collected via tracheal cannulation and the samples were stored at -70°C .

2.7. Cytokine quantification

Levels of IL-5, IL-13 and IL-33 in BALF were determined using an ELISA kit (Abcam, USA), in accordance with the manufacturer's instructions.

2.8. RNA extraction and quantitative real-time PCR

Total RNA was isolated from BALF cells using TRIzol. RNA was then reverse-transcribed to produce cDNA using a First Strand cDNA Synthesis Kit (Maxima First Strand cDNA Synthesis Kit). Quantitative PCR to measure mRNA expression was done and analyzed using a Rotor-Gene SYBR Green PCR Kit and a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany). The primer sequences are shown in Table 1. The latter were obtained from previous studies with BLAST search being performed using the BLAST and IDT OligoAnalyzer (version 3.1) to confirm their specificity [22].

2.9. Histological evaluation

On day 31, after euthanasia of the mice, the lungs were isolated and fixed by immersion in 10% neutral buffered formalin and then embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) to enable observation of the effects of NSE extracts on changes in lung histology following OVA exposure in mice. The ratio of mucus production was determined in two separate examinations by visually scoring the intensity of PAS stain in 10 randomly selected microscopy fields on histological sections at $400\times$ magnification by two pathologists. The absence of any observable PAS stain was scored 0 and a low level of Periodic acid–Schiff (PAS) stain in negative-control histological sections was scored between 0.0 and 0.5 by two pathologists. Moreover, the number of goblet

cells (PAS-positive cells) per 100 epithelial cells was determined by two pathologists in several randomly selected microscopy fields on histological sections at $400\times$ magnification. The number calculated was the Goblet Cell Index (GCI). The GCI was classified using 4 scores as follows: score 0: $\text{GCI} < 5\%$, score 1: $5\% \leq \text{GCI} < 25\%$, score 2: $25\% \leq \text{GCI} < 50\%$, score 3: $50\% \leq \text{GCI} < 75\%$, score 4: $75\% \leq \text{GCI} \leq 100\%$. Eosinophils (Eos) were examined in histological sections of lung tissue at $1000\times$ magnification by two pathologists with five repeats per pathologist. Absence of Eos or presence of only few Eos was scored 0, an incomplete layer was scored between 0.1–1, one complete layer of peribronchial/perivascular Eos was scored 1, the presence of two complete layers was scored 2, the presence of three complete layers was scored 3, and the presence of more than three complete layers of Eos was scored 4. Photomicrographs were taken with an Olympus B $\times 50$ microscope equipped with a Leica DFC 320 digital Camera [22].

2.10. Statistical analysis

Values were expressed as mean $\pm$ SD and analyzed using one-way analysis of variance (ANOVA) followed by a Newman–Keuls test using SPSS software, version 17.0. The results were considered statistically significant for $P < 0.05$.

3. Results

3.1. Effect of NSE on OVA-induced eosinophils in BALF and blood

Fig. 2 shows that for the positive control group, eosinophils in blood ($3.5 \pm 0.8\%$) and BALF ($63 \pm 8\%$) were markedly elevated compared with the corresponding values for the PBS-challenged negative control group in blood ($0.5 \pm 0.1\%$) and BALF ($5 \pm 2\%$). The eosinophil counts were significantly ($P < 0.05$) decreased by NSE in blood ($1.4 \pm 0.3\%$) and BALF ($22 \pm 5\%$). No significant difference ($P < 0.05$) was found in the number of eosinophils for the asthmatic group treated with NSE compared to Theo group in blood ($1.6 \pm 0.4\%$) and BALF ($19 \pm 4\%$).

Table 1
Primer sequences.

Gene Symbol	5'-3'	Sequence (5'-3')	Length (bp)	Amplicon Size (bp)
IL-4	Sense	AGATCATCGGCA TTTGAACG	21	67
	Antisense	TTTGGCACATCCATCTCCG	19	
IL-5	Sense	ACATTGACCGCCAAAAAGAG	21	62
	Antisense	ATCCAGGAAGTGCCTCGTC	19	
IL-13	Sense	GGTCCACACAGGGCAACT	18	43
	Antisense	AATAAGATCAAGAAGAAATGTGCTCAA	27	
Muc5	Sense	CAGGACTCTCTGAAATCGTACCA	23	128
	Antisense	AAGGCTCGTACCACAGGGA	19	

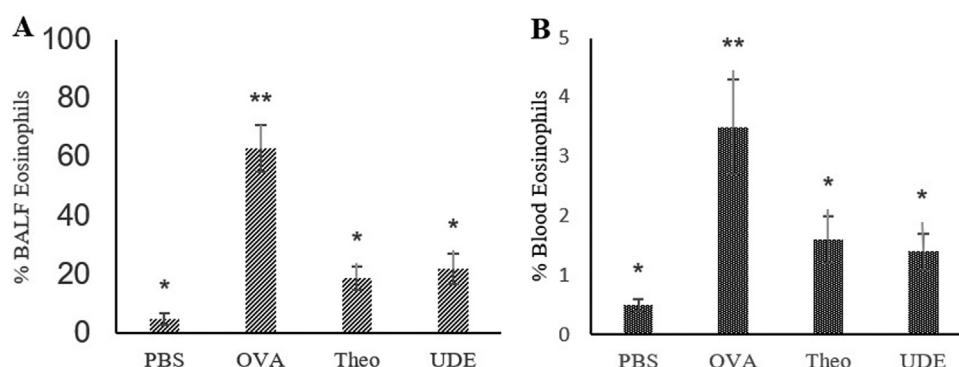


Fig. 2. Effect of treatment on eosinophils in BALF (A) and blood (B). Percentage eosinophils are shown as mean $\pm$ SD for 7 animals per group ($P < 0.05$).

3.2. Effect of NSE on Th2 cytokine levels in BALF

As shown in Fig. 3, OVA-challenged mice presented significantly elevated levels of IL-5 (109.47 ± 5.1 vs. 20.98 ± 3.1 pg.ml⁻¹), IL-13 (159.20 ± 3.8 vs. 52.23 ± 2.9 pg.ml⁻¹) and IL-33 (25.16 ± 0.9 vs. 3.02 ± 1.1 ng.ml⁻¹) in BALF compared with the negative control group ($P < 0.05$). The increases in levels of IL-5, IL-13 and IL-33 in the asthmatic group treated with NSE (43.12 ± 3.5 , 63.76 ± 3.3 pg.ml⁻¹, 10.40 ± 0.8 ng.ml⁻¹, respectively) and Theo (62.38 ± 9.9 , 96.65 ± 3.8 pg.ml⁻¹, 10.18 ± 1.9 ng.ml⁻¹, respectively) were significantly reduced ($P < 0.05$). The groups treated with NSE showed similar levels to the control group (PBS), demonstrating an anti-inflammatory effect.

3.3. Effects of NSE on gene expression of cytokines and mucin

In the OVA group, mRNA expression of IL-4 (8.00 ± 2.1), IL-5 (2.98 ± 0.1), IL-13 (2.00 ± 0.1) and Muc5 (12.00 ± 1.4) was significantly increased compared to the PBS group (1.00 ± 0.1 , 1.00 ± 0.01 , 1.00 ± 0.02 and 1.00 ± 0.4 , respectively) ($P < 0.05$). In the oral NSE group, there was a marked attenuation in mRNA expression of IL-4 (1.56 ± 0.3), IL-5 (1.42 ± 0.18), IL-13 (1.58 ± 0.04) and Muc5 (1.83 ± 0.2) compared to the OVA group ($P < 0.05$). The results show

that NSE extract significantly ($P < 0.05$) lowered the elevated levels of mRNA expression of IL-4, IL-5, IL-13, and Muc5 (Fig. 4).

3.4. Effects of NSE on lung histopathology

Histological analysis of tissue sections (Fig. 5 and Table 2) demonstrated that sensitization and challenge with OVA promoted considerable peribronchial and perivascular infiltration of inflammatory cells. No obvious inflammation was observed in the lungs of mice in the negative control group. NSE caused remarkable attenuation of perivascular and peribronchial infiltrates at the 3.3 g.kg⁻¹ dose. The OVA-exposed group exhibited a marked increase in mucus hypersecretion. The NSE group exhibited significantly reduced mucus production at levels similar to those found in the Theo group ($P < 0.05$). Goblet cell hyperplasia was found in the lungs of the positive control group compared with the negative control group. NSE extract significantly suppressed goblet cell hyperplasia in the mice exposed to OVA ($P < 0.05$). Theo, the standard drug used in this experiment, yielded values similar to those obtained with NSE.

4. Discussion

In this study, the protective effect of nettle seeds was demonstrated in a murine model of allergic asthma. NSE reduced the percentage number of eosinophils and was effective in reducing OVA-induced asthma-associated Th2 cytokine expression and in decreasing Th2 cytokine concentrations. NSE reduced OVA-induced mucus hypersecretion in lung tissue and goblet-cell hyperplasia in the airways, and NSE treatment of OVA-sensitized and OVA-challenged mice was as effective as Theo treatment with regard to all parameters measured.

Asthma is regarded as an inflammatory disease of the lung characterized by inflammatory mediator release, eosinophil recruitment, mucus hypersecretion and bronchoconstriction [22].

Eosinophil infiltration of lung in samples from asthma patients has also been reported, and pulmonary eosinophil count thus correlates with asthma severity [23]. We showed that in asthmatic mice, the eosinophil count in BALF and mucus hypersecretion were notably increased, while NSE treatment significantly decreased eosinophil count and mucus hypersecretion ($P < 0.05$). Further, such results have been reported with use of *Cassia occidentalis* and *Bupleurum chinense*, and are consistent with our findings [24,25].

It is well known that Th2 cell-associated cytokines, including IL-4, IL-5, IL-13 and IL-33, play an important role in the inflammatory response in asthma [26]. We showed that Th2 cell-associated cytokines were significantly elevated in mice immunized and challenged with ovalbumin ($P < 0.05$), but that NSE significantly decreased levels of Th2 cell-associated cytokines ($P < 0.05$).

In common with NSE, *Ocimum basilicum* reduced gene expression and concentrations of IL-4, IL-5, IL-13 in asthmatic mice [27]. These cytokines promote mast-cell growth, induce eosinophilopoiesis, and regulate eosinophil function [28]. IL-4 is essential to the initiation of allergic airway responses and plays a role in regulating Th2 cell proliferation and survival, as well as IgE synthesis [29].

Treatment with nettle-leaf extract reduces IL-4 concentrations in BALF to 14.3% [19], compared with an 80.5% decrease for nettle-seed extract. Nettle seeds are thus more effective than leaves. IL-13 is thought to play a more important role in the effector phase of the response and to be sufficient to induce the main signs of allergic disease, including mucus production and impairment of airway smooth muscle [29]. IL-5 is a key cytokine for induction of eosinophil maturation [30]. Nettle significantly reduced eosinophil counts and IL-5 levels ($P < 0.05$). It may thus suppress eosinophil

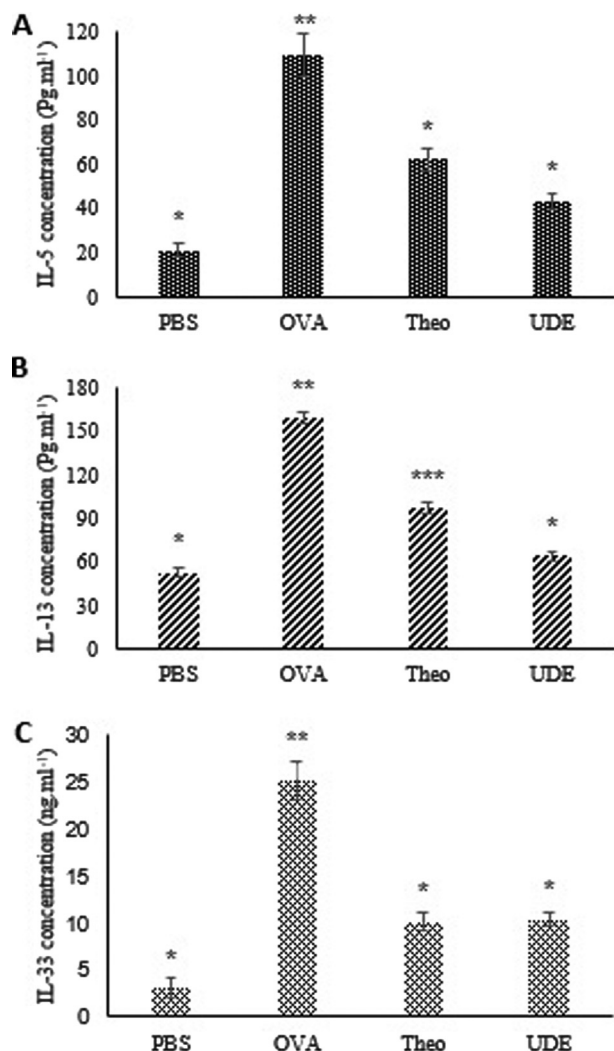


Fig. 3. Effect of NSE on IL-5 (A), IL-13 (B), and IL-33 (C) concentrations in BALF. Values are expressed as mean $\pm$ SD for 7 animals per group ($P < 0.05$).

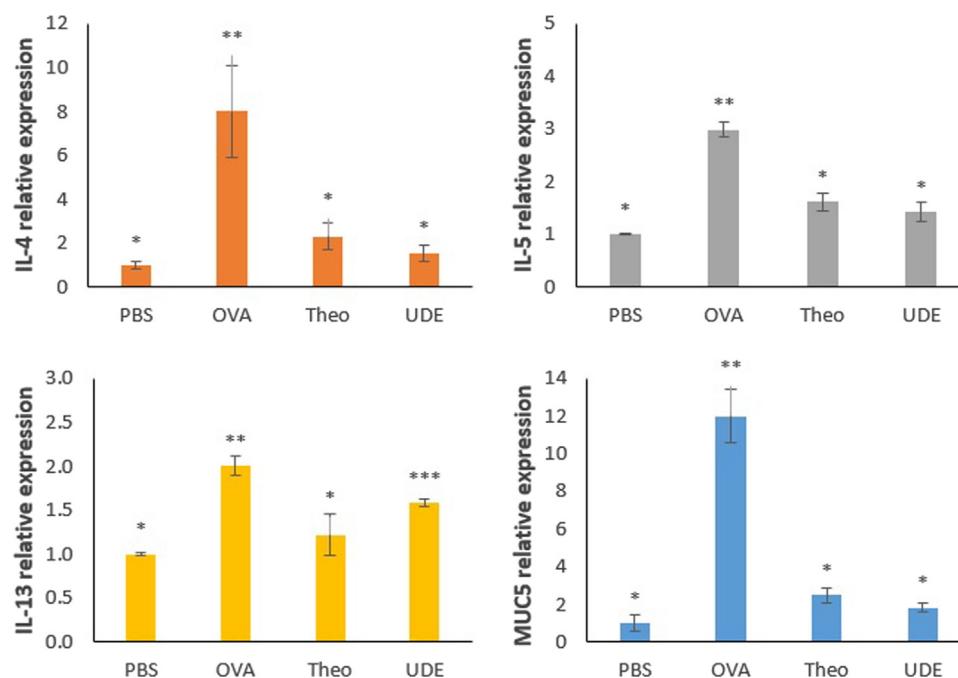


Fig. 4. Effect of NSE on mRNA expression of cytokines (IL-4, IL-5, IL-13) and muc5 in BALF determined using quantitative RT-PCR. Values are expressed as mean $\pm$ SD for 7 animals per group ($P < 0.05$).

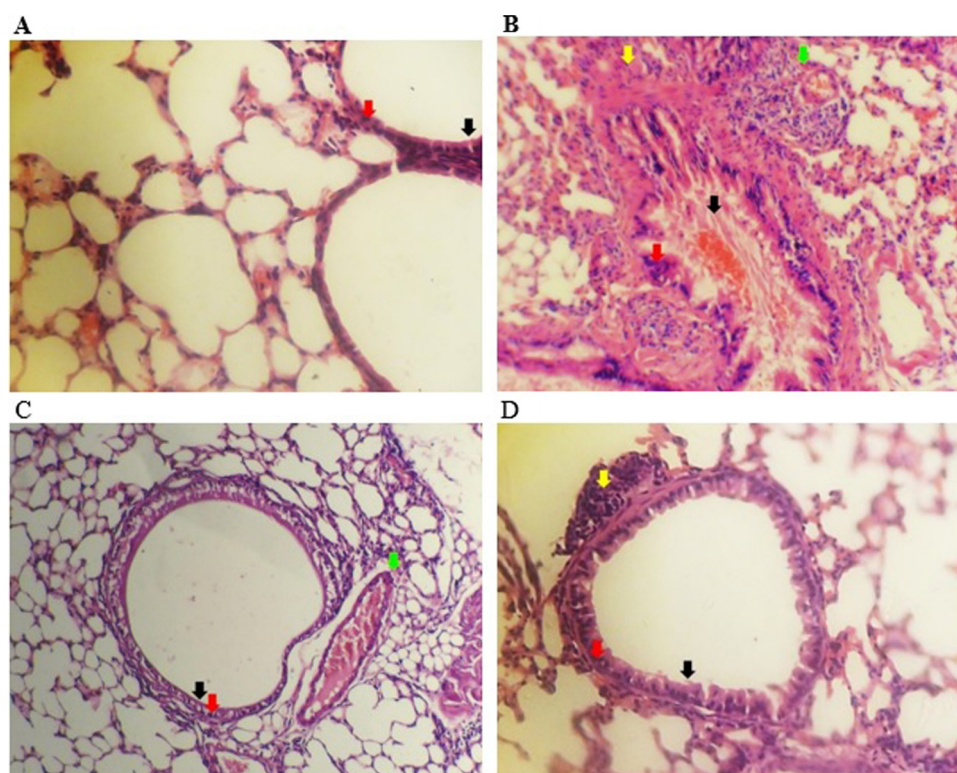


Fig. 5. Histological changes in lung sections stained with hematoxylin-eosin. A: PBS; negative control group challenged with PBS; B: positive control group sensitized and challenged with ovalbumin; C: group sensitized and challenged with OVA and treated with 3.3 g.kg⁻¹ nettle seed extract (NSE); D: group sensitized and challenged with OVA and treated with 1% theophylline (Theo). Goblet-cell, mucus, peribronchial inflammation and perivascular inflammation in sections are indicated by red, black, yellow and green arrows, respectively.

maturation, thereby alleviating inflammation and asthma. These results are in agreement with those of Xu et al. [25].

These findings further support our results that nettle extracts ameliorate allergic asthma by suppressing Th2-mediated cytokines. Consequently, we believe that nettle may also be able to play a therapeutic role in Th2 cell-mediated immune diseases.

Morphological changes in epithelium (including goblet-cell hyperplasia and mucin hypersecretion) and an increase in respiratory blood vessels (referred to as airway remodeling) occur in allergic asthma [31]. Histological analysis of pulmonary tissue sections showed that sensitization and challenge with OVA promoted a significant increase in peribronchial and perivascular infiltrates

Table 2
Histological analysis of lung tissue.

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*
NSE	1.5 ± 0.2*	1 ± 0.3*	1.5 ± 0.2*	25 ± 10*

The mice were sensitized and challenged with OVA (positive controls) or else challenged with PBS (negative controls). The groups sensitized and challenged with OVA were treated with 1% theophylline (Theo) or 3.3 g.kg⁻¹ nettle seed extract (NSE). Changes are shown in goblet-cell hyperplasia, peribronchial and perivascular inflammation, and mucus hypersecretion. Values are expressed as mean ± SD for 7 animals per group (*: $p < 0.05$, **: $p < 0.01$)

($P < 0.05$), while significant reductions were observed in these infiltrates and in mucus production in the group treated with NSE ($P < 0.05$).

Theophylline (a phosphodiesterase inhibitor), the standard drug used in all experiments, showed similar results to NES. Although theophylline (a phosphodiesterase inhibitor) is a commercially available drug, associated toxicity is a frequent clinical problem since life-threatening cardiovascular and neurologic toxicity can occur [32]. NSE may thus be a good candidate for clinical trials.

We concluded that nettle seed ameliorates allergic asthma, as evidenced by the reduction in Th2 cytokines, mucin, inflammatory cell infiltration, and mucus hypersecretion in lung tissue. Consequently, this plant may possibly constitute an accessible natural source of therapy of allergic asthma. The available literature contains scant information about the constituents of nettle seeds. Further experiments have been proposed to elucidate the mechanism of action.

Disclosure of interest

The authors declare that they have no competing interest.

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