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Evaluating the Immunoreactivity of *Ailanthus Altissima* (the Tree of Heaven) Pollen Extract in Atopic Patients

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ABSTRACT

IgE-mediated hypersensitivity reaction to pollens is a common health problem in atopic patients. In this regard, the assessment of the allergenicity of highly pollinating plants would be demanding. Based on the increment of *Ailanthus altissima* (*A. altissima*) tree in some parts of Iran and considering its probable role in respiratory allergy, in this study, we aimed to investigate its IgE-immunoreactivity and in diagnostic applications.

One hundred and twenty-five allergic rhinitis patients who were diagnosed as high IgE responders and demonstrated seasonal rhinitis or rhinoconjunctivitis, as well as 20 healthy controls (HCs) with no allergic symptoms, were enrolled in this study. Total protein extract was prepared from *A. altissima* pollens and subjected to quality control experiments and finally used in ELISA and western blotting studies.

Approximately 24% of the atopic patients (30 from 125) showed positive immunoreactivity to *A. altissima* extract. The median (IQR) of absorbance (450 nm) of the specific IgE against *A. altissima* pollen extract in HCs and positive groups were 0.33 (0.28-0.42) and 0.59 (0.36-0.79), respectively (p<0.001). Receiver operating characteristics (ROC) curve analysis of the specific ELISA results, revealed a cut-off value of 0.46 and a sensitivity of 70% and specificity of 100%. Western blotting with the sera positive cases revealed that the main immunoreactive proteins range from 10 to 70 kDa.

This study revealed that some of *A. altissima* pollen proteins ranging from 10 to 70 kDa show IgE-reactivity in atopic patients and may play a role in their allergic reaction symptoms.

Keywords: Allergy; Atopy; *Ailanthus altissima*; ELISA; IgE-reactivity; Western blotting

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INTRODUCTION

It is believed that the emerging improvements in the lifestyle of modern societies, especially in terms of good hygiene and clinical health care, have increased the incidence of hypersensitivities. The spectrum of type I allergic reactions ranges from mild symptoms such as seasonal rhinorrhea and sneezing to life-threatening conditions such as anaphylaxis. Although those symptoms are usually mild, they tend to become chronic and may interfere with the quality of life. Hence, characterization of the causative molecules could help in diagnostic and treatment steps.[1-3] Moreover, the application of allergen immunotherapy, as a specific approach for management of allergic reactions requires identification and characterization of the allergens and production and purification of a standard form of those proteins in a semi-industrial scale. Once a standard form of the target allergen is prepared, it might be used whether as part of a diagnostic tool or as a tolerance inducing agent in immunotherapy protocols.[4]

Plant pollens are regarded as common inhalant allergens and due to their scattering nature may be found everywhere.[5] Many reports emphasize the cross-reactivity of allergens from various pollen sources, confirming that the atopic patients who are initially sensitized with a specific pollen allergen, may be sensitive to pollens from other species, too.[6] In this regard, plants such as *Ailanthus altissima* (*A. altissima*) that have high pollination potency should be further considered. This tree is native to China, however, based on its fascinating appearance as an ornamental plant (as indicated by its common name, the tree of heaven), as well as its adaptability to many climate conditions, has been distributed intercontinental to different parts of the world and nowadays can be found almost anywhere from Asia to America.[7]

Some reports point out to the allergenicity of the pollens of *A. altissima*.[8-11] In 2016 Mousavi et al reported type I hypersensitivity to *A. altissima* in a 31-year-old woman, explaining strong serum immunoreactivity of the patient with the 42 and 52 kDa proteins of the pollen extract.[11] Later on, in a proteomics study, 13 immunoreactive protein spots were characterized using two-dimensional immunoblotting of the pollen extract with sera from asthmatic patients. They concluded that this plant might contain several allergic proteins, from which enolase and pectatelyase might be the most prominent ones.[12] However, it seems that the proposed allergenic proteins in this study are not merely specific to *A. altissima* pollens and could be found in other well-known allergenic sources and might augment allergic symptoms in atopic patients. Given the vast distribution of *A. altissima* in different parts of Iran as a common ornamental tree, and according to a previous study regarding the distribution of common plant aero-allergens in Iran, including *A. altissima*;[10] we got fascinated to assess the clinical relevance of *A. altissima* pollen extract in terms of IgE-immunoreactivity in atopic patients. Moreover, no clinical testing on atopic patients has been conducted regarding the allergenicity of *A. altissima* pollen extract, so far.

