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Immunochemical Characterization of *Ligustrum Vulgare* (Privet) Pollen Allergens: Study of Common Allergenic Plant in Iran

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ABSTRACT

Ligustrum vulgare (Privet) pollen proteins are responsible for allergies in susceptible individuals in many regions of the world. This study investigated the immunochemical characterization of Privet pollen extract and the occurrence of skin prick test reactivity to Privet and other allergenic pollen grains in allergic rhinitis patients.

All subjects experienced a skin prick test with twenty-two allergen extracts. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separated Privet pollen extract, IgE-immunoblotting, and specific ELISA procedures determined the allergenic profile on forty-five Privet allergic patients.

A positive allergic reaction to *L. vulgare* pollen extract was observed in forty-five (31.4%) out of 145 patients. Ten resolved protein fractions were found on SDS-PAGE, ranging from 10 to 80 kDa. IgE-specific antibodies interacted with several allergenic protein bands from Privet-allergic patients in the immunoblotting assay. The most significant interaction was observed in proteins with molecular weights of approximately 15, 18, 43, and 66 kDa.

Privet pollen is regarded as a potent allergen composed of IgE-binding constituents. Considering the high allergenicity of Privet pollen grains and since many countries are rich in this plant, identification and production of recombinant forms of common allergens in this species can be used for developing more efficient diagnostic, therapeutic, and preventive approaches.

Keywords: Allergens; Iran; Ligustrum; Prevalence; Pollen

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INTRODUCTION

Over the past century, increasing pollen allergy prevalence and related incidence have become a

pandemic health problem. Pollen proteins, which comprise 2.5% to 61% of their dry mass, are estimated to account for up to 40% of respiratory allergic disorders,[1] including rhinitis and asthma,[2] in nearly half a billion people in the world.

The genus *Ligustrum* includes approximately 50 species.[1] *Ligustrum* spp. are evergreen shrubs or small trees that belong to the Oleaceae family, which is one of the families rich in allergens in the world.[3,4] One of the major sources of inhalant allergens related to allergic respiratory diseases in different regions is *Ligustrum vulgare* pollen.[5] The flowering rate of Privet is so high that white blooms fully cover the plant during the flowering season and their small white flowers are collected in blooming terminal tufts.[6]

Ligustrum spp. pollen grains can lead to allergic reactions in susceptible individuals in northern Europe, South Asia, the Mediterranean area, and the United States.[5-7] They are resistant to changing climate conditions and predominantly grow in urban areas. They are widely used as either ornamental plants or hedges in parks and gardens in most parts of Iran, especially in the northern half of the country and in some areas of Tehran city and suburbs.[8]

Although allergenic features of Privet are known in Iran and other countries, a few documented studies have investigated and characterized Privet pollen extract. It is essential to identify allergenic constituents in pollen grains for allergen-specific immunotherapy designs, diagnostic procedures, and illustrate the sensitization mechanisms to different allergens.[9,10]

The present research aimed at exploring the immunochemical characterization of *L. vulgare* pollen and measuring the prevalence of sensitization to this aeroallergen and other allergenic pollens in the sample population.

MATERIALS AND METHODS

Pollen Collection and Protein Extraction

Due to the distribution of *L. vulgare* in the provinces of Iran, which were shown as potentially high-risk areas of related allergies in figure 1, we collected the plant inflorescences from *L. vulgare* with a fresh flower (Figure 2), in an urban green area of Tehran (Iran) during the *L. vulgare* pollination season (mid-April to end- June). The collected *L. vulgare* species were approved by a botanist. Samples were dried at 37°C to separate the impurities from the pollen grains and then sequentially sieved through filters with pore diameters of 300, 200, and 100 µm. To extract protein, we collected pollen particles impurities less than 5% and purities higher than 95%. Diethyl ether buffer was used several times to defatted grains. Storage of samples was done at a temperature of -20°C for subsequent analysis. Pollen grains of *L. vulgare* were stained with basic fuchsin according to a previous study.[11] *L. vulgare* defatted pollen grains were rotated (17 h, 4°C) in phosphate-buffered saline (PBS) 0.01M (pH 7.4) (400 mg: 1 mL) for protein extraction.[12] Samples were centrifuged (13,000 ×g, 4°C, 20 min), followed by dialysis of supernatant against PBS overnight. Then, lyophilization was performed and, the product was stored at -70°C for further use. The Bradford Protein Assay was used for measuring the total protein content of the pollen extract, bovine serum albumin (BSA, Sigma, USA) was used as a standard.[13]

Selection of Subjects and the Skin Prick Test (SPT)

The present research was approved by The Medical Ethics Committee (IR.IUMS.REC.1397.943). All subjects completed a written informed consent form. Allergic rhinitis cases were taken from Alavi Zanjani Charity Asthma and Allergy Clinic in Tehran from March 2019 to February 2020. Out of 145 of the patients that were skin prick tested, 45 subjects were chosen based on the result of the test (positive SPT only for Privet and with the symptoms of allergic rhinitis) and 10 healthy individuals with negative SPT and without specific IgE to *L. vulgare* pollen extract were selected as negative controls.

The SPT solution contained twenty-two common allergen extracts (GREER, USA) from nine types of trees, including Pine (*Pinus palustris*), Willow (*Salix nigra*), Birch (*Betula populifolia*), Ash (*Fraxinus excelsior*), Sycamore (*Platanus orientalis*), Elm (*Ulmus crassifolia*), Acacia (*Acacia spp*), Olive (*Olea europaea*), Privet (*Ligustrum vulgare*), four types of grasses pollen extracts such as Meadow (*Festuca elatior*), Bermuda grass (*Cynodon dactylon*), Ryegrass (*Lolium perenne ssp*), Timothy grass (*Phleum pratense*), and seven types of weeds extracts consisted of *Russian thistle* (*Salsola kali*), Lamb's Quarters (*Chenopodium album*), Pigweed (*Amaranthus retroflexus*), Plantain (*Plantago lanceolate*), Ragweed (*Ambrosia artemisiifolia*) Mugwort (*Artemisia vulgaris*), and Alfalfa (*Medicago sativa*).

Figure 1. *Ligustrum* spp. plant distribution map in Iran based on the recent (2016 and after) reports and statistics by forests and rangelands organization, jihad schools of agriculture and natural resources, Green space organization, and municipalities of 31 provinces of Iran. It is displayed in three shades of green. The color saturation gradient corresponds with high, intermediate, and low-density areas.

Figure 2. *Ligustrum vulgare* (Privet). (a, b) Privet plant flowering in growing seasons (The place of plant in the picture is the school of medicine, Iran University of Medical Sciences, Iran, Tehran). (c) Bloom (d) fruit (e, f) pollen grain without and with basic fuchsin stain under the light microscope (40x objective), respectively.

At the time of the study, these patients did not receive corticosteroids, antihistamines, or immunotherapy. For the SPT, allergen extracts were put on the forearm, followed by punching the skin with a lancet.[14] we used glycerin and histamine (10 mg/mL) as negative and positive controls. A skin wheal diameter size more than 3 mm within 20 min of administrating the test was considered positive (reactive). Serum samples from all subjects were stored at –20°C for further use. Patients were selected based on symptoms and positive SPT results. Supplementary Table is given inside Supplementary 1 (S1) shows the recorded patient's demographic data including gender, age, and allergic reactions to Privet pollen extract.

Specific IgE Assay

An indirect ELISA approach was established and optimized for evaluating levels of specific IgE antibodies against the pollen extract proteins of *L.vulgare*.[15,16] Briefly, 100 µL of *L. vulgare* pollen extract in carbonate buffer (15 mM Na2CO3 and 35 mM NaHCO3, pH 9.6, Sigma, USA) was incubated per well of 96-well microtiter plate, Maxisorp, for overnight at 4ºC. Then, each well was blocked with 150 µL of BSA 2% at 37ºC for 1 h followed by incubation with serum (100 µL) at ambient temperature for 3 h. After that, each well was incubated with a biotinylated goat anti-human IgE antibody (1:4000) (Seracare, USA) at room temperature for 2 h. Washing was performed five times with TPBS. A dilution (1:20000) of horseradish peroxidase-conjugated streptavidin (Seracare, USA) (100 µL) was added to each well, which was then loaded with 100 µL of the chromogenic substrate, following five washes. An ELISA reader was used for reading the optical density (OD) of the plate at 450 nm wavelength. For negative control, sera from 10 control cases were utilized. It was considered that IgE-specific antibodies were positive in samples that their ODs at 450 nm wavelength was three times higher than the mean of the negative control (negative control mean=0.04).

SDS-PAGE

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE) and Coomassie brilliant blue staining were used for the analysis of proteins. The method of previous work was followed,[17] and proteins were mixed with loading buffer (5% (v/v) 2-mercaptoethanol (2-ME, Sigma, USA), Tris-Hcl (pH:6.8, Merck, Germany), SDS 1% (Sigma, USA), Bromophenol blue (Sigma, USA), Glycerol Sigma, USA),) and then added into 15% acrylamide separation gels (after heating at 90°C, 4 min), under non-reducing and reducing circumstances. Separate gels were used for reduced and non-reduced samples (loading buffer without 2-ME). Coomassie Brilliant Blue R250 (Sigma, USA) was used for staining the total soluble protein bands so that an electrophoretic profile of *L. vulgare* can be obtained.

IgE-immunoblotting Assay

Then, was transferred total protein profile in SDS-PAGE to the PVDF membrane (Immun-Blot, Bio-Rad) using Tris, glycine transfer buffer in a wet transfer cell (Bio-Rad, USA) at 300 mA for 25 min. After transferee, membranes were blocked by incubating in BSA (2%) overnight.

A three-hour incubation was imposed on PVDF membranes with sera (45 sensitive patients') diluted to 1:5 in PBS for immunodetection. The membranes were washed three times washing in PBS -Tween 20, and then they were incubated for another 3 h with biotinylated Goat anti -Human IgE (Seracare, USA) diluted to 1:4000. Membranes were rewashed and incubated for 1 h with streptavidin-HRP (Seracare, USA) diluted to 1:20000. By incubation of the membrane in peroxidase substrate for enhanced chemiluminescence by HRP substrate luminal (Parstous Co, Iran) and imaged by a G-Box gel documentation system (Syngene, UK), the enzyme-substrate reaction was completed. Then, a comparison of images against those from Coomassie-stained gels was made.

RESULTS

Patients and SPTs

A total of one hundred forty-five allergic rhinitis patients participated in this study. In allergic rhinitis, the cardinal clinical manifestations include sneezing, itching, runny nose, stuffiness, watery, itchy eyes, headaches, swollen eyelids, wheezing, and cough.

Positive SPT to minimum one of the pollen aeroallergens was observed in 145 cases (61 females and 84 males, with a mean age of 28.3±3.11 years). Ten negatives (4 females and 6 males, with a mean age

of 27.5±2.37 years) were the test control. Among the 22 pollen extracts studied for testing, the most common allergenic weeds, grasses, and trees were *Russian thistle* (*Salsola kali*) (57.2%), Bermuda grass (*Cynodon dactylon*) (35.9%), and Sycamore (*Platanus occidentalis*) (49.7%), respectively (Figure 3).

Out of 145 positive patients, 45(31.4%) showed positive allergic reactions to *L. vulgare* pollen extract (Table 1). The mean diameters of reactive positive wheal sizes were 8.9±3.15. Table 1 shows the patient's clinical characteristics, specific IgE values, and skin prick test reactivity to *L. vulgare* extract. The particular IgE values to *L. vulgare* pollen extract were surveyed in 145 individual patients' sera. 45 individuals (positive SPT patients to Privet pollen extract) had detectable particular IgE levels responding to *L. vulgare* pollen proteins (Table 1).

Protein Profile of *L. vulgare* Pollen

The SDS-PAGE method was used to separate the protein composition of *L. vulgare* extract. Then, the sample was stained with Coomassie Brilliant Blue staining (Figure 4). The reducing SDS-PAGE conditions indicated at least ten protein bands with a molecular weight range from10 to 80 kDa in *L. vulgare* pollen extract. The highest molecular weights of reactive bands were around10, 15, 18, 43, and 66 kDa (Figure 4).

The rest of the bands showed molecular weights of about 20, 26, 30, 36, and 85 kDa. Higher intensity bands were observed in the regions of about 10-20 and 40-45 kDa.

Additionally, SDS-PAGE separation of *L. vulgare* pollen extract in non-reducing circumstances showed six protein bands with molecular weights around 10-50 kDa (figure 4).

IgE- binding Profile of *L. vulgare* Pollen Extract

The IgE interaction was evaluated for each separate protein band. *L. vulgare* pollen extract was assessed by using immunoblotting and sera from Privet allergic cases. Figure 5 indicates these specific IgE binding protein bands probed with sera from all the forty-five (31.03%) patients with Privet allergy.

As shown by the finding, molecular weights of the IgE antibody-reactive protein bands were 10 to 80 kDa. Proteins that had molecular weights of approximately 15, 18, 43, and 66 kDa presented the highest interaction. Molecular weights of other IgE antibody-reactive protein bands were about 10, 25, and 35 kDa, and there was not any interaction between any protein bands and the pooled sera of negative controls in the immunoblotting assay.

In terms of binding intensity and frequency of identification by the sera, the major IgE binding bands parallel the major protein-stained sites in MW are as around 15 (44.4%), 18 (40%) around 43 (42.2%), and 66 kDa (60%) (Figure 6).

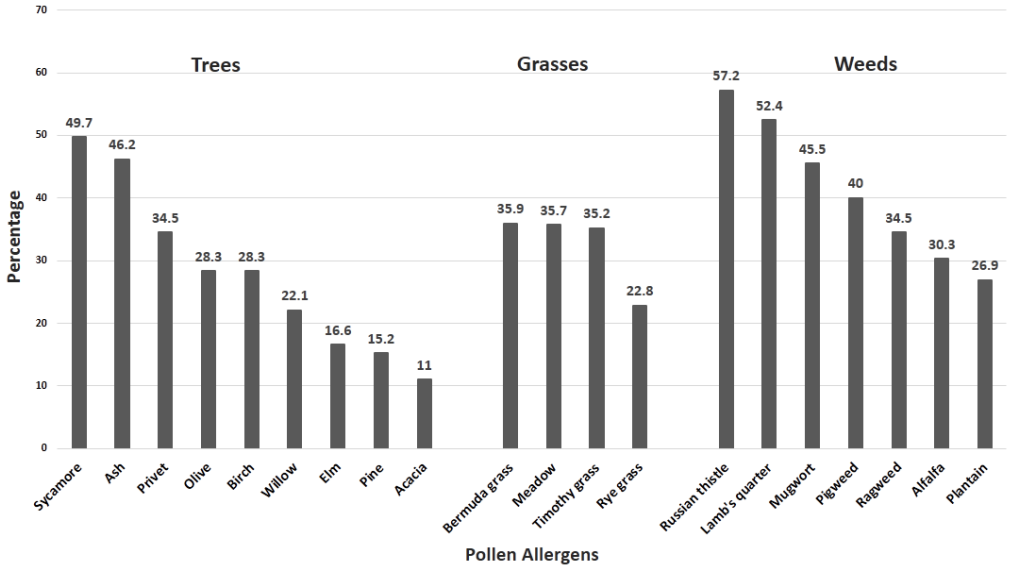

Figure 3. Frequency of positive allergenic pollen extracts skin prick tests on allergic rhinitis patients (145 cases). Allergic rhinitis cases were taken from Alavi Zanjani Charity Asthma and Allergy Clinic in Tehran (March 2019 - February 2020).

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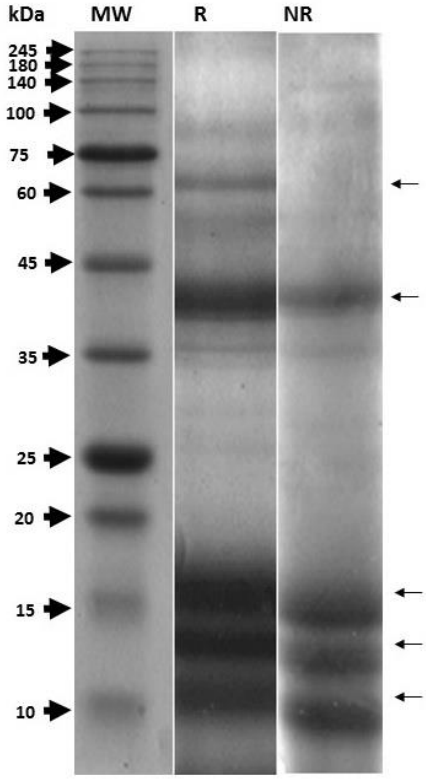

Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *L. vulgare* pollen extract. Coomassie brilliant blue-stained SDS-PAGE of the crude extract of *L. vulgare* pollen extract under reducing (10-80 kDw, the highest molecular weights of reactive bands were around 10, 15, 18, 43, and 66 kDa) and non-reducing (around 10-50 kDa) circumstances on 15 % acrylamide gel. MW, molecular weight marker (SMOBiO PM2600); NR, non-reducing condition R; reducing condition.

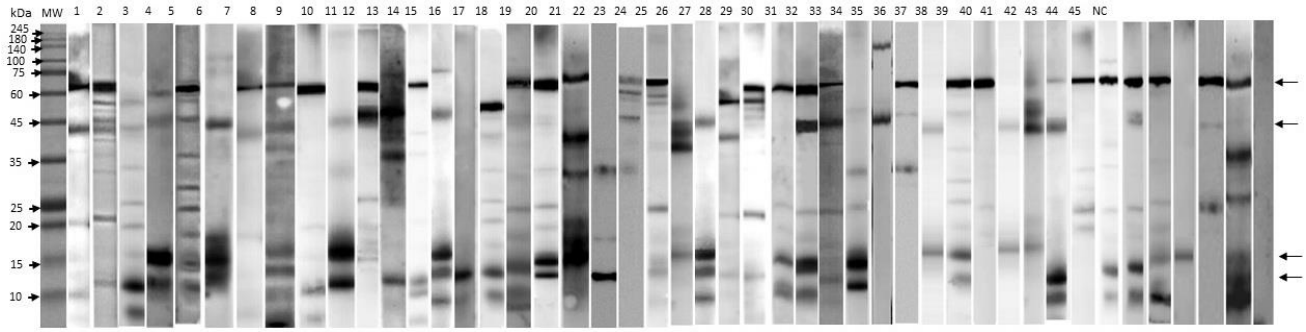

Figure 5. Immunoblotting of *L. vulgare* pollen extract (with reducing SDS-PAGE). Firstly, strips were blotted by *L. vulgare* pollen extract. Then, they were then incubated with sera of allergic cases (1 to 45), followed by probing for IgE reactive protein bands. Proteins that had molecular weights of approximately 15, 18, 43, and 66 kDa presented the highest interaction. NC, negative control. MW, low molecular weight.

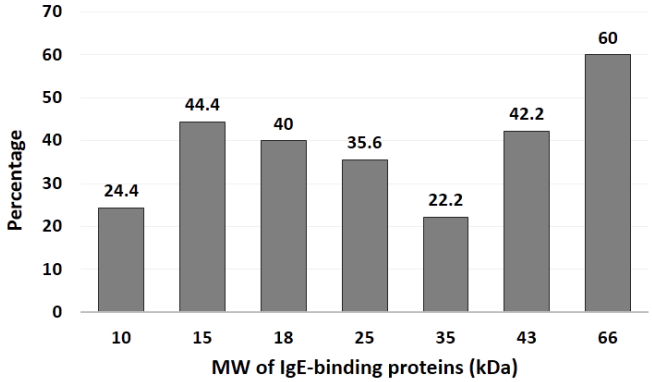

Figure 6. Recognition frequencies of the IgE -binding constituents identified in *L. vulgare* pollen extract. The frequencies were obtained from the results of the examination of all 45 sera.

DISCUSSION

In this research, the immunochemical characterization of *L. vulgare* pollen extract, a part of the Oleaceae family, was performed to recognize the IgE-binding proteins of *L. vulgare* allergic patients. The prevalence of sensitization to allergenic pollen was also determined in patients with allergic rhinitis.

The results showed that 31.4% (45/145) of patients had a positive allergic reaction to *L. vulgare* pollen extract. The SDS-PAGE of *L. vulgare* pollen extract demonstrated ten main protein fractions that ranged from 10 to 80 kDa. Moreover, the IgE-immunoblotting assay revealed that the major-specific IgE-binding proteins had molecular weights of around 15, 18, 43, and 66 kDa.

Based on our study, 145 subjects had a positive SPT to a minimum of one allergen. Following previous studies,[18-21] the most common allergenic pollen belonged to weeds family members *Salsola kali* (57.2%) and *Chenopodium album* (52.4%) which are botanically close (the Amaranthaceae/Chenopodiaceae family) and have an excessive degree of cross-reactivity, followed by trees, and grasses.

A prevalence rate of 34.5% was found for sensitivity to the Privet in our study. To our knowledge, there are no prior studies on the prevalence of allergic reactions to Privet in Iran. In Argentina, the most common cause of allergic diseases among children is an allergy to *Ligustrum* pollen (61.5%).[22] Furthermore, the prevalence of sensitization to Privet was 36.6% in Mexico City.[23] The role of *Ligustrum* species in hypersensitivity has been miscalculated it is because the large dimension of its pollen grains prevents long-distance dispersion, and the incidence of allergic disorder through *Ligustrum* allergens has been poorly investigated.

The allergenic prevalence of some tree pollens, including Sycamore (49.7%) and Ash (46.2%), were similar to previous studies in Iran[3,18,19] and other reports from Spain,[24] Turkey,[25] and Saudi Arabia.[26]

Privet is a member of the highly allergenic Oleaceae family. A high prevalence of sensitivity to Privet pollen is anticipated as the plant is abundantly planted in cities. Along with other members of the Oleaceae family such as Ash, this pollen has been confirmed as an essential origin of aerial allergens in many regions, playing a key role in worsening respiratory allergies.[3-5,22,23,27] However, there are a few international studies conducted on the allergenicity of *Ligustrum* spp. pollen proteins.

Forty-five out of 145 patients showed a skin wheal with a diameter above 3 mm after the SPT. as shown in the present study, most cases with a positive SPT to *L. vulgare* showed a high level of specific IgE antibody to proteins available in *L. vulgare* pollen extract.

The reducing SDS-PAGE conditions indicated at least ten protein bands with a molecular weight range from 10 to 80 kDa in *L. vulgare* pollen extract. Furthermore, under non-reducing conditions, the SDS-PAGE of *L. vulgare* pollen extract indicated six protein bands that had molecular weights of about 10 to 50 kDa. Bonds in the protein structure are disulfide by the presence of cysteine amino acid, resulting in reducing conditions by 2ME buffer, which is implied by these distinctive band patterns under reducing and non-reducing circumstances. Because of disulfide bonds, many allergens, especially food allergens, are resistant to denaturation.[28] It has been indicated that disulfide bond degradation induces the availability of IgE-bound

epitopes in allergen molecules, helping with allergenicity and recognition in immunological processes.[17,29,30] According to these results, it can be stated that specific proteins in *L. vulgare* pollen extract show sensitivity to degradation due to exposure to a reducing condition or proteolysis. According to the studies in the literature, specific pollen proteins, like methionine synthase, break down into two fragments in reducing conditions.[31]Besides, the strong conditions and preparation of pollen extract might influence the size and amount of degraded proteins.[32]

According to immunoblotting findings, proteins that have molecular weights ranging from 10 to 80 kDa showed the highest reactivity with the IgE antibody available in sera of allergic subjects. Also, it should be mentioned that subjects with molecular weights of approximately15, 18, 43, and 66 kDa show the greatest specific IgE-reactivity. Moreover, other IgE antibody-reactive protein bands showed molecular weights of about 10, 25, and 35 kDa. Severe responses were given by 44.4% of sensitive patients to *L. vulgare* to about15kDa proteins. Previous studies on pollens of some allergenic plants, including *Salsola kali*, *Amaranthus retroflexus*, *Populus nigra*, *Fraxinus excelsior, and Acacia farnesiana,* showed that the protein bands with a weight range around 14 kDa to 15 kDa can be associated with the profilin plant family.[31,33-36] Research is currently underway to determine the nature of this allergen.

The Ole e 1-related protein family with 18 to 20 kDa, has been identified as an allergen in some of the allergenic plants, including *F. excelsior* (Fra e 1),[37] *O. europaea* (Ole e 1),[38] and *S. vulgaris* (Syr v 1).[39] Here, an 18-kDa IgE-reactive protein was also identified in 40% of the patients sensitive to *L. vulgare* pollen. Previously, this around 18-kDa protein in *L. vulgare* pollen was named Lig v 1. which is a heterogeneous protein containing 145 amino acids and two non-glycosylated (18.5 kDa) and glycosylated (20 kDa) variants.[40]

In the present study, two other important IgE-binding proteins in *L. vulgare* pollen extract were those with molecular weights around 43 and 66 kDa. In previous studies, proteins with MWs about 43 to 45 kDa have been reported from allergenic sources such as tomato, Oilseed rape, Platanus, Japanese cedar, and grasses.[6,41,42] Moreover, an IgE-binding protein about of 65 to 66 kDa was described as the main IgE-binding protein in different allergenic pollen grains belonging to various plant species.[17,21,32, 43-46]

In conclusion, the results clarify and demonstrate the *L. vulgare* pollen as a strong allergen origin with various IgE-binding constituents. The exact nature of proteins from *L. vulgare* pollen extract and the IgE cross-reactivity of these proteins with different members of the Oleaceae family and other common allergenic plants were not determined in this study. The production and identification of recombinant forms of common allergens in Privet pollen grains, and investigating the pattern of cross-reaction with other allergenic sources will lead to new diagnostic, therapeutic, and preventive guidelines in controlling allergic reactions to Privet pollen. Attempts to clone and produce major allergens from Privet pollen grains are currently underway.

CONFLICT OF INTEREST

No conflict of interest concerning the publication of this paper was declared by the authors.

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