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Evaluation of electroelution and immunodiffusion as methods for purification and identification of the allergenic proteins of *Ailanthus excelsa* Roxb. pollen

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Abstract

The total soluble proteins from the pollen of *Ailanthus excelsa* Roxb., a potentially allergenic plant, was extracted and separated on a 12% SDS-Polyacrylamide gel with an aim to identify the allergenic determinants responsible for causing respiratory disorders. The protein profile revealed nine prominent bands, which were designated as AE1 to AE9 lying within a short molecular weight range of 42.7 kDa to 97.4 kDa. Each protein fraction was recovered from the gel by electroelution and sent for skin tests to identify the allergenic fractions. All the protein fractions except AE3 (85.3 kDa), were found to be capable of inducing reactivity in sensitive patients. However, AE6 (59.7 kDa) and AE8 (45.0 kDa) proved to be the major allergenic determinants, while AE1 (97.4 kDa), AE2 (92.6 kDa), AE4 (73.3 kDa), AE5 (64.8 kDa), AE7 (52.4 kDa) and AE9 (42.7 kDa) are the minor contributors of allergenicity. Immunodiffusion analysis with pooled sera of patients sensitive to the total extract also revealed similar results.

The role of plants serving as purifiers of air has been known to us, since times immemorial. To cope with the gradually increasing levels of toxic pollutants, tree plantation programs have been undertaken in different countries of the world to help in environmental clean up. *Ailanthus excelsa* Roxb. (Simaroubaceae) is one such exotic avenue tree, the plantation of which has been encouraged under social forestry programs for large-scale tree plantation in different densely populated cities and towns of India. Large areas of several districts are now covered with monoculture plantations of this exotic tree, as a result of which the pollen of this tree has emerged as a major contributor to pollen loads in these areas. Unfortunately, this has posed a real problem and a serious threat as an important aeroallergen to patients suffering from naso-bronchial disorders and sensitive to the pollen of *A. excelsa*. The allergenic potential of the pollen of *A. excelsa* in causing several respiratory disorders in sensitive patients has been reported earlier by several workers (Acharya, 1980; Tilak, 1989; Mondal & Mandal, 1997).

Allergen extracts have been long used to diagnose and treat allergy. Immunotherapy has been the most successful treatment so far. However despite the use of allergenic extracts from pollen in immunotherapy in millions of patients annually, little is known about the contents of these extracts with respect to allergen and antigen species, as well as their effectiveness in desensitizing patients. Immunotherapy as practiced today consists of injecting small but repeated and increasing doses of allergen extract, either to cure or ameliorate the symptoms. However the allergen preparations used in immunotherapy contain non-allergenic protein antigens and irritants as well as active proteins. Thus it may contain the relevant allergens in minute, irreproducible amounts leading up to 100X variation in allergenic potency. There is always the associated risk of fatal side reactions including systemic anaphylaxis, as the contaminating constituents unrelated to the few allergens to which a patient is actually sensitive, can lead to the induction of IgE antibodies with new specificities (Mohapatra, 1994). Further, the allergen content and composition

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of extracts prepared by different companies may vary considerably (Vijay, 1994). This is the reason why immunotherapy is usually not favored, as pharmacological treatment can give fast results compared to the protracted injection regime required for immunotherapy even though a lifetime of medication may be required and the underlying allergy is not treated (Thomas, 1994). Thus proper standardization of allergen extracts for clinical use, and identification and purification of the allergenic constituents is of prime importance for proper reproducibility of treatment and quantitation of sensitization. With these points in mind, the present study was undertaken to identify, isolate and partially characterize the allergenic components of the pollen of *A. excelsa*.

Material and methods

Pollen collection

Pollen grains of *Ailanthus excelsa* were collected in bulk from the plants growing in Santiniketan, West Bengal, Eastern India. The pollen from the anthers was sieved using different meshes (100, 200 and 300 μm). Microscopic analysis of the samples was done to check pollen purity of 90 to 95%.

Protein extraction

Protein was extracted from defatted pollen in 0.2 M Tris HCl buffer, pH 7.4 according to the method of Singh et al. (1993) with slight modification (Mondal et al., 1997) by continuous stirring at 4°C for 20 h and the extract after centrifugation at $15\,000 \times g$ for 25 min at 4°C was subjected to 80% ammonium sulphate precipitation and the protein repelleted by centrifuging at $10\,000 \times g$ for 15 min. at 4°C. The precipitate was re-suspended in 0.1 M Tris HCl buffer, pH 7.4 and desalted by dialyzing against distilled water for 48 h at 4°C and the samples stored at -20°C until further use.

Protein estimation

The protein concentration in the extract, as well as in various eluted fractions, was estimated by the modified method of Lowry (Lowry et al., 1951). A calibrated solution of bovine serum albumin was used as a standard.

Gel electrophoresis

SDS-Polyacrylamide gel electrophoresis was performed in 12% gel according to the method of Laemmli (1970) with modifications. Ten microlitres of the protein sample containing 70 μg of protein

was heated with equal amount of sample buffer [0.06 M is HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β -mercaptoethanol, 0.01% Bromophenol Blue] at 100°C for 3 min and loaded into the well of the gel and electrophoresis performed using Laemmli buffer system [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature at 70V (mini gel – 8 $\times$ 7 cm) (Slab Gel System-mini, Model No. 05-01-00 obtained from Biotech R & D Laboratories, YERCAUD, South India) for 2 h 30 min. The gel was calibrated with a marker mixture consisting of Myosin, rabbit muscle (M.W. 205 kDa), β -Galactosidase, *E. coli* (M.W. 116 kDa), Phosphorylase b, rabbit muscle (M.W. 97.4 kDa), Albumin, Bovine (M.W. 66 kDa), Albumin, Egg (M.W. 45 kDa) and Carbonic Anhydrase, Bovine Erythrocytes (M.W. 29 kDa) obtained from Sigma Co.; USA. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R 250 and destained with methanol:acetic acid:water (4:1:5) mixture.

Recovery of protein from gel

The individual protein fractions were separated by directly eluting from the gel following the method of Wilson and Goulding (1986) instead of going into the time consuming and expensive methods of column chromatography. After electrophoresis, using the same 8 $\times$ 7 cm Slab Gel System (Biotech DUAL-mini, Model No. 05-01-00) one side of the gel (covering two lanes) was cut vertically and stained in order to ascertain the banding positions. Only those portions corresponding to the protein bands to be recovered were cut out with a sterile scalpel from the other half of the gel which was not stained. The gel pieces were then transferred into pre-treated dialysis bags filled with electroelution buffer (containing 0.05 M Tris and 0.192 M Glycine, pH 8.4) so that each gel piece was surrounded by small amount of buffer with no air bubbles. These dialysis bags were immersed in electrode buffer in an electrophoresis tank of a horizontal gel apparatus (Mini system, Model No. 03-01-00, Biotech). Electric current (120 V) was passed through the bags for nearly 3 h to elute the proteins out of the gel. At the end, the current was reversed for 30 s in order to release the protein from the wall of the sac. The buffer containing the eluted proteins was then transferred into a cotton plugged Eppendorf tube and centrifuged for 2 min to remove contaminating gel particles. The entire process was repeated several times to extract sufficient quantity of each protein fraction for loading in the gel and for further skin tests. The protein in each fraction was quantified and each fraction was again electrophoresed to check its homogeneity.

Preparation of antigenic extracts for skin prick tests

In order to further purify the eluted protein fractions from any contaminating constituents of the gel, the protein fractions were subjected to fractional precipitation by ammonium sulphate (100% saturation) to precipitate out the proteins. The pellet after centrifugation at $10\,000 \times g$ for 5 min at 4°C in a microcentrifuge was re-suspended in 0.05 M Phosphate buffered saline, pH 7.4 in such a way that each antigenic fraction had the concentration of 1 in 500 (w/v) i.e. 1 part by weight of protein in 500 parts by volume of buffered saline and dialysed against distilled water for 48 h at 4°C until all the salt was removed. Each fraction was then sterilized by filtering through a Millipore Filter Paper (0.22 μm) and stored in sterile vaccine vials at 4°C . Prior to testing these extracts on patients, their sterility was checked for microorganisms by transferring a few drops of the antigenic extract to Leuria broth agar plate and incubating at 37°C for 24 h. The total antigen was extracted according to the method of Sheldon et al. (1967) with slight modifications. Pollen were first defatted and the antigens were then extracted from the defatted pollen in 0.05 M Phosphate buffered saline, pH 7.4 in 1:10 ratio (w/v) by continuous stirring at 4°C for 20 h and then centrifuged at $15\,000 \times g$ for 25 min at 4°C . The supernatant containing the antigen was dialysed against distilled water. After dialysis, the sample was again centrifuged and the supernatant used for skin tests after sterilization.

Skin prick tests

The skin tests with the individual protein fractions were performed on patients earlier found to be sensitive to the total pollen extract of *A. excelsa* (Mondal & Mandal, 1997). Negative and positive controls were also performed (Shivpuri, 1980). The negative control was the buffer saline in which the allergen was re-suspended and positive control was histamine acid phosphate injection diluted with buffered saline to 0.1 mg/ml. Results were recorded after 15 to 20 min and depending on the wheal diameter, the tests were graded 1+ to 4+. Size of the wheal and erythema were taken into account, for grading the skin reactions as -ve (same as the control), 1+ (wheal = 1–2 mm; erythema = 1–5 mm), 2+ (wheal = 2–3 mm; erythema = 6–10 mm), 3+ (wheal = 3–5 mm; erythema = 11–15 mm) and 4+ (wheal > 6 mm; erythema > 15 mm). This grading was done by relating wheal size to the histamine control.

Preparation of antiserum

Blood was collected from the patients showing positive response to the total extract of *A. excelsa*

and the blood samples were allowed to clot by standing for 3–4 h at room temperature according to the method of Sadasivam and Manickam (1996). The serum was then separated from the clot by low speed centrifugation and stored at -20°C in the presence of 0.1% sodium azide as antibacterial agent.

Immunodiffusion

Ouchterlony immunodiffusion was performed according to the method described by Johnstone and Thorpe (1982). Equal protein loadings of each fraction (50 μg total protein) were placed into the outer wells and 25 μl of the mixture of the antiserum (150 μg total protein) of *A. excelsa* pollen sensitive patients was placed in the central well. The plates were incubated for 48 h at 25°C and washed with 0.9% (w/v) NaCl (saline) several times, rinsed thoroughly in distilled water and dried to observe the precipitin arcs.

Results*Protein concentration*

Protein content in the pollen extract of *A. excelsa* was found to be 14.6 mg ml^{-1} .

SDS-PAGE

The SDS-PAGE of the protein extract of the pollen of *A. excelsa* revealed nine prominent bands (Figures 1 & 2), which were designated as AE1 to AE9. These bands were found to lie within a very short molecular weight range of 42.7 kDa to 97.4 kDa (Table I).

Skin tests

According to our earlier report, skin tests with total antigenic extract, which was performed on 115 patients, induced positive response in 46.95% of the patients (Mondal & Mandal, 1997). Among these sensitive patients 35.19% were females between the ages of 16–45 years while 64.81% were male between the ages of 12–65 years (Table V). 13.04% showed 2+ reaction while 3+ reaction was observed in only 0.87% of the patients. None of the patients showed 4+ reaction (Table II). The total antigen was also tested on 20 normal persons of whom three (15%) showed 1+ reaction. The individual antigenic fractions were further tested on 54 sensitive patients (Tables III & IV, Figures 3&4). The highest incidence of skin reactivity was observed in AE6 (59.7 kDa) and AE8 (45.0 kDa), which induced

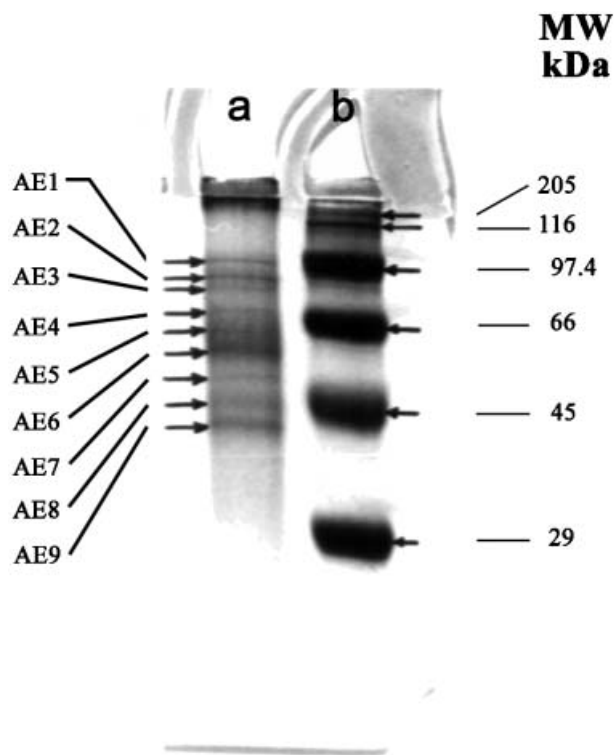


Figure 1. SDS-PAGE pattern of pollen extract of *Ailanthus excelsa* collected from full bloomed flowers (a) contains 46 µg of 0–80% ammonium sulphate pellet fraction of crude homogenate. It represents AE1 to AE9 (b) represents marker proteins.

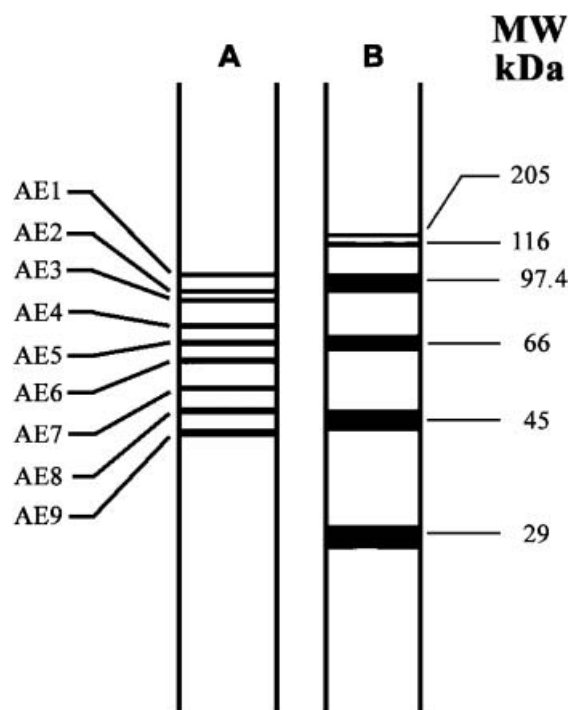


Figure 2. Diagrammatic representation of the SDS-PAGE pattern of pollen extract of *Ailanthus excelsa* collected from full bloomed flowers (A) represents pollen sample [AE1 to AE9] (B) represents marker proteins.

Table I. Molecular weights of various protein bands of the antigenic extract of *Ailanthus excelsa* pollen.

Fraction	Molecular weights (kDa)
AE1	97.4
AE2	92.6
AE3	85.3
AE4	73.3
AE5	64.8
AE6	59.7
AE7	52.4
AE8	45.0
AE9	42.7

positive reaction in all the 54 patients proving them to be the major allergenic determinants. AE6 also induced 4+ reaction in 1.89% of the patients. A lower incidence of skin reactivity was found in AE1 (97.4 kDa), AE2 (92.6 kDa), AE4 (73.3 kDa), AE5 (64.8 kDa), AE7 (52.4 kDa) and AE9 (42.7 kDa) which induced only 1+ and 2+ reaction in fewer number of patients except AE5 which also induced 3+ reaction in 3.45% of the positive patients. AE3 (85.3 kDa) however did not induce any skin reactivity.

Immunodiffusion

Immunodiffusion of the pooled antiserum of the 54 patients also helped to identify the allergenic fractions as well as to compare their degree of allergenicity (Figure 5 a, b). Similar results were observed as in case of skin tests with precipitin arcs being obtained in case of all the fractions except AE3. However, the intensities of the precipitin arcs differed with the maximum amount of precipitate being observed in case of AE6 and AE8.

Discussion

With the discovery of IgE antibodies, new in vitro IgE-binding assays like Western blot and ELISA have developed to measure specific IgE antibodies in the serum. However, immunodiffusion with the protein fractions and serum proved to be a simple and quick method to identify allergenic protein fractions. Electroelution on the other hand proved to

Table II. Results of skin tests against the antigen of *Ailanthus excelsa* in patients of naso-bronchial allergy (n=15) and in nonatopic subjects (n=15).

Total number of tests	Patients				
	–ve %	1+ %	2+ %	3+ %	4+ %
Allergic 115	53.05	33.04	13.04	0.87	0.0
Nonatopic 15	85.00	15.00	0.0	0.0	0.0

Table III. Results of skin tests against total extract and individual antigenic fraction of *Ailanthus excelsa* pollen on sensitive patients.

Patient No.	Age (yrs)	Sex	Total protein	AE1	AE2	AE3	AE4	AE5	AE6	AE7	AE8	AE9
1.	24	F	1+	-ve	1+	-ve	-ve	1+	1+	-ve	1+	-ve
2.	20	M	2+	1+	-ve	-ve	-ve	-ve	2+	-ve	2+	-ve
3.	26	M	1+	-ve	-ve	-ve	1+	-ve	1+	-ve	1+	-ve
4.	28	F	2+	-ve	-ve	-ve	1+	1+	2+	1+	3+	-ve
5.	32	M	1+	1+	1+	-ve	1+	1+	2+	-ve	1+	-ve
6.	41	M	1+	-ve	1+	-ve	-ve	-ve	2+	1+	1+	1+
7.	62	M	1+	2+	1+	-ve	-ve	1+	2+	1+	1+	1+
8.	30	F	1+	-ve	1+	-ve	1+	-ve	1+	-ve	1+	1+
9.	51	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	2+	1+
10.	42	M	2+	2+	1+	-ve	1+	1+	3+	1+	3+	2+
11.	46	M	1+	1+	-ve	-ve	1+	1+	1+	-ve	1+	2+
12.	32	F	2+	1+	-ve	-ve	-ve	1+	2+	-ve	2+	2+
13.	38	M	1+	1+	-ve	-ve	1+	1+	1+	-ve	1+	-ve
14.	47	M	2+	-ve	1+	-ve	-ve	1+	3+	2+	2+	-ve
15.	36	F	1+	-ve	1+	-ve	1+	-ve	2+	-ve	1+	2+
16.	42	F	1+	2+	-ve	-ve	1+	-ve	1+	1+	2+	2+
17.	41	F	1+	-ve	-ve	-ve	-ve	-ve	1+	1+	1+	1+
18.	27	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
19.	29	M	2+	1+	1+	-ve	-ve	1+	2+	2+	3+	-ve
20.	31	M	1+	1+	1+	-ve	-ve	1+	2+	-ve	1+	-ve
21.	27	F	1+	-ve	1+	-ve	-ve	1+	1+	-ve	1+	-ve
22.	28	M	1+	-ve	1+	-ve	1+	1+	2+	-ve	1+	1+
23.	29	F	1+	1+	-ve	-ve	1+	2+	1+	-ve	1+	-ve
24.	30	M	1+	1+	-ve	-ve	-ve	-ve	1+	1+	1+	-ve
25.	36	F	1+	-ve	-ve	-ve	1+	-ve	2+	-ve	1+	-ve
26.	22	M	2+	-ve	-ve	-ve	1+	-ve	3+	1+	2+	-ve
27.	16	F	1+	1+	-ve	-ve	-ve	1+	1+	-ve	1+	-ve
28.	56	M	2+	2+	1+	-ve	-ve	2+	2+	-ve	2+	2+
29.	51	M	1+	1+	-ve	-ve	-ve	-ve	2+	-ve	1+	-ve
30.	46	M	2+	2+	1+	-ve	1+	-ve	2+	1+	2+	2+
31.	45	F	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
32.	28	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
33.	27	M	1+	-ve	1+	-ve	-ve	-ve	2+	1+	1+	-ve
34.	30	F	1+	-ve	-ve	-ve	1+	1+	1+	1+	1+	1+
35.	32	M	2+	1+	1+	-ve	1+	1+	3+	1+	1+	1+
36.	39	M	1+	-ve	-ve	-ve	1+	1+	1+	-ve	1+	1+
37.	12	M	1+	-	-	-	-	-	-	-	-	-
38.	65	M	2+	1+	1+	-ve	1+	1+	2+	-ve	2+	1+
39.	48	M	1+	1+	-ve	-ve	-ve	1+	2+	-ve	1+	1+
40.	24	M	2+	1+	-ve	-ve	1+	1+	3+	1+	2+	1+
41.	26	M	2+	1+	-ve	-ve	1+	1+	3+	2+	2+	-ve
42.	36	F	1+	1+	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
43.	41	M	2+	-ve	1+	-ve	-ve	2+	2+	-ve	3+	-ve
44.	52	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
45.	58	M	1+	1+	1+	-ve	-ve	-ve	1+	-ve	1+	-ve
46.	42	F	1+	1+	1+	-ve	-ve	-ve	1+	1+	2+	1+
47.	60	M	1+	-ve	-ve	-ve	-ve	1+	2+	-ve	2+	1+
48.	28	F	1+	2+	1+	-ve	-ve	1+	2+	-ve	1+	-ve
49.	48	M	3+	2+	1+	-ve	1+	3+	4+	2+	2+	2+
50.	30	F	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	1+
51.	47	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	-ve
52.	31	F	1+	-ve	-ve	-ve	-ve	1+	2+	-ve	1+	-ve
53.	43	F	2+	1+	1+	-ve	1+	2+	2+	1+	2+	-ve
54.	46	M	1+	-ve	-ve	-ve	-ve	-ve	1+	-ve	1+	1+

be effective as a simple and inexpensive method for separating and purifying the protein fractions from the total crude antigenic extract.

Although gel filtration can be an easier and more sophisticated technique to separate or isolate individual proteins, the protein fraction isolated by electroelution and subsequent fractional precipitation

by ammonium sulphate followed by dialysis, helped purify the fractions from any contaminating constituents of the gel. The purity of the fractionated antigens was shown by repeated SDS-PAGE runs and immunodiffusion. The atopic patients as well as the normal persons were first tested with the total crude protein extract to establish the potentiality of

Table IV. Frequency distribution of allergenicity of the total extract and individual allergens of *Ailanthus excelsa*.

Antigen tested	Number of patients +ve	% Showing			
		1+	2+	3+	4+
Whole	54	70.37	27.78	1.85	0.0
AE1	27	74.07	25.93	0.0	0.0
AE2	23	100.00	0.0	0.0	0.0
AE3	0	0.0	0.0	0.0	0.0
AE4	22	100.00	0.0	0.0	0.0
AE5	29	82.76	13.79	3.45	0.0
AE6	53	45.28	41.51	11.32	1.89
AE7	19	78.95	21.05	0.0	0.0
AE8	53	64.15	28.30	7.55	0.0
AE9	24	66.67	33.33	0.0	0.0

the pollen of *A. excelsa* as an effective aeroallergen before being tested with the individual isolated antigenic fractions. The incidence of positivity in the normal persons could be due to irritants present in the extracts or may be due to the contaminating constituents unrelated to the actual allergen to which a person has been actually sensitized, as the total pollen extracts are heterogeneous crude extracts containing proteins, enzymes, lectins, glycoproteins, lipoproteins, etc. and may contain the relevant allergen in minute and irreproducible amounts as pollen allergens generally comprise 0.5 to 1.0% of the total extractable proteins (Stanley & Linskens, 1974). Some of the non-atopic subjects showed a positive response only when tested with the total antigenic extract, but they were not tested with the individual purified antigenic fractions.

Each extract of pollen may contain several antigenic proteins of which only a limited number of these may be allergenic. Further only limited areas in the antigen (antigenic determinants or epitopes) take part in forming immune complexes. Therefore, the contaminating constituents unrelated to the few allergens need to be removed before preparation of standardized vaccines for adequate diagnosis and immunotherapy. The human serum of an allergic subject can also react to allergens of related species.

Table V. Age-wise distribution of patients showing positive skin response to *Ailanthus excelsa* pollen antigen.

Age group	Male	Female	Total
11-20	2	1	3
21-30	10	8	18
31-40	5	5	10
41-50	10	5	15
51-60	6	0	6
61-70	2	0	2
Total	35(64.81%)	19(35.19%)	54(100%)

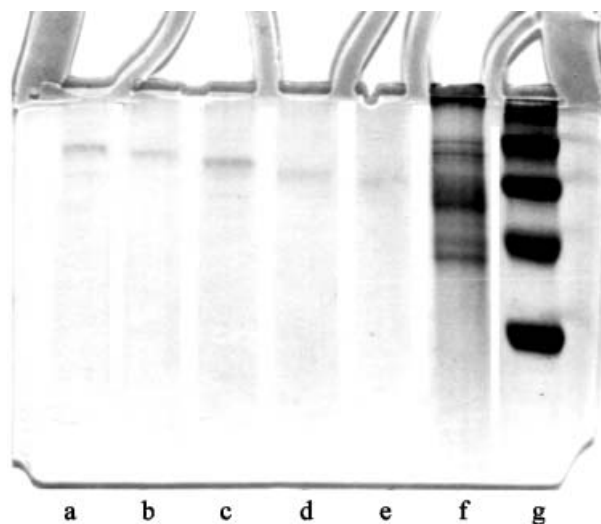


Figure 3. SDS-PAGE of the gel eluted fractions of the pollen extract of *Ailanthus excelsa* (a) to (e) contains 10 µgm of 0-100% ammonium sulphate pellet fraction of the eluted proteins of the first 5 bands i.e. AE1 to AE5; (f) contains 46 µgm of 0-80% ammonium sulphate pellet fraction of total homogenate; (g) represents marker proteins.

Therefore subjects sensitized to allergens of other plants may also show a positive skin prick test reaction to allergens of *A. excelsa*. This aspect has however not been dealt with in the present paper.

Conclusions

The present study reveals that the pollen of *A. excelsa* is a highly potential allergen with almost all the protein fractions except AE3 (85.3 kDa) to be capable of inducing reactivity in sensitive patients.

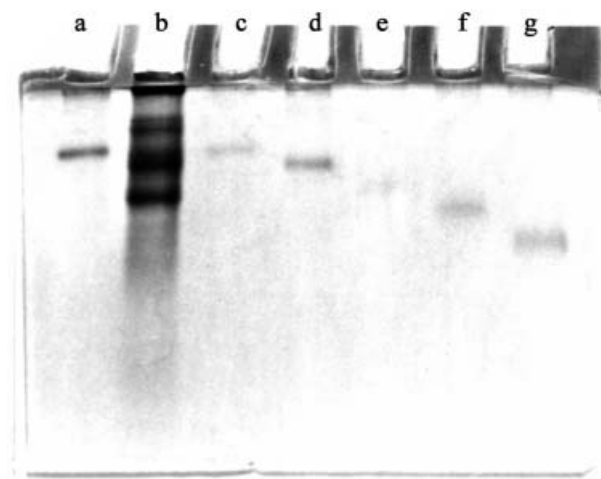


Figure 4. SDS-PAGE of the gel eluted fractions of the pollen extract of *Ailanthus excelsa* (a & c-g) contains 10 µgm of 0-100% ammonium sulphate pellet fraction of the eluted proteins of the last 6 bands i.e. AE4 to AE9 (b) contains 50 µgm of 0-80% ammonium sulphate pellet fraction of total homogenate.

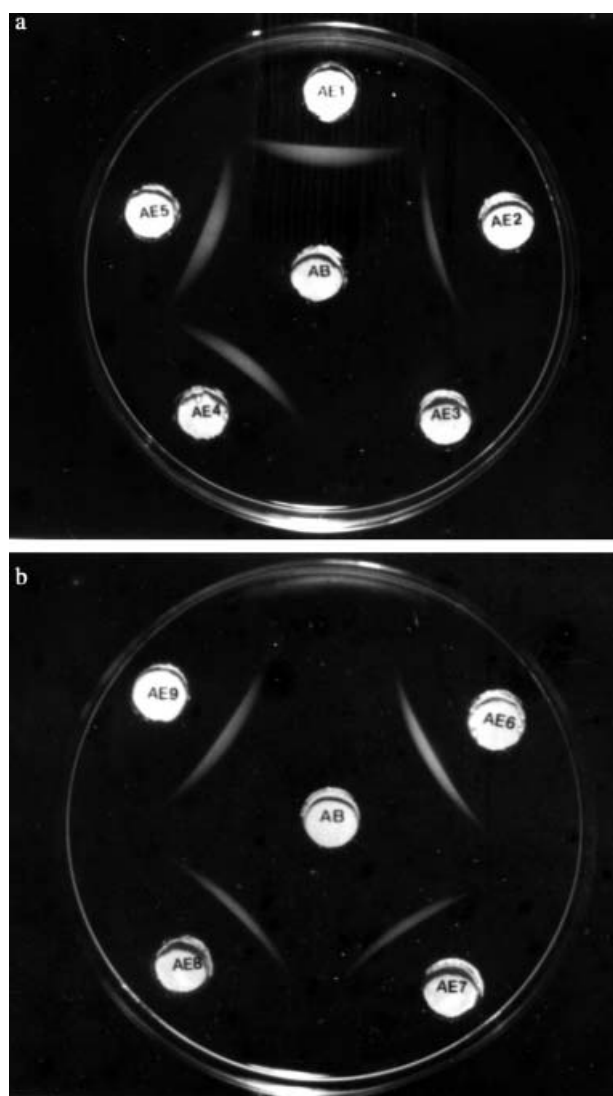


Figure 5. Serological precipitation reaction between pooled antiserum of *Ailanthus excelsa* pollen sensitive patients and individual protein fractions of *Ailanthus excelsa*. AB represents pooled human antiserum (25 μ l containing 150 μ gm total protein) and (a) AE1 to AE5; (b) AE6 to AE9 containing 50 μ gm of protein fractions.

However, AE6 (59.7 kDa) and AE8 (45.0 kDa) are the major allergenic determinants, while AE1 (97.4 kDa), AE2 (92.6 kDa), AE4 (73.3 kDa), AE5 (64.8 kDa), AE7 (52.4 kDa) and AE9 (42.7 kDa) are the minor contributors of allergenicity. Further, although being an entomophilous plant, *A. excelsa* has been found to have a high pollen productivity (ca 1 635 180 pollen per flower) [Mondal et al., 1998], and thus may release large amount of pollen in the air, which generally in case of entomophilous plants remain concentrated around their source plants (Tilak, 1989). Several districts are now covered with monoculture plantations of this exotic tree, the pollen of this tree can prove to be a major contributor to pollen loads in

these areas, particularly during their peak flowering period between the months of January to April (Mondal & Mandal, 1997).

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