MATERIALS AND METHODS

Patients Selection

One hundred-twenty-five allergic patients with seasonal rhinitis or rhinoconjunctivitis attending in 2016-2018 at the allergy clinic of Rasul e Akram general hospital, Tehran, Iran, who stated a positive history of the mentioned symptoms and showed a positive (wheal greater than 3 mm) skin prick test (SPT) to a set of common local aeroallergens, were included in this study by filling a consent form following a physical examination by an expert clinician. Patients with asthma and other medical complications were excluded from the study to merely focus on commonly observed symptoms. Twenty non-allergic and healthy individuals were also included as negative control (Table 1). Sera were collected from all participants and kept at -40°C until use. For the healthy control group, sera were pooled and subsequently used as the negative control. This research was in compliance with the Ethics Committee acceptance in Iran University of Medical Sciences (IR.IUMS.REC1395.9211126203).

Pollen Gathering

The male flowers of local *A. altissima* trees were picked from April to September in 2016 and 2017 in Tehran, Iran. The anthers were removed and meshed through a 250-micron mesh so as to release the pollens. The purity and uniformity of pollens were inspected by microscopic examination and acceptable batches (with purity higher than 98%) were stored at -20°C.

Protein Extraction

The pollens were defatted and used for total protein extraction. In brief,0.5 gram of the pollen was suspended in ethyl-ether at the ratio of 1:10 and following an initial 1-hour shaking, passed through a filter membrane (Whatman 3) to discard. The collected pollens were then re-suspended in a fresh defatting solution at the same condition and shaken at 4°C, overnight. Following another filtration step, the defatting solution was discarded and the pollens were collected, dried and suspended in 5 mL of 150 mM phosphate-buffered saline (PBS) pH 7.2 and was shaken overnight. We also took advantage of a brief sonication step for better releasing of the protein contents. After extensive centrifugation, the supernatant was dialyzed against 20 mM potassium phosphate buffer, pH 7.2, and then filtered through a 0.22 µm filter membrane. The quality and quantity of the protein contents were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford's colorimetric methods, respectively. Usually, the resulting concentration of the total protein in the extract was approximately 6 mg/mL which regarded as suitable for IgE-reactivity analysis.

Indirect ELISA

The ELISA technique was used to determine IgE-reactivity against *A. altissima* proteins. In brief, wells of the Maxisorb ELISA plates (Nunc, Denmark) were coated with 2 µg of the pollen total extract in 0.2M bicarbonate buffer pH 9.0, overnight at 4°C. Thereafter, the wells were blocked using 300 µL of 2% bovine serum albumin (BSA) in PBS solution for 3 hours. After washing, 1:5 diluted sera of the patients and controls were added to each well and the plate was incubated for another 3 h. After an extensive wash, 1:2000 dilution of biotinylated anti-human IgE antibody (KPL, MA, USA) in BSA 1% was added to the wells and the plate was incubated for 2 h. After washing, 1:3000 diluted HRP-conjugated streptavidin (Sigma-Aldrich, MA, USA) was added to the wells and the plate was incubated for 1 h. After an extensive wash, the H_2O_2/TMB solution was added as a substrate/chromogen. The enzymatic reaction was stopped after 15 min with 2N H_2SO_4 and the optical densities (OD) were measured by ELISA reader at 450 nm versus 620 nm as a reference filter. All incubations were performed at 37°C, except for the coating step, and all serum samples and antibody reagents were loaded at 100 µL volumes.

Western Blotting

The protein pattern of the total extract was determined by SDS-PAGE. Moreover, the resolved proteins were transferred on polyvinylidenedifluoride (PVDF) membranes (Millipore, Bedford, USA) at 0.8 mA/cm2 within 50 min using a semi-dry blotting apparatus (PeQlab Biotechnologie GmbH, Germany). After cutting the membrane into separate lanes, each lane was incubated with 2% BSA to block the empty sites. Then, each lane was incubated with 1:5 diluted sera from the patients and controls to determine the reactive bands. Afterward, the lanes were probed by 1:2000 dilution of biotinylated anti-human IgE for 2 h, followed by a washing step and addition of 1:3000 dilution of HRP-conjugated streptavidin for 1 h. Finally, the chemiluminescent substrate (Amersham™ ECL™ Select, UK) was used to capture the image of the reactive bands using the Chemi-documentation System (Labtech, FUSION FX, England).

Statistical Analysis

Graph Pad Prism was employed for the statistical analysis; data are shown as a percentage (%), mean±SD, and median (IQR). Quantitative variables were analyzed by the Mann-Whitney U test and also the receiver operating characteristic curve (ROC) analysis was used to characterize the area under the curve (AUC) and the best cut-off points of ELISA results. p values lower than 0.05 were regarded as significant.

RESULTS

In this study, 30 cases with immunoreactive sera against the total extract of *A. altissima* pollen, based on ELISA results, and 20 people as healthy controls (HCs) participated. The mean ± SD age of age in HCs and seropositive group were 34.9±9.9 and 33.6±9.6, respectively. Female to male ratio for HCs and seropositive individuals were 10:10 and 22:18, respectively (p=0.78). The median (IQR) of total IgE (IU/mL) for HCs and seropositive groups were 52 (36-67.25) and 505 (149-940), respectively (p<0.001). History of family allergy was positive for 22 (55%) of seropositive cases. Patients' positive SPT reactions to respiratory allergens are presented in figure 1 A.

Sera of the studied groups were subjected to allergen-specific ELISA and western blotting analysis. The median (IQR) of absorbance (450 nm) of the specific IgE against *A. altissima* pollen extract in HCs and seropositive cases were 0.33 (0.28-0.42) and 0.59 (0.36-0.79), respectively (p<0.001) (figure 1B). The optimum cut-off OD was 0.46 which was capable to discriminate positive sera with the highest sensitivity (70%) and specificity (100%). The AUC (confidence intervals) for positive sera versus HCs was 0.796 (0.68-0.91). The AUC and p-value were presented in figure 1C.

IgE-immunoblotting of 30 seropositive samples with *A. altissima* pollen crude extract revealed immunoreactive protein bands ranging from 10 to180 kDa, with a majority ranging within 10-70 kDa. This not only confirms the previous results but also helps to gain a better insight into the molecular features of the *A. altissima* pollens and crude extract (Figure 2).

Table 1. Demographic data for healthy controls and sera positive groups

Group	Number N	Gender Ratio (Female: Male)	Age Mean±SD	Total IgE (IU/ml) Median (IQR)	Familial history of allergy (%)
Sera positive group	30	1.2 (22:18)	33.6 ±9.6	505 (149-940)	22 (55%)
Healthy Controls	20	1 (10:10)	34.9 ±9.9	52 (36-67.25)	0 (0%)

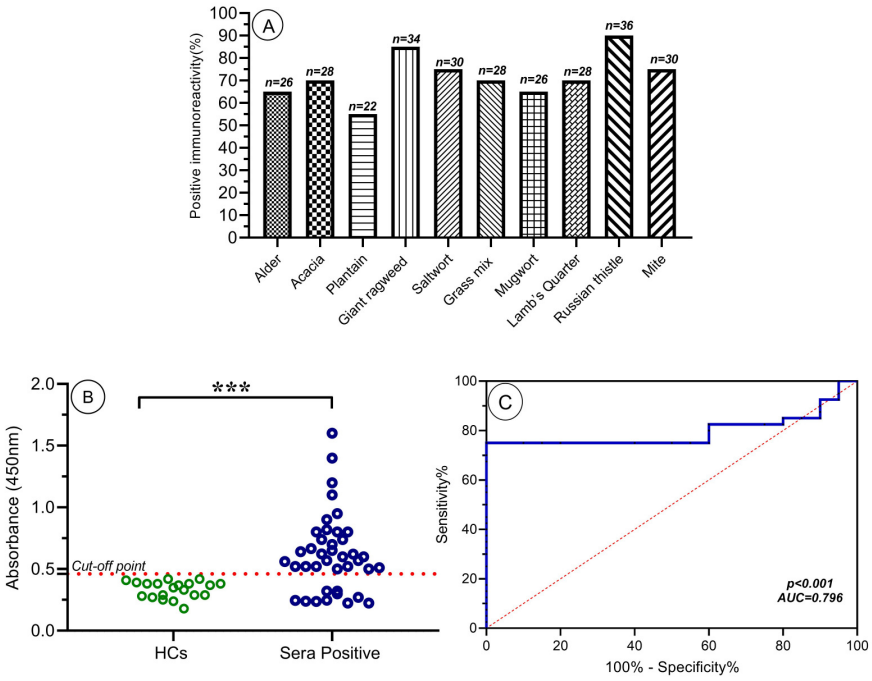

Figure 1. A. Frequency of positive skin prick test results to common respiratory allergens in patients who showed seropositivity to *A. altissima* extract in ELISA; B. Comparison of the absorbance (450 nm) of IgE antibodies against *A. altissima* total protein extract in HCs and positive cases; C. Receiver Operating Characteristic (ROC) curve analysis for IgE antibodies of seropositive groups in comparison to HCs (AUC= 0.796, p<0.001).

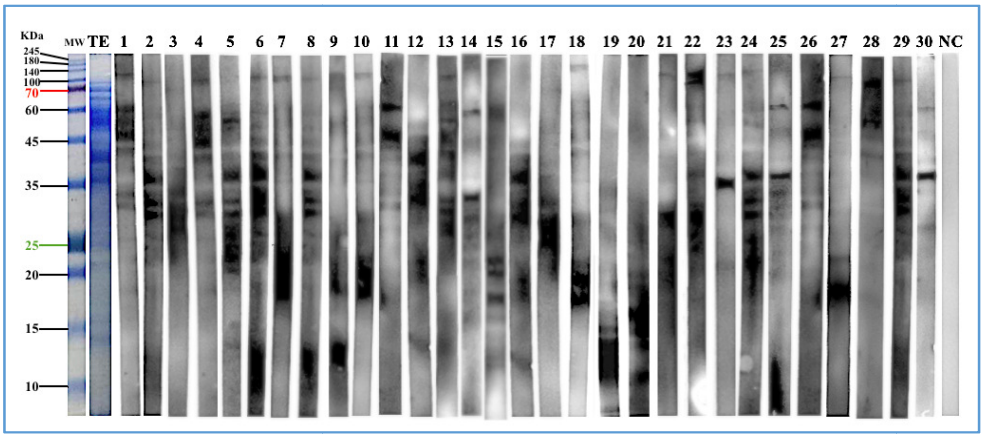

Figure 2. Western blot analysis of seropositive group using *A. altissima* total protein extract. Lane MW: molecular weight marker (Smobio, Taiwan); Lane *TE*: Coomassie Brilliant Blue stained SDS-PAGE of the crude extract of *A. altissima* pollen on 15% polyacrylamide gel; Lane 1 to 30: Each strip was first blotted with *A. altissima* pollen extract. All strips were then incubated with sera from patients showing a positive reaction in specific ELISA and subsequently, IgE-reactive protein bands were visualized; NC: negative control.

DISCUSSION

Small size and weight, confer the capability to pollens to easily scatter and stay suspended in the air and expose susceptible individuals. Pollinosis or hay fever is the common manifestation of an allergy that affects many people worldwide. Allergenicity of *A. altissima* pollens was recently proposed;[11] furthermore, there is a report of acute contact dermatitis with *A. altissima* on forearms of a patient.[13] Meanwhile, the clinical and pharmaceutical benefits of this tree such as its anti-tumor activities should not be forgotten.[14]

Despite a recent publication, none of the available studies have thoroughly addressed the molecular nature and characteristics of *A. altissima* allergens.[12] However, the allergenicity of *A. altissima* pollens and their clinical relevance was not clear to decide whether it is necessary to introduce it into clinical settings.

In this study, atopic patients were chosen based on their clinical symptoms and high IgE titer. Sera from the patients were tested for specific IgE using ELISA, and then the positive sera were further studied by western blotting. It was observed that the sera from a considerable number of patients reacted with several protein bands from *A. altissima* pollen crude extract. This observation was in line with the findings of an immunoproteomics study in which the reactivity of *A. altissima* proteins was examined by sera from allergic asthma patients.[12] Moreover, the pattern of the immunoreactivity bands obtained with the sera of asthmatic patients was different from atopic patients who participated in our study. Of course, this difference could be due to the scarcity of the number of samples.

In fact, 30 out of 125 patients, demonstrated immunoreactivity with *A. altissima* pollen extract. The sera positive cases showed high levels of Total IgE as well as a higher titer of specific IgE in ELISA tests comparing with the controls. Moreover, the cut-off value of 0.46 was capable to differentiate the sensitive patients to *A. altissima* from the non-responders. It is noteworthy to state that one of the authors of this essay showed strong type I allergy reactions to the pollens of *A. altissima* during pollen collection; most probably due to close contact with the pollens of *A. altissima*, the mentioned individual manifested severe signs of contact dermatitis on both hands and face, resembling anaphylaxis, as previously reported by Bennett et al.[13]

In addition, type I hypersensitivity reaction needs a prior exposure and sensitization of the immune system which consequently results in IgE class switching.[15] The *A. altissima* pollens possess a rough sheath which hinders their prompt release due to the mentioned physical characteristic.

Although, it is deduced from the immunoblotting results that these pollens are potentially IgE-reactive, due to having a rigid sheath they might not pose a significant risk of allergenicity to the near-by individuals.

Surely, pollens from other plants and even other organisms such as fungi may share allergenic epitopes with this tree pollens, thus cross-reactivity might affect

the way that this tree induces allergy in the people. Therefore, sensitization with other allergenic sources which shares similar components with *A. altissima*, and vice versa should be considered. So that, exposure to *A. altissima* could enhance or worsen the symptoms of an already allergic person.

It is almost accepted that this tree does not have a unique or specific allergen. In brief, Balero et al examined the allergenicity of the *A. altissima* using it's protein extract in RAST and skin prick tests and concluded that this tree possesses allergens that are not confined to this tree and are shared by other common allergen sources.[8]Mousavi et al reached a similar conclusion, too, confirming that this tree does not pose any unique allergen and only contains IgE-reactive proteins that are shared by other sources as well.[12] Moreover, it must be taken into account that the pollination of this tree occurs from April to June which is also shared by other plant species too. It could be presumed that there is a chance, those cases who are allergic to the pollens of *A. altissima* were individuals who were also in close contact with other tree pollens.[11] This raises the idea that unless being in close exposure to the pollen contents, there is not enough chance to develop an allergy to its pollens. Furthermore, the rather large pollens of this tree usually fall to the ground and don't get dispersed in the air, nor distributed by the wind. In fact, the pollination of this tree is mediated by the insect pollinators attracted to its heavy unpleasant odor.[16] Altogether, these considerations might significantly influence the risk of allergenicity by *A. altissima*.

Interestingly, in spite of some data regarding *A. altissima* allergenicity, some studies are pointing out the anti-allergy and anti-inflammatory features of *A. altissima* which must be taken into consideration. Namely, Kim et al have found some canthinone alkaloids, especially canthin-6-one, in the stem barks of *A. altissima* which are capable of inhibiting nitric oxide synthase induced by LPS in the culture of a macrophage cell line.[17] Besides, Cho et al in an in vitro study on LPS-stimulated macrophages showed that the canthin-6-one is capable to interfere with the actions of many inflammatory mediators such as inducible nitric oxide synthase, prostaglandin E2, cyclooxygenase-2, TNF-α, and downregulation of the AKT and NF-κB pathways.[18] Moreover, Kumar et al found that the barks of relative species to *A. altissima*, called *A. excelsa* and *A. roxb* show bronchodilator activity and this might be applied to the *A. altissima* as well.[19] They also proposed a significant antihistamine activity for the aqueous extract of the barks of *A. roxb*, too; which again could be held for *A. altissima*.[20] Kang et al showed that *A. altissima* possesses an anti-anaphylactic and anti-inflammatory effect through inhibiting mast cell degranulation mechanisms, namely by decreasing NF-κB *rel* A in the nucleus of mast cells and also decreasing the degradation of inhibitor of κB alpha.[21] Moreover, Jin et al showed that in an ovalbumin-induced asthma mouse model, luteolin-7-O-glucoside extracted from the *A. altissima* was able to significantly decrease the eosinophil infiltration, as well as prostaglandin E2 and IgE levels in the bronchoalveolar lavage fluid of asthmatic mice; besides, it was shown that the gene expression of hallmark cytokines of Th2 including IL-4, IL-5, and IL-13 was downregulated after treatment with the luteolin-7-O-glucoside.[22] It should also be kept in mind that regarding its proposed clinical benefits, including anti-tumor, anti-inflammatory, anti-fungal, anti-bacterial, and herbicide activity,[23-27] the *A. altissima* which was initially employed in the Chinese traditional medicine, now could pave its way in the industry of natural remedies all around the world and people should be aware of its potential ability of allergenicity. Also, it is recommended to conduct further researches on larger groups and populations and taking advantage of any possible means or technic in order to thoroughly address all aspects of *A. altissima* possible allergenicity until reaching an established and robust conclusion. This study has several limitations, including a low sample size for the immune reactive group, not specified immunoreactivity of extracted pollen, and qualitative results.

Taken together it seems that the *A. altissima* tree could be regarded as a potential allergenic source in terms of high distribution and pollination capability; particularly when intervention comes across and one is in close contact with the trees pollen. Hence the *A. altissima* could pose the risk of allergenicity for the nearby individuals. Given the vast distribution of this tree, as well as the various benefits that are attributed to this tree as a natural remedy, caution must be taken when working with this tree especially regarding the risk of contact dermatitis. Overall, considering the advantage of *A. altissima* pollen extract in immunological diagnostic and immunotherapy protocols is recommended.

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