

## Immunochemical Characterization of *Prosopis Juliflora* Pollen Allergens and Evaluation of Cross-Reactivity Pattern with the Most Allergenic Pollens in Tropical Areas

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### ABSTRACT

Allergy to *Prosopis juliflora* (mesquite) pollen is one of the common causes of respiratory allergy in tropical countries. Mesquite is widely used as street trees in towns and ornamental shade trees in parks and gardens throughout arid and semiarid regions of Iran. The inhalation of mesquite pollen and several species of *Amaranthus*/*Chenopodiaceae* family is the most important cause of allergic respiratory symptoms in Khuzestan province. This study was designed to evaluate IgE binding proteins of mesquite pollen extract and its IgE cross-reactivity with other allergenic plants.

Twenty patients with allergic symptoms and positive skin prick tests (SPT) for mesquite pollen extract participated in the study. Crude pollen extract was prepared from local mesquite trees and used for the evaluation of allergenic profiles of *P. juliflora* pollen extract by Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-immunoblotting.

There were several protein bands in mesquite pollen extract using SDS-PAGE with the approximate range of molecular weight of 10-85 kDa. The most frequent IgE reactive bands among the patients' sera were approximately 20 and 66kDa. However, there were other IgE reactive protein bands among the patients' sera with molecular weights of 10, 15, 35, 45, 55 and 85kDa. Inhibition experiments revealed high IgE cross-reactivity between mesquite and acacia.

There are several IgE-binding proteins in *P. juliflora* pollen extract. Results of this study indicate that proteins with a molecular weight of 10 to 85 kDa are the major allergens in *P. juliflora* pollen extract.

**Keywords:** Allergen; Cross Reaction; Pollen; *Prosopis*

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## INTRODUCTION

*Prosopis juliflora* (Mesquite) is a species of flowering plants belonging to the Fabaceae family which is found in warm climate in arid and semi-arid regions throughout the world. In Khuzestan province, southwest Iran, mesquite is widely used as a street tree in towns and ornamental shade tree in parks and gardens. They need little or no watering and can survive on limited rainfall. *P. juliflora* flowers twice a year, in February-March and August-September and during the period large amounts of pollen debris are deposited underneath trees. Pollen from the mesquite tree is an important source of respiratory allergy in tropical countries, including the United States,<sup>1</sup> Saudi Arabia,<sup>2</sup> South Africa,<sup>2,3</sup> Kuwait,<sup>4</sup> United Arab Emirates (UAE)<sup>5</sup> and India.<sup>6</sup>

Protein analysis of mesquite pollen revealed several components ranging from 11 to 99 kDa.<sup>7-9</sup> Furthermore, previous studies reported that different allergic components with molecular weight such as 20, 52, 59 and 66 kDa as dominant allergens of mesquite pollen.<sup>7,9-11</sup>

In Iran and other neighboring countries, the importance of *P. juliflora*, *Acacia farnesiana*, and the most allergenic members of Amaranthaceae/Chenopodiaceae family (*Salsola kali*, *Amaranthus retroflexus*, *Chenopodium album* and *Kochia scoparia*) pollens in causing respiratory allergy have also been well ascertained.<sup>3,5,12-14</sup> Moreover, there are several allergenic proteins with approximately similar molecular weight such as 14-15, 18-20, 66 and 95-97 kDa, in pollen extract of mesquite and selected plants. However, despite high prevalence of sensitivity to proteins with this similar molecular weight in these plants, there is no information about probable cross-reactivity among allergens of these common allergenic plants. Therefore, we evaluated proteins of mesquite pollen extract which are specifically reactive to the immunoglobulin E (IgE) of patients with skin test positive for mesquite and other selected plants. In addition, IgE cross-reactivity among *P. juliflora* and the selected members of Amaranthaceae/Chenopodiaceae were assessed using in vivo and in vitro experiments.

## MATERIALS AND METHODS

### Patient's Population and Skin Prick Test (SPT)

Twenty patients with respiratory allergy were

included in this study. These patients presented to the Immunology and Allergy department of Ahvaz Jundishapur University of Medical Sciences. They were asked to complete a detailed questionnaire. The patients were considered as having a history of allergy if they reported at least one ocular, nasal, or respiratory symptom to common allergens such as house dust mite, domestic animals, food, or pollen. They were also evaluated by clinical examination and SPT with mesquite, acacia and the most allergenic members of Amaranthaceae/Chenopodiaceae family. Five healthy subjects who presented with negative SPTs and no specific IgE to the *P. juliflora* pollen extract were assigned as negative controls. The Human Ethics Committee of the institute approved the study protocol with informed written consent from each participant.

SPTs were performed by an experienced nurse under physician's supervision. In this test, *P. juliflora* and the most allergenic pollen extracts (*A. farnesiana*, *S. kali*, *C. album*, *A. retroflexus*, and *K. scoparia*) were put on the patients' inner forearms and irritation of the epidermis was caused by prick method. The result was observed after 15 minutes. Next, the mean diameter of wheal reaction in every patient was measured and compared with negative (Glycerol saline) and positive (Histamine, 10mg/ml) controls. Patients with a wheal diameter >3 mm were considered positive compared to negative and positive controls, and were asked to donate a serum sample. Serum samples of patients were stored at -20°C before use.

### Preparation of Pollen Extract

Polleniferous materials were collected from *p. juliflora*'s flowers during February-April 2012 throughout Ahvaz city, a tropical region in southwest Iran with a tropical climate and a population of more than 1.4 million.<sup>12</sup> The pollen from five other selected allergenic plants was collected during flowering season.

Collection and processing of pollen materials were carefully done by trained pollen collectors. Pollen grains were separated by passing the dried materials through different sieves (100, 200 and 300 meshes) successively. The final fine powder was subjected to a purity check for pollen content using a microscope. Pollen materials with more than 95% pollen and less than 5% floral parts of the same plant were taken for protein extraction.

Pollen materials were defatted using repeated changes of diethyl ether. Pollen was extracted as described previously.<sup>15</sup> In brief, two grams of pollen

was mixed with 10ml phosphate-buffered saline (PBS) 0.01 M (pH 7.4) by continuous stirring for 18 hours at 4°C. The supernatant was separated by centrifugation at 14,000 g for 30 min, filtered and the supernatant collected. The extract was then freeze-dried. The protein content of the extract was measured by Bradford's method.<sup>16</sup>

### SDS-PAGE and IgE- Immunoblotting

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of mesquite pollen extract was performed according to Laemmli<sup>17</sup> using 12.5% acrylamide separation gels under reducing and non-reducing conditions. Reducing and non-reducing sample buffers were the same except that the final reducing sample buffer contained 2-mercaptoethanol (2-ME). The molecular masses of protein bands were estimated with Image Lab Analysis Software (Bio-Rad, USA) by comparison with protein markers of known molecular weights (Amersham Low molecular weight Calibration Kit for SDS electrophoresis, GE Healthcare, UK). Separated protein bands from the electrophoresis of mesquite pollen extract were electro-transferred to Polyvinylidene difluoride (PVDF) membranes (GE Healthcare, UK), as described earlier.<sup>15</sup> In brief, after blocking and washing, membranes were incubated with a 1/5 dilution of serum pool or individual sera from patients with mesquite allergy or with control sera for 3 hours. Biotinylated goat anti-human IgE (Nordic Immunology Co., Netherlands) (1:500 v/v in PBS) was added to the blotted membrane strips and incubated for 2 hours at room temperature. The unbound antibodies were removed from blots by washing with TPBS (PBS containing 0.05% Tween 20) and followed by incubation with 1:10,000 v/v in TPBS-HRP-linked streptavidin (Sigma-Aldrich, USA) for 1 hour at room temperature.

After several washes with TPBS, strips were incubated with Supersignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, USA) for 5 min, and proteins were then visualized by chemiluminescence using ChemiDoc XRS+ system (Bio-Rad, USA).

### Measurement of Total IgE and Specific IgE

Total serum IgE levels were measured by means of a commercially available ELISA kit according to the manufacturer's instructions (EUROIMMUN,

Germany).

To measure the levels of specific IgE to mesquite pollen in patients' sera, an indirect ELISA was developed as described previously.<sup>18</sup> Briefly, 0.1 µg of mesquite pollen extract in 100 µl carbonate buffer (15 mM Na<sub>2</sub>CO<sub>3</sub> and 35 mM NaHCO<sub>3</sub>, pH 9.6) was incubated at 4 °C overnight per well of a 96-well microtiter plate (Nunc MaxiSorp, Denmark). Each well was then blocked for 1 hour at 37°C with 150 µl of 2% BSA (Bovine serum albumin) in PBS following by incubation for 3 hours with 100 µl of serum at room temperature with shaking. Each well was then incubated for 2 hours at room temperature with 1:1000 dilution of biotinylated goat anti-human IgE antibody (Nordic Immunology Co., Netherlands) in PBS. Each incubation step was followed by five washes with TPBS. Wells were added by 100 µl of a 1:5000 dilution of horseradish peroxidase-conjugated streptavidin (Bio-Rad, USA). Following five washes, 100 µl of chromogenic substrate was added to each well and the plate was incubated for 15 min in the dark. The plate was read at 450 nm with an ELISA reader. Optical density (OD<sub>450</sub>) greater than four times the median values of the negative controls were considered to be positive.

### In Vitro Inhibition Experiments

ELISA inhibition assays were performed as described above, except that a pooled serum (1:2 v/v) from mesquite allergic patients (Nos. 2, 6, 8, 10, 12 and 18) was preincubated for one hour at room temperature with either 1000, 100, 10, 1, 0.1 or 0.01 µg of the selected pollen extract (including *A. farnesiana*, *S. kali*, *C. album*, *A. retroflexus* and *K. scoparia*) as inhibitors or with BSA as a negative control. Percentage of inhibition was calculated using the following formula: (OD of sample without inhibitor - OD of sample with inhibitor / OD of sample without inhibitor) × 100.

To study the cross-reactivity between mesquite pollen and selected allergenic plants, the IgE-immunoblot inhibition experiment was performed. SDS-PAGE resolved mesquite pollen proteins were transferred to PVDF membrane. After blocking, membrane strips were kept for 3 hours at room temperature with a mix of 100 µl of pooled sera (1:10 v/v) (from patients 2, 6, 8, 10, 12 and 18) which were preincubated with pollen extracts from the selected allergenic plants, as well as BSA (negative control).

## RESULTS

### Patients

Twenty patients, 12 males and 8 females (mean age, 30.95±11.49 years; age range 13-56 years), were included in present study (Table 1). All patients suffered from respiratory allergies and seasonal rhinitis without asthma. The patients were all positive by SPT with *P. juliflora*, *A. farnesiana*, *S. kali*, *A. retroflexus*, *C. album* and *K. scoparia* pollen extracts (Table 1). A serum pool of 5 non-allergic subjects was used as a negative control.

### SPT

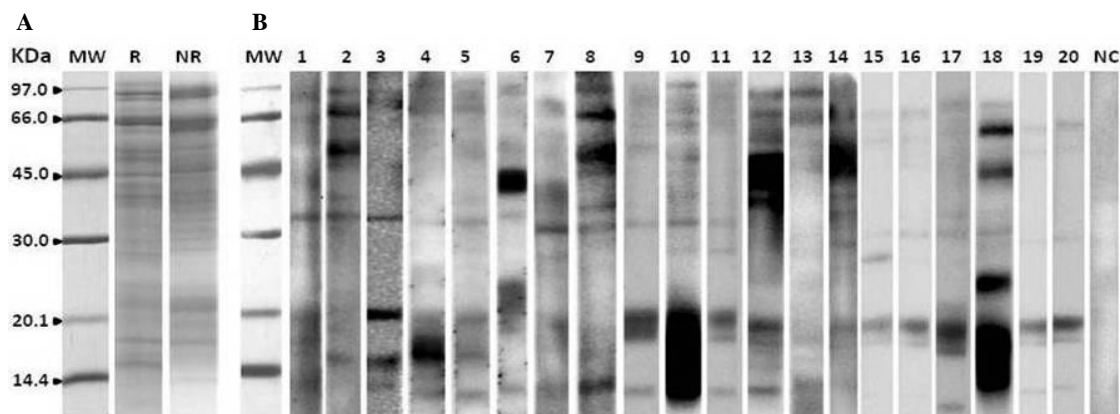
Mean diameters of positive wheal sizes were: *P. juliflora*: 7.95±3.12 mm; *A. farnesiana*: 6.70±1.45 mm; *S. kali*: 9.95±2.87 mm; *C. album*: 8.85±2.90 mm; *A. retroflexus*: 8.80±2.58 mm, and *K. scoparia*: 8.80±2.68 mm. Positive correlation coefficients were attained between the *P. juliflora* and *A. farnesiana* ( $r=0.65$ ,  $p=0.0008$ ), *P. juliflora* and *S. kali* ( $r=0.65$ ,  $p=0.0008$ ), *P. juliflora* and *C. album* ( $r=0.48$ ,  $p=0.01$ ), *P. juliflora* and *A. retroflexus* ( $r=0.33$ ,  $p=0.1$ ), and *P. juliflora* and *K. scoparia* ( $r=0.39$ ,  $p=0.09$ ) pollen extracts for wheal diameter on SPT.

Table 1. Clinical characteristics, total and specific IgE values and skin reactivity of allergic patients

Sample No.	Age (years)/Sex <sup>1</sup>	Symptoms <sup>2</sup>	Mesquite Specific IgE <sup>3</sup> (OD)	Total IgE (IU/ml)	Diameters (mm) of the wheal obtained by prick test <sup>4</sup>					
					<i>P. juliflora</i>	<i>A. farnesiana</i>	<i>S. kali</i>	<i>C. album</i>	<i>A. retroflexus</i>	<i>K. scoparia</i>
Patients										
1	32/M	N,E	0.7	181	5	5	8	7	9	10
2	46/M	L,N,E	1.1	430	9	8	10	10	12	9
3	39/M	L,N	0.9	179	7	5	8	9	11	10
4	16/F	L,N,E	0.8	165	7	7	8	11	5	12
5	18/M	N,E	0.8	123	5	5	4	3	3	5
6	25/M	L,N,E	1.4	313	8	6	10	8	10	7
7	26/F	L,N,E	1.0	345	6	7	8	5	7	4
8	43/F	N,E	1.98	679	9	6	10	11	12	10
9	21/M	N,E	2.07	381	8	7	9	9	10	11
10	29/M	L,N,E	2.5	598	15	9	16	11	9	12
11	23/F	N,E	0.7	180	5	5	7	8	7	6
12	29/F	L,N,E	2.4	623	13	6	15	10	12	10
13	30/M	N,E	0.9	172	6	7	10	7	7	8
14	21/F	L,N,E	1.8	320	10	8	8	12	10	10
15	56/M	L,N,E	0.9	430	7	6	12	10	11	12
16	43/M	N,E	1.4	212	6	8	11	10	8	8
17	32/F	L,N,E	1.1	289	7	6	10	15	11	13
18	46/M	L,N,E	2.6	323	15	10	10	10	7	8
19	34/F	N,E	0.6	160	5	5	5	3	5	4
20	13/M	N,E	1.2	420	6	8	12	8	10	7
Controls										
21	32/ F	-	0.06	48	0	0	0	0	0	0
22	23/ M	-	0.05	67	0	0	0	0	0	0
23	40/F	-	0.05	15	0	0	0	0	0	0
24	28/M	-	0.09	78	0	0	0	0	0	0
25	18/M	-	0.1	55	0	0	0	0	0	0

<sup>1</sup> M, male; F, female. <sup>2</sup> L, lung symptoms (breathlessness, tight chest, cough, wheeze); N, nose symptoms (sneezing, runny, blocked); E, eye symptoms (itching, dryness). <sup>3</sup> Levels of specific IgE to *A. retroflexus* pollen extract by ELISA (optical density at 450nm).

<sup>4</sup> The mean wheal diameters are displayed in mm. Histamine diphosphate (10 mg/ml)-positive control; Glycerin-negative control.



**Figure 1.** A. Coomassie Brilliant Blue stained SDS-PAGE of the crude extract of *P. juliflora* pollen in reducing and non-reducing conditions on 12.5% acrylamide gel. Lane MW, molecular weight marker (GE Healthcare, UK); R, reducing condition; NR, non-reducing condition. B. Immunoblotting of mesquite pollen extract. Each strip was first blotted with *mesquite* pollen extract. All strips were then incubated with allergic patients' sera and IgE reactive protein bands were detected. Lane MW, low molecular weight. NC, negative control.

### Protein and Allergenic Profile

The protein composition of *P. juliflora* extract was analyzed by Coomassie Brilliant Blue staining (Figure 1A). The reducing SDS-PAGE separation of the pollen extract showed several resolved protein bands in the *P. juliflora* extract with molecular weights in the range of approximately 10 to 95 kDa. The most prominent bands showed MWs of approximately 15, 18, 45 and 85 kDa. Other predominant bands were identified at 39, 52, 55 and 66kDa. Non-reducing SDS-PAGE of mesquite pollen extract showed several protein bands with relatively similar molecular weights with those in reducing condition (Figure 1A).

IgE-reactivity of the separated protein bands from the electrophoresis of mesquite pollen extract was determined via immunoblotting assays. Specific IgE binding fractions probed with sera from all 20 allergic patients are shown in the figure 1B. The results showed several IgE reactive bands ranging from about 10 to 85 kDa. Figure 1B shows the apparent MW of each protein fraction and the prevalence of each one among all 20 allergic patients. The most frequent IgE reactive bands among the patients' sera were approximately 20 and 66kDa. However, there were other IgE reactive protein bands among patients' sera with molecular weights 10, 15, 35, 45, 55 and 85kDa (Figure 1B). No band was detected when a negative control serum pool was assayed.

### Specific IgE Levels and Inhibition ELISA

The sera of 20 allergic patients were evaluated for

specific IgE binding to total mesquite pollen extract. All allergic patients had detectable specific IgE levels to the total extract of mesquite pollen (Table 1). These results are consistent with those obtained in the IgE immunoblotting assays using the pollen extract of mesquite (Figure 1B). To investigate cross-reactivity and allergenic potency among *P. juliflora* and acacia, *S. kali*, *C. album*, *A. retroflexus*, and *K. scoparia*, ELISA inhibition with their extracts was done (Figure 2). Almost complete inhibition (92%) was attained with 100µg/ml of mesquite pollen extract as positive control. Pre-incubation of pooled serum with 100 µg/ml of acacia revealed significant inhibition of IgE binding to allergenic proteins of *P. juliflora* pollen extract (75%) (Figure 2). However, when the other plant pollen extracts were employed as inhibitor, low inhibition occurred, which ranged between 39% for *S. kali* to about 25% for *K. scoparia*.

### Immunoblot Inhibition Assays

In order to evaluate the IgE cross-reactivity between mesquite and five other allergenic pollen extracts, an immunoblot inhibition was carried out with the mesquite pollen extract as the solid phase. As shown in Figure 3, IgE reactivity to most of the allergenic proteins of mesquite pollen was inhibited when the mesquite pollen extract was used as an inhibitor (control negative lane 2). Approximately complete inhibition of IgE binding to 20, 55, and 66 components of mesquite extract occurred when the sera were preincubated with acacia extract at 100 µg/ml.

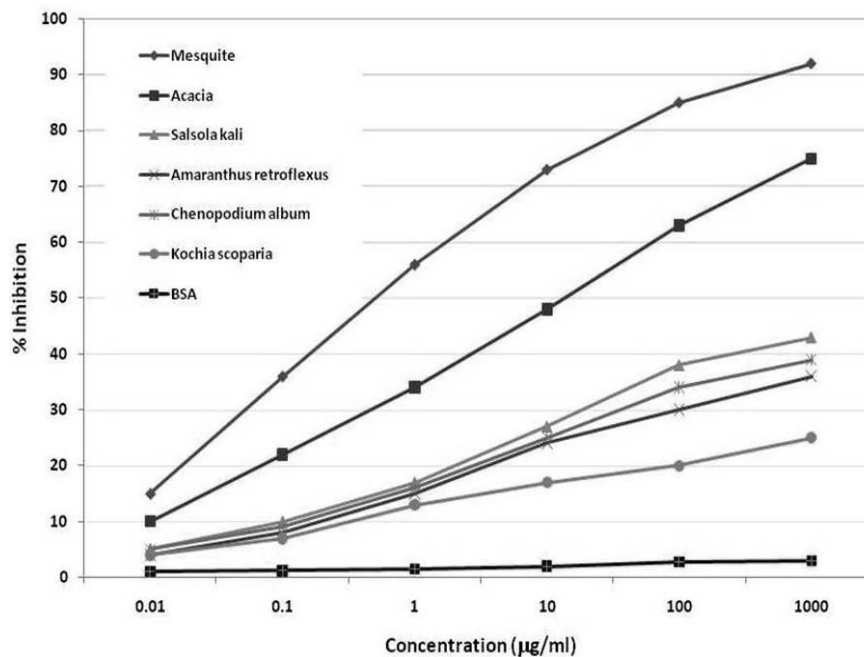


Figure 2. Inhibition of IgE-Binding to *p. juliflora* pollen extract by ELISA using pollen extract from the most allergenic plants: *A. farnesiana*, *S. kali*, *C. album*, *A. retroflexus* and *K. scoparia*. Control experiments were performed with BSA.

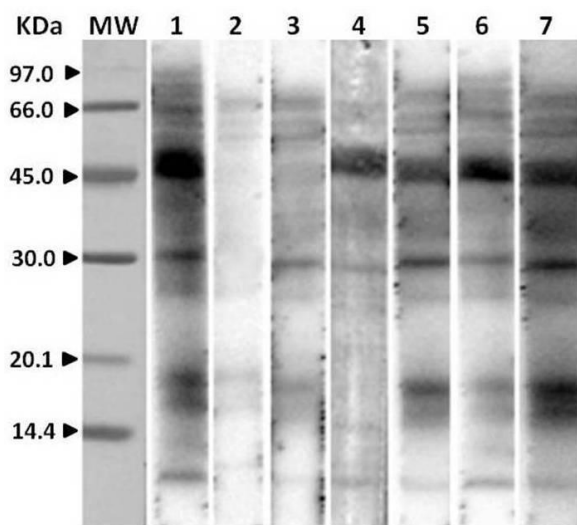


Figure 3. Immunoblotting inhibition assays. Lane MW, molecular weight marker (GE Healthcare, UK); Lane 1, Mesquite protein strip incubated with pooled serum without inhibitor (negative control). Lane 2, Mesquite protein strip incubated with pooled serum containing 100 µg of mesquite pollen extract as inhibitor (positive control). Lane 3-7, Mesquite protein strip incubated with pooled serum containing 100 µg acacia, *S. kali*, *C. album*, *A. retroflexus* and *K. scoparia*, respectively, as inhibitor.

Meanwhile, the three bands at about 10, 15 and 85 kDa were partially inhibited. However, when *S.kali*, *C. album*, *A. retroflexus*, and *K. scoparia* pollen extracts were used as inhibitors, the 15 and 66 kDa bands were inhibited whereas the 35 and 55 kDa bands almost were not inhibited (Figure 3).

## DISCUSSION

Mesquite is one of the major allergenic trees throughout arid and semiarid areas of Iran and neighboring countries particularly Kuwait, Saudi Arabia and UAE.<sup>2,5,12,19</sup> SDS-PAGE revealed several bands from the mesquite pollen extract with the estimated MWs from 10 kDa to 85 kDa (Figure 1A). Among those bands, eight IgE binding protein fractions with apparent MWs of 10, 15, 20, 35, 45, 55, 66 and 85 kDa were detected from the blot (Figure 1B). In previous studies the proteins with apparent MWs 20, 26, 59, 66 and 85 kDa have been the most allergenic proteins in the plants.<sup>6,7,9,20</sup>

There are some inconsistency in molecular weight of mesquite pollen proteins of our research and

those reported by previous studies.<sup>7,8,20</sup> This inconsistency could be due to differences in pollen extract, serum samples, concentration of the gels and method of calculation of molecular weight.<sup>7,8</sup> However, in accordance with the earlier studies,<sup>6,7,20</sup> the results of immunoblots showed that in the allergenic profile of mesquite pollen extract, the proteins with apparent MWs of 20 and 66 kDa are the major reactive proteins.

The results of SDS-PAGE also revealed that the overall pattern of migration of mesquite pollen proteins was not considerably changed under both non-reducing and reducing conditions. However, on the non-reduced gel, the content of the five bands with the apparent MWs of 24, 55, 66, 85 and 95kDa were greater than those on the reduced gel. It was thus suggested that cysteine residues of the pollen proteins may not be associated with interchain disulfide bonds. Moreover, in the previous studies, it was demonstrated that some proteins in pollen extract such as methionine synthase of *S. kali* or *A. retroflexus* was partially degraded into two fragments with approximate MWs of 45 and 39 kDa.<sup>21-23</sup> Taken together, these observations suggested that some proteins in mesquite pollen extract may be susceptible to degradation as a result of proteolysis or exposure to a reducing condition. In addition, it is possible that the number or size of the products of these proteins' degradation depends on the conditions of pollen extract preparation and storage. Nevertheless, additional studies are required to elucidate the patterns of degradation and the number and the size of the cleavage products.

In general, the knowledge of pollen cross-reactivity is crucial for diagnostics as well as formulation of immunotherapy vaccines. Cross-reactivity among pollens belonging to the same genus and/or different genera has been demonstrated earlier.<sup>24</sup> Emerging evidence is available about cross-reactivity of mesquite with other plants. In one study, cross-reactivity among mesquite pollen components with some tree species such as *Ailanthus excels*, *Cassia siamea*, *Holoptelea integrifolia*, *Putranjiva roxburghii* and *Salvadora persica* pollen allergens has been described.<sup>7</sup> Several studies reported that proteins with apparent MWs of 44-45 and 66 kDa are allergenic in the pollen extracts of mesquite<sup>7,8</sup> and the selected member of Amaranthaceae/Chenopodiaceae family.<sup>15,22</sup> The results of immunoblotting inhibition revealed that the IgE binding reactivity of the allergenic proteins with 15 and

66 kDa from the mesquite pollen extract was more or less inhibited by all five pollen extracts. As shown in the figure 3, IgE reactivity to most of the allergenic proteins of mesquite pollen was inhibited when *A. farnesiana* pollen extract was used as an inhibitor. This indicates a significant IgE cross-reactivity between the two pollens and is also in line with the results of the SPTs.

The results of SPTs indicated highly significant correlations between the wheal diameters from mesquite pollen extract and those from *A. farnesiana*, *S. kali* and *C. album* pollen extracts. These results suggest IgE cross-reactivity among these pollens, and related to acacia is concurrent with the results of immunoblotting and inhibition experiments. These results collectively suggest that in these pollen extracts, protein components with molecular weight at 15 and 66 kDa may play a greater role in cross-reactivity compared to others. Earlier studies had also indicated one of the most allergenic proteins of *S. kali* pollen, named Sal k 1, with a MW of 40-43 kDa.<sup>25, 26</sup> Sal k 1 displayed pectin methylesterase (PME) properties and is considered a major allergen of *S. kali* pollen.<sup>18,25,26</sup> Some cross-reactive proteins have been genetically engineered and are found to have potential for use in immunotherapy.<sup>27</sup>

Another dominant IgE binding protein band with an estimated molecular weight of 15 kDa was also detected by immunoblotting of mesquite pollen extract. Earlier, the allergens belonging to the profilin family with apparent MWs of 14 to 15 kDa were found from *S. kali*, *A. retroflexus* and *C. album* pollens.<sup>28-30</sup> It may be that the 15 kDa- protein of mesquite pollen is homologous with the 15-kDa IgE reactive band in these plants. However, further studies are required to prove the nature of this allergenic protein of mesquite pollen.

In conclusion, mesquite pollen is a potent allergenic source with several IgE binding components. The observations suggest that 20 and 66 kDa proteins could be used as diagnostic and therapeutic reagents for patients allergic to mesquite and acacia. Regarding the extensive cross reactivity between these two trees and the abundance of them in tropical and subtropical regions, identification and production of the recombinant forms of common allergens of this pollen may lead to the exploration of new guidelines for diagnostic, therapeutic, and preventive purposes. Efforts are now underway to clone cDNAs encoding allergenic cross-reactive proteins from mesquite pollen.

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