

Assessment of allergenicity to *Mallotus Phillipensis* pollen in atopic patients in India: A new allergen

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Summary

Objective: Pollen grains of the Euphorbiaceae family are well known causative agents of respiratory allergies in India, European countries and USA. *Mallotus phillipensis* belongs to the same family and may have some common allergenic properties. It has thus been evaluated for the first time in Indian population for its pollinosis causing properties.

Methods: Pollen antigen of *Mallotus phillipensis* (MP) was extracted and characterized for its protein components by biochemical methods. Pollinosis potency of crude extract of MP pollen was evaluated by skin prick test on population residing in different parts of India. Specific IgE binding characteristics of the extract were determined by ELISA and Immunoblot.

Results: Marked skin reactivity in 5.7% atopic population was recorded and subjects constituting 23.8% of the total patients tested showed skin sensitivity to the MP pollen antigen. Significantly raised specific IgE against MP pollen were recorded in 50% of the skin test positive patients. A number of protein bands were detected in a wide Molecular weight range as well as in acidic pI range, by SDS-PAGE and IEF, respectively. A total 11 protein fractions were detected by the specific IgE antibodies on immunoblotting with patient's sera and were considered allergenic.

Conclusion: Patients from different geographical regions have shown sensitization to MP pollen antigen. Many proteins have similar molecular weights and pI as other allergenic members of the family (*Ricinus communis* and *Putranjiva roxburghii*) found in India, which constitutes a good reason for studying cross reactivity among the members of family Euphorbiaceae, in the future.

Key Words: *Mallotus phillipensis*, allergenicity, atopic patients, Immunoblot, Cross reactivity

Introduction

Pollen grains are the earliest known allergens and the major source of morbidity among the atopic subjects [1- 4]. A number of tree pollen grains are considered to be important constituent of the spectrum of allergy triggering agents and are part of the local vegetation [5 - 9]. Different eco-geographic regions in India support different allergenic pollen flora [10 - 12] and many taxa require to be investigated for their allergenic properties in local population.

Although a sizable population is regularly exposed to *Mallotus phillipensis* (MP) pollen recorded from different regions of India including the Himalayas, information on its allergenic potential has been completely lacking. This was substantiated by the fact that many patients do not respond to a battery of allergens being routinely used for diagnosis of respiratory allergy. Family Euphorbiaceae (spurge) includes important allergenic plants such as *Ricinus communis* and *Putranjiva roxburghii* from India, [13-16] and *Mercularis annua* and *Haevea brasiliensis* from

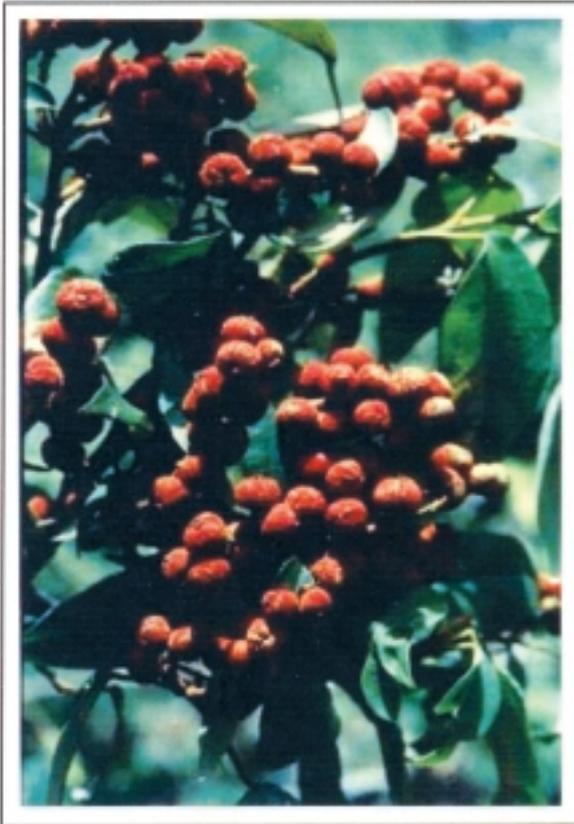


Figure 1. Twig of *Mallotus phillipensis*. With bloomed flowers.

Europe [17- 20]. Some of these also show cross reactivity [14,15,18]. *Mallotus phillipensis* also belongs to same family and is a small much branched evergreen tree with thin gray bark (Figure 1). It is part of Himalayan vegetation and also distributed throughout India. Pollen of *Mallotus* is also anemophilous, as many other members of the family Euphorbiaceae and are reported from air in different parts of the country [12, 21].

Considering the distribution of the *Mallotus* pollen all over India and suspecting that there might be some common properties comparable to the pollen of the other members of the family *Mallotus*, we investigated the crude antigen of the *Mallotus phillipensis* pollen for their antigenic and allergenic properties.

Materials & Methods

Pollen collection: The polliniferous material from *Mallotus phillipensis* (MP) tree was collected during the pollen season (October to December, 1995) from Shivalik ranges in Himalayas. To procure pollen, dried flowers were crushed gently and the pollen thus released was sieved through 100, 200, 300 mesh/cm² sieves. Pollen samples with a > 95% purity were processed for antigen preparation.

Defatting: Pure MP pollen were defatted to remove the lipids and non specific irritants, using diethyl ether 3 - 4 times the volume of pollen material by repeatedly changing the ether for a fresh lot till the ether became colourless. Defatted pollen was then dried in vacuum desiccators containing calcium chloride for 24 - 48 hrs. and then stored in dry airtight container at 4° C, till antigen extraction.

Extraction of Antigen: Defatted pollen was suspended in 1:20 (w/v) ratio in phosphate buffer saline (pH 7.8) at 4°C by continuous stirring for 20 hrs. The suspension was then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was dialyzed against distilled water in visking dialysis tubing (cut off point of 7000 Daltons) for 24 hrs with frequent changes of distilled water. After dialysis, the extract was centrifuged again for 30 min at 4°C. The supernatant was then passed through a nitrocellulose membrane filter (0.22 µ) lyophilized in small aliquots and stored at -70° C for future experimentation.

Estimation of protein in pollen antigen

The soluble protein content of MP pollen extract was estimated by Lowry's method with slight modification [22]. The soluble proteins were precipitated using 15% phosphotungstic acid. Serial dilutions of BSA were used as standard to calibrate the optical density (OD) values for measuring the amount of protein present in the pollen extract. Reading for colour development was taken at 740 nm using spectrophotometer (Beckman, DU 640 B).

Characterization of antigens

The characterization of protein profile of MP pollen antigen was carried out by iso-electric focussing (IEF) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Isoelectric Focussing (IEF)

The method described by Garfin [23] was used for separation of protein by IEF. Ampholine PAG plates (Pharmacia, Sweden) with pH gradient 3.5 - 9.5 were used according to the manufacturer's manual. Proteins with known isoelectric points between 3.5 to 9.3 (pI markers) were electro-focussed along with the MP pollen extract. After electro - focussing, the gel was fixed in a solution of 11.4% trichloroacetic acid and 3.4% sulphosalicylic acid for one hr minimum and then washed with destaining solution (25% ethanol in 80% glacial acetic acid) for 30 min. The gel was then stained with Coomassie Brilliant Blue R 250 (0.13% in destaining solution) for 3 hrs. It was then destained till clear bands were obtained.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using polyacrylamide gel (12%) containing 0.1% SDS in conjunction with tris-glycine buffer (pH 7.4) using Mini Electrophoretic Apparatus (Bio Rad) [24]. The gel was calibrated with marker proteins (Sigma, USA). Electrophoresis was carried out at a constant current of 4 mA / well. The gel was stained overnight by keeping it in the staining solution (10% glacial acetic acid and 0.25% Coomassie Brilliant blue in 95% ethanol). The gel was then destained in 5% glacial acetic acid and 95% ethanol for varying periods till protein bands appeared clear.

Immunoassay of antigenic extracts

Clinical Studies

For assessment of allergenicity to MP pollen antigen, patients with nasobronchial symptoms reporting to allergy treatment centres at Kolkata, Chandigarh, Thiruvananthapuram and Delhi, were included randomly during 1996 - 1998. All the patients selected for skin prick test were above 5 years of age. Their detailed medical history was taken and diagnosed as a case of nasobronchial allergy. Along with MP, other common inhalant allergens such as *Cynodon dactylon*, *Artemisia*

scoparia, *Amaranthus spinosus*, *Chenopodium album*, *Morus alba*, *Prosopis juliflora*, and *Ricinus communis* were also tested to establish their atopy. Skin prick test (1:10 w/v concentration) was performed with the help of 23 G sterilised disposable needles. The reaction was allowed to develop for 15 - 20 minutes. Skin reaction was graded as compared to negative (buffer saline) and positive (histamine hydrochloride 1 mg/ml) control as per the criteria outlined by Singh et al [25]. A diameter of 3 mm and above was considered positive.

Serum samples: Blood samples were collected from the patients showing positive skin reactions to antigen of MP pollen antigen with their consent. Blood was also collected from healthy volunteers to act as control. Blood was allowed to clot and the expressed sera stored in small aliquots at -70°C for future use.

Enzyme Linked Immuno Sorbent Assay (ELISA)

Specific IgE antibodies in the sera of the patients against MP pollen antigen was estimated by indirect ELISA method outlined by Sepulveda et al [26]. Polystyrene microtiter plates (Nunc, A/S Roskilde, Denmark) were coated with 100 μl of pollen extracts (20 $\mu\text{g}/\text{ml}$ protein) in 0.1M carbonate buffer (pH - 9.6) and incubated overnight at 4°C . The plates were thoroughly washed with PBS (pH - 7.4) followed by blocking the free sites with 1% BSA. Patient's sera,

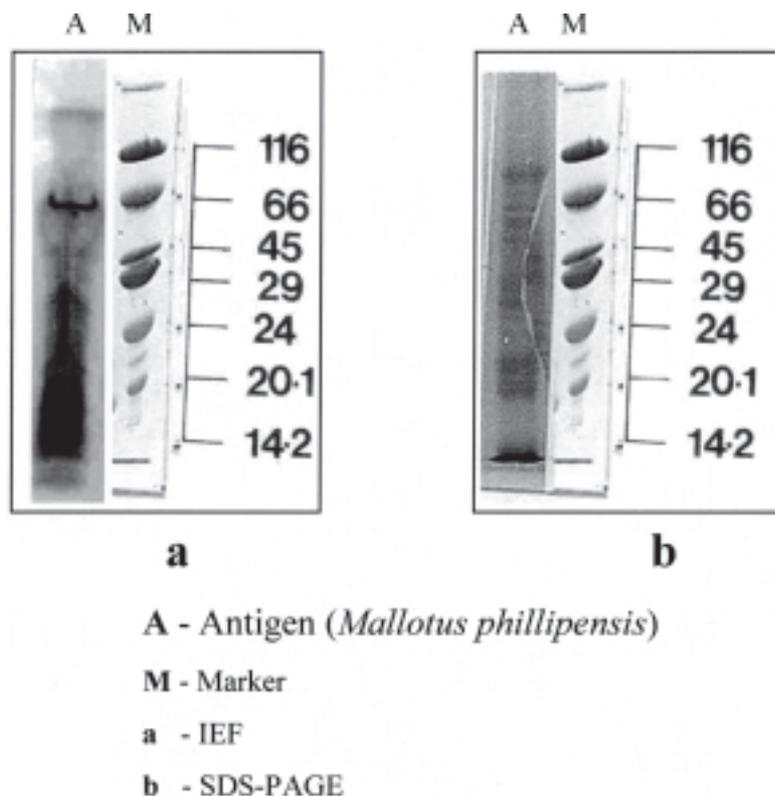


Figure 2.

a) IEF protein profile of *Mallotus phillipensis* showing multiprotein fractions separated on precasted PAG-plate between 3.5-9.5 pI range.

b) SDS-PAGE profile of *Mallotus phillipensis* showing multiprotein fractions in molecular weight range of 14-131 kD.

diluted to 1:10 were added to each well and incubated overnight at 4° C. After repeated washings, 100 µl of alkaline phosphatase labeled antihuman IgE antibodies (diluted 1: 1000) were added to each well and incubated at 37° C for 4 hrs. For colour development p-nitrophenyl phosphate (p-NPP) was added to each well and incubated at 37° C for 45 min. Optical density (OD) was measured at 410 nm with Immuno-reader (Dynatech Medical Products Ltd., Guernsey, UK).

OD values for different serum samples were analyzed according to the criteria laid down by Kauffman et al [27] with slight modifications. Depending on percent binding, OD values were categorized into 2 groups i.e. < 15% and > 15%. Sera showing more than 30% binding were considered to have significantly raised ($p < 0.05$) specific IgE against MP pollen antigen.

Immunoblot (Western Blot)

Protein bands separated onto SDS-PAGE were electrophoretically transferred to the NC membrane following the method of Towbin et al [28]. Proteins were then blotted to NC membranes by electro transfer, using Tris glycine buffer (pH 8.3). After blotting for 5 hrs, unbound sites on the NC membranes were blocked by incubating them with 3% BSA. After washing the NC membrane strips were incubated with pooled as well as individual sera (1:5) of ELISA positive patients. Pooled sera from healthy volunteers showing negative skin reactivity and ELISA were used as control. After washing, the strips were incubated with antihuman IgE peroxidase conjugate(1:000) (Sigma, USA). DAB (Diaminobenzidine) solution was used as substrate for colour development.

Results

The outcome of the investigation has been discussed under two main headings: Biochemical characterization and Immunological Studies.

Biochemical characterization

The total soluble protein in the crude MP pollen extract was estimated to the tune of 1.32 mg/ml in 1:20 (w/v) concentration of crude extract.

Iso Electric Focussing: A total 28 bands were detected by Coomassie blue staining in the antigenic extract of MP and they were distributed between pI points of 3.6 to 9.3 (Figure 2a). Five of the proteins were focused in alkaline region within 7.5 to 9.3. Of these, fractions with pI 9.3 and 7.5 are more prominent than the other three. Thin and sharply focused bands represent most of the proteins. Proteins at pI 4.2, 4.4, 4.75, 6.4 and 6.2 were prominent and deeply stained

with Coomassie blue. Among other proteins with pIs, 3.6, 5.3, 6.75, 7.4 were also clearly stained. The bands with 6.95, 7.1 and 8.95 pI were diffused.

SDS-PAGE: A total 25 of protein bands were detected in the extract of MP by Coomassie blue stain (Figure 2b). Molecular wt of separated proteins ranged from 11.7 kD to 140 kD. All the fractions were evenly distributed throughout the profile. Four high molecular weight fractions were also detected with molecular weights of 109, 116, 120 and 140 kD. Among them 120 kD fraction is prominent. Protein bands with molecular weight of 36, 69 and 71 kD were darkly stained and prominent. Most of the other proteins separated on SDS-PAGE were represented by well stained bands, but some low molecular weight (13.7, 17.2, 17.7 and 18 kD) proteins were diffused.

Immunoassay studies

Allergenicity of the MP pollen

The total 425 patients were tested against antigenic extracts of MP. Among them the male/female ratio was 268: 157, and 67.7% of the patients were older than 15 years. All the patients tested at different medical centers were diagnosed in three groups based on the nature of their symptoms. More than 50% (52.9%) of the patients were suffering from both allergic rhinitis and bronchial asthma. However, 23.5% patients each were suffering from either allergic rhinitis or asthma. Seasonality of symptoms of patients was categorized into three groups i.e. seasonal, perennial or irregular. Seasonal allergic cases were 40.5%, whereas, perennial cases were 26.1% against 33.4% who had irregular symptoms during the year.

Among the 425 patients tested, a maximum number i.e. 218 atopic subjects were tested at Kolkata and maximum sensitivity of 32.5% (1+ and above skin reaction) was recorded. It was followed by 25.3% at Thiruvananthapuram and 13.1% at Chandigarh. But only 5% subjects showed 1+ and above skin reaction at Delhi. However, marked skin positivity was 5.7% of the subjects at all the centers. Again at Kolkata a maximum number of patients showed marked skin test positivity (2+ and above) i.e. 7.3% followed by 4.7% and 3.6% at Thiruvananthapuram and Chandigarh, respectively. Only one patient showed marked positivity at Delhi. (Table 1).

Specific IgE concentration against M.P. pollen allergen

Out of 20 sera tested, 10 were considered negative as they had less than 15% binding with MP antigen. Out of the rest, 7 subjects had significantly raised ($p < 0.05$) specific IgE against MP pollen antigen. However 50% of the serum samples showed more than 15% binding with MP antigen (Table 2).

Table 1. Skin prick test results against *Mallotus phillipensis* pollen antigen tested on the patients in different parts of India

Places	No of Patients tested	Skin reactivity			Skin sensitivity		Skin positivity	
		1+	2+	3+	1 + to + 3		2 + to 3 +	
					No	%	No	%
Kolkata	218	55	16	-	71	32,5	16	7.3
Chandigarh	84	7	3	1	11	13.1	4	3.6
Thiruvana-thapuram	63	13	2	1	16	25.3	3	4.7
Delhi	60	2	-	1	3	5.0	1	1.6
Total	425	77	21	3	101	23.8	24	5.7

Immunoblot

Immunoblot profile of MP is provided in Figure 3. Altogether 11 fractions were recognized as IgE binding among 16 fractions transferred to NC membrane, detected with pooled sera of the ELISA positive patients. Molecular weights of these fractions varied from 12 - 131 kD. Four low molecular weight fractions were bound to IgE in the patient's sera with molecular weights of 12, 21, 29.3 and 32 kD. Four high molecular wt proteins i.e. 116, 121, 126, 131 kD were also recognized as allergenic. The rest of the three IgE binding fractions had molecular wt of 45, 52 and 59 kD.

In the sera of 12 individual patients (Figure 3), a maximum 11 fractions were detected to have IgE binding in one patient. Most of the IgE binding fractions fall in

the molecular wt range of 12 to 59 kD (Table 2). The rest 4 had high molecular wt (116, 121, 126 and 131 kD) protein epitopes. Another 2 serum samples showed binding with 10 fractions each. Sera of 5 patients' had shown binding with 9 fractions and 3 serum samples had specific IgE binding with 8 fractions.

As far as IgE binding of each fraction was concerned, 3 proteins with molecular wt 12, 21 and 121 kD showed binding with 12 sera (100%). Another three fractions with molecular mass 32, 45 and 59 kD showed binding to specific IgE in 10 patients (92.3%). 126 and 29.3 kD proteins had 84.6% and 69.2% binding in 9 and 8 persons respectively. Serum samples of 6 (54.5%) patients showed binding with 116 kD protein whereas 52 kD band had 45.5% binding. Three protein fractions of 85.6, 90 and 103 kD did not show binding with any serum.

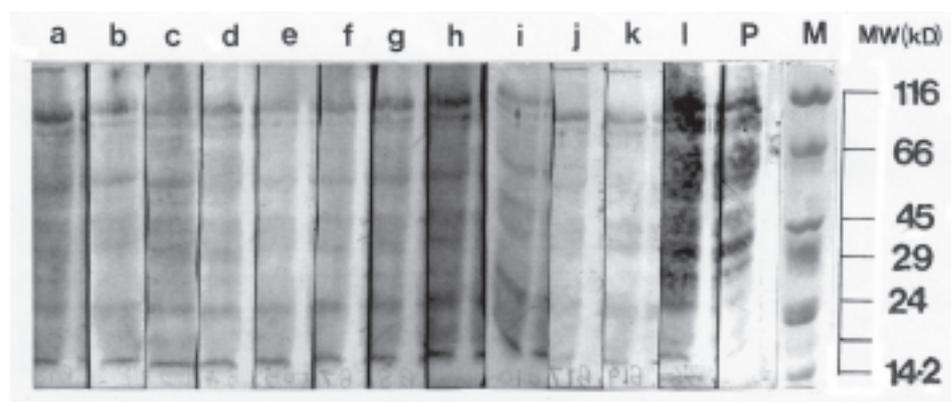


Figure 3. Specific IgE binding fractions of *Mallotus phillipensis* pollen antigen detected by immunoblot when probed with sera from 12 individual atopic patients and pooled together
a-I - Individual patient's sera
P - Pooled sera
M - Molecular weight marker protein (kD)

Table 2. Percent binding of allergen specific IgE antibodies estimated by ELISA in the sera of skin test positive patients against *Mallotus phillipensis* pollen antigen

Antigen used	No of sera tested by ELISA	% Binding				No of ELISA (>15%) + ve sera	Correlation with SPT (%)
		0-15%	15-30%	30-60%	>60%		
Mallotus phillipensis	20	10	3	2	5	10	50.0

Of all the separated proteins fractions with molecular weight 12, 21, 32, 45, 59 and 121 kD are considered major allergens in MP pollen extract showing 100% binding.

Discussion

In the past few decades the reported cases of the allergic rhinitis and other related diseases like asthma and urticaria have grown at tremendous rate as recorded from various epidemiological studies carried out all over the world [29, 30]. In India, the volume of respiratory disease has increased from 10% in 1960 [31] to 30% in the year 2000 [12, 32]. The pollen grains constitute one of the important components of air and are considered as major causative agent of respiratory disease, as is evident by the spurt of literature from across the world during last few decades [2,3, 7, 8, 12, 33].

Since the industrial revolution, there has been continuous transformations in the vegetation of the localities at micro as well as macro levels, due to climatic changes and the anthropogenic activities. In Italy, a recent study showed a new emerging spectrum of allergens which includes a number of new pollen types, not known as allergens earlier [33]. Similar studies have been carried out on all India level in order to identify the new allergens at different ecogeographic regions of the country, that supports a varied spectrum of pollen flora [12]. Among the different species from Himalayas, under investigation, pollen of MP is one of the important cosmopolitan genus.

Sensitization to multiple pollen species is a frequent diagnostic event as several allergenic molecules with high level of homology have been identified in the pollen of the same family [34, 35] due to the phenomenon of cross reactivity. It has been reported that atopic patients with a history of grass pollen allergy often develop sensitivities to a variety of species of grasses [36]. This could be true for pollen of MP also since other members

of the Euphorbiaceae family are well established allergens. Strong sensitizing plants of this family cross-react between pollen and seeds of *Ricinus communis*; pollen of *Ricinus communis* and *Putranjiva roxburghii*; and pollen of *Mercurialis annua*, *Ricinus communis*, and latex from *Hevea brasiliensis* have been reported [14, 15, 18, 37].

MP is a small much branched evergreen tree with thin grey bark. It is distributed throughout India, occasionally ascending to 1,500 msl in the outer Himalayas, commonly found in Sal and mixed forests. Pollen of MP is reported from air in different parts of the country [12, 21]. Pollen is tricolporate, as many other members of the family, 22 - 24 in diameter and easily respirable by the human beings. We investigated the *Mallotus phillipensis* pollen for the allergenic and antigenic properties of the crude pollen antigen. Sensitization pattern of the MP pollen antigen was assessed among atopic population, and its biochemical characterization was carried out.

MP pollen induced an average sensitivity in 5.7% in the atopic patients from different parts of the country, a new record from India. A large number of the patients showed 1+ skin reaction against MP pollen at Kolkata and Thiruvananthapuram. They constituted the risk group patients, as they might develop allergy in future due to repeated exposure to the allergens and/or could be showing non-specific reaction. Sensitivity of the patients can increase due to prolonged exposure to MP pollen or while interacting with other environmental factors. However, other members of the family Euphorbiaceae (*Putranjiva roxburghii* and *Ricinus communis*) are known allergens in India. Skin sensitivity against *Ricinus communis* is recorded to be 9% - 46% in patients whereas *Putranjiva roxburghii* is reported to elicit allergic reaction in 0.37% to 12% patients as revealed by different studies from India [15, 16, 38,39]. Therefore it is suspected that skin test reaction recorded against MP could also be due to cross reactivity with other members of this family.

MP is a cosmopolitan genus and part of the vegetation all over India. Higher skin sensitivity at Kolkata, Chandigarh and Thiruvananthapuram can be explained on the basis of its plantation in different parts in the country in addition to Himalayan region. However, MP does not form part of Delhi's vegetation, therefore poor sensitization in the population is expected.

Among all the 24 skin test positive patients at all the centers, higher number of males as compared to females showed skin reactivity. Subjects above of 15 years of age were recorded to be higher in number with skin reactivity as compared to children. Most of the patients were suffering from allergic rhinitis and bronchial asthma as well. Also most of the patients had seasonal or perennial symptoms. Many of the patients are reported to have symptoms during pollen season or exaggerated symptoms in months following pollen season to which they were allergic. It is in conformity with the findings that prevalence of hay fever symptoms together with positive skin test to pollen was significantly higher in the exposed (13.6%) vs. the less exposed community [40].

Allergenic activity is dependent on the ability of the proteins to interact with specific IgE antibodies present in the atopic patients. Generally, proteins are responsible for causing allergies in susceptible individuals but carbohydrates and lectins also act as allergenic components but only occasionally. The efficacy of any pollen antigen used for allergy diagnosis and immunotherapy always depends on the quality of the extract used, therefore we have characterized the MP pollen extract.

The total soluble protein content of MP pollen antigens was 1.32 mg/ml and it seemed to be low as compared to the tree pollen allergens from plains as observed by us [12, 13]. However, low protein content was also reported in *Alnus incana* pollen [41], and *Platanus* (850 µg/ml), and 1250 µg/ml in *Dactylis glomerata* [42]. However, total protein is not considered to be the yardstick for biological potency of the extracts and the presence of IgE recognizing protein fractions is important.

The molecular separation of pollen antigens by IEF revealed multiple protein fractions in pI range of 3.1 - 9.6 with majority of fractions in acidic pI range as also reported in *Ricinus communis* and *Putranjiva roxburghii* allergens [15,19,43]. However, presence of basic proteins is also not a unique phenomenon in MP as 2 proteins in *Ricinus communis* were also recorded in the basic region [43]. The pollen of MP, separated into multiple proteins, between 11.7 and 140 kD. protein epitopes of 20, 25, 29, 36 and 54 kD detected in MP antigen are observed to be common to *Ricinus communis* antigen [42] and may be considered cross reacting allergens.

To substantiate the allergy diagnosis, skin test could be taken as a primary diagnostic test. To confirm IgE mediated hypersensitivity, estimation of specific IgE levels in the sera of the patients against allergens is

employed as an in vitro assay. Moderate (50%) correlation between skin positive patients and presence of raised specific IgE antibodies against MP antigen was observed but such results are also reported by other authors [44, 45]. This could be due to poor exposure to MP pollen in these patients as the concentration of the MP pollen in air is not very high and moreover it coexists with many of the other pollen grains of Euphorbiaceae, including *Ricinus communis* and *Putranjiva roxburghii*, and there could be homology between their biochemical characteristics. The elevated antibodies in the patients may be against other related genera but also recognized epitopes in MP pollen antigens with some common properties. Another possibility could be that some of the patients were not tested in the pollen season (October to December) of MP. Therefore, as blood samples were collected only after skin tests, low titer of the antibodies in the sera, thus not detected by ELISA, could be a possibility.

Immunoblot analysis has been carried out to identify the allergenic components in MP pollen antigens by probing the protein profile with the pooled sera of the patients showing positive ELISA. Immunoblot analysis has also been used to study the heterogeneity in the binding of antibodies to major protein fractions of MP pollen. Immunoblot analysis showed 11 IgE binding fractions in MP antigen. Among these, five proteins with 21, 32, 116, 121 and 126 kD molecular weight showed strong immunostaining with MP antigen from pooled sera. Multiple allergenic fractions are also reported from other Euphorbiaceae members, *Ricinus communis* (13 fractions) and *Putranjiva roxburghii* (12 fractions) in molecular weight range of 14 - 92 kD [13, 15]. Multiple allergens in *Mercurialis annua*, another member of Euphorbiaceae from Mediterranean Europe, has been reported [18] with 2 major allergens of low molecular weight (15.8 kD and 14.1 kD).

The MP antigen also revealed a number of IgE binding fractions, when probed with individual patients sera. Proteins with 100% binding are 12, 21 and 121 kD fractions, considered major allergens. Another 4 fractions (32, 45, 54 and 126 kD) showed more than 50% binding, thus they can be considered important allergenic fractions, while others showed poor binding (see Table 3). Singh et al [13] highlighted 66 and 70 kD fractions in *Ricinus communis* as the principle proteins in the extracts, whereas in *Putranjiva roxburghii*, five proteins with MW 30, 43, 55, 80 and 92 kD were recognized by specific IgE binding proteins present in the patients sera [16]. Shared allergenicity between *Ricinus* and *Putranjiva* pollen extracts with 6 common proteins is also confirmed by immunoblot inhibition studies of *Mercurialis annua* which showed cross reactivity with *Ricinus communis* pollen antigen [18]. Therefore, crossreactivity to MP pollen with *Ricinus communis* and *Putranjiva roxburghii* is to be studied in order to further establish MP as an important allergen from India. However, the identification of allergenic fractions in MP antigen, which has been investigated

Table 3. Specific IgE binding protein fractions of *Mallotus phillipensis* antigens with pooled and individual sera of patients

	Mol. W t	12.0	21.0	29.3	32.0	36.8	45.0	52.0	59.0	66.8	85.6	90.0	103.0	116.0	121.0	126.0	126.0	131.0
Pooled	+	+	+	+	-	+	+	+	+	-	-	-	-	+	+	+	+	11
1	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	11
2	+	+	+	+	-	+	-	+	+	-	-	-	-	-	+	+	-	8
3	+	+	-	+	+	+	+	+	+	-	-	-	-	+	+	+	-	10
4	+	+	+	+	-	+	-	+	+	+	-	-	-	+	+	+	-	10
5	+	+	-	+	+	+	-	+	+	+	-	-	-	-	+	+	-	9
6	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	9
7	+	+	-	+	-	+	-	+	+	-	-	-	-	+	+	-	-	7
8	+	+	+	-	-	+	+	+	+	-	-	-	-	+	+	+	-	9
9	+	+	+	+	-	+	+	+	+	-	-	-	-	+	+	-	-	9
10	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-	9
11	+	+	+	+	-	-	-	+	+	+	-	-	-	-	+	+	-	8
12	+	+	-	+	+	+	-	+	+	-	-	-	-	-	+	+	-	8
	13	13	9	12	5	12	6	12	3	0	0	0	0	7	13	11	2	

12, 21 and 121 kD fractions had 100% binding
 32, 45, 59 kD protein fractions showed 92.3% binding
 126 kD fraction had 84.4% binding

for the first time, will be useful for diagnosis of allergy patients and manufacture of allergens in the country.

In conclusion, the pollen of *Mallotus phillipensis* has been studied for its allergenicity in Himalayas and other parts of the country and its important IgE binding proteins have been identified by partial characterization and made a basis to study the cross reactivity among the Euphorbiaceae family members. The results discussed in this study will provide a basis for further molecular characterization and standardization of MP extract with particular reference to Type I hypersensitivity, and will add to the information regarding the characterization and standardization of the allergenic extracts from India to be used for diagnosis and therapy of millions of allergy sufferers.

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References

1. Singh, A.B. and Singh, A. Pollen Allergy- A global scenario. In: Recent trends in Aerobiology, Allergy and Immunology. Ed. S.N. Agashe, Oxford IBH, 1994.
2. Potter P C and Cadman A. Pollen allergy in South Africa. Clinical and Exp Allergy; 1996. 26: 1347 - 1354.
3. D' Amato G, Spiekma F Th M, Liccardi G, Jagar S, Russo M, Kontou - Filli K, Nikkels H, Wuthrich B and Bonini S. Pollen related allergy in Europe. Position paper. Allergy; 1998. 53: 567 - 578.
4. Singh A B and Rawat A. Aeroallergens in India: current status. In Asthma and Allergy. 2000. Ed Dr. Rajendra Prasad.
5. Eriksson N E, Wihl J A, Arrendal H and Strandhede S O. Tree pollen allergy II. Sensitization to various tree pollen allergens in Sweden- a multicentre study. Allergy; 1984. 39: 610-617.
6. Bucholtz G A, Lockey R F and Serbousek D. Bald cypress tree (*Taxodium distichum*) pollen, an allergen. Ann Allergy; 1985. 55: 805 - 810.
7. Park J W, Ko S H, Kim C W, Jeoung B J and Hong C S. Identification and characterization of the major allergen of the *Humulus japonicus* pollen. Clin. Exp. Allergy; 1999. 29: 1080 - 1086.
8. Ezeamuzie C I, Thomson M S, Al-Ali S, Dowa, Khan M and Hijazi Z. Asthma in the desert: spectrum of the sensitizing aeroallergens. Allergy; 2000. 55: 159 -162.

9. Rawat A, Singh A, Gaur S N, Kumar L, Roy I, Ravindran P, and Singh A. B. Clinical and immunologic evaluation of *Cedrus deodara* pollen: a new allergen from India. *Allergy*; 2000. 55: 620-626.
10. Rao M, Prakash O and Subbarao P V. Reaginic allergy to *Parthenium* pollen evaluation by skin test and RAST. *J Allergy*; 1985. 15: 449 - 454.
11. Kumar P R, Mathew P M and Ravindran P. Studies on the allergenicity of nine tropical pollen allergens. *Grana*; 1998. 37: 185-188.
12. Anonymous. All India Coordinated Project on Aeroallergens and Human Health. Report. 2000. Ministry of Environment and forests, New Delhi.
13. Singh A B, Malik P, Parkash D and Gangal S V. Identification of specific IgE binding protein fractions in Castor Bean (*Ricinus communis*) pollen obtained from different source materials. *Grana*; 1993. 3: 376 - 380.
14. Singh A, Panzani R C, Singh A B. Specific IgE to castor bean (*Ricinus communis*) pollen in sera of clinically sensitive patients to seeds. *J. Invest Allergol Clin Immunol*; 1997. 7 (3): 169 - 174.
15. Singh B P, Verma J, Sridhara S, Rai D, Makhija N, Gaur S N and Gangal S V. Immunochemical characterization of *Putranjiva roxburghii* pollen extract and cross reactivity with *Ricinus communis*. *Int Arch Allergy Immunol*; 1997. 114: 251-257.
16. Parui A, Mondal A K, and Mandal S. Identification and partial characterization of the allergenic properties of *Ricinus communis* L. pollen - a new approach. *Grana*; 1999. 38: 311-15.
17. Ariano R, Panzani R C, Falagiani P, Chiapella M and Augeri G. Respiratory allergy to the pollen of *Mercurialis annua* (Euphorbiaceae). *Ann. Allergy*; 1993.10: 249 - 254.
18. Vallverdu A, Garcia-Ortega P, Martínez J, Esteban M I, de Molina M, Fernández- Tavora L, Fernández J, Bartolome B and Palacios R. *Mercurialis annua*: characterization of main allergens and cross-reactivity with other species. *Int Arch Allergy Immunol*; 1997. 112 (4): 356 - 364.
19. García - González J J, Bartolomé - Závala B, Del Martrigo - Pérez M, Barceló - Muñoz J M, Fernández - Meléndez S, Negro - Carrasco M A , Carmona - Bueno M J, Vegta - Chicote J M , Muñoz - Roman C, Palacios - Peláez R, Cabezudo- Artero Band Martínez-Quesada J. Pollinosis to *Ricinus communis* (castor bean): an aerobiological, clinical and immunochemical study. *Clin Exp Allergy*; 1999. 29 : 1265 - 1275.
20. Tomei F; Marini A, Marcellini L, Riservato R. Latex allergy. History, diagnosis and prevention. *Paminerva Med*; 2000. 43 (3): 217- 222.
21. Gaur R D and Kala S P. Studies on the aerobiology of a Himalayan alpine zone, Rudranath, India. *Arctic Alpine Res*; 1984. 16: 173 - 183.
22. Lowry O, Rosenborough N, Farr A, Randall R. Protein measurement with Folin phenol reagent. *J Biol Chem*; 1951. 193: 263 - 275.
23. Garfin D E. Isoelectric Focussing. In *Methods in Enzymology*, Ed Deutscher Mp; 1990. 182: 459 - 477.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*; 1970. 227: 680 - 685.
25. Singh A B, Malik P, Parkash D and Gupta C K. Biological standardization of pollen allergens from India. *Asia Pacific J Allergy Immunol*; 1992.10: 103 - 109.
26. Sepúlveda H J, Longbottom J and Pepys J. Enzyme linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of *Aspergillus fumigatus*. *Clin Allergy*; 1979. 9: 359 - 357.
27. Kauffman H F, Bearmount F, Meurs H, vander Hede and Sand de veries K. Antibody measurements against *Aspergillus fumigatus* by means of double diffusion and Enzyme linked immunosorbent assay (ELISA). *J Allergy Clin Immunol*; 1983. 72: 255 - 261.
28. Towbin H, Staehalin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA*; 1979. 76: 4350 - 4354.
29. Schafer T and Ring J. Epidemiology of allergic disease. *Allergy*; 1997. 52 (Sup 38): 14 - 22.
30. Anonymous. World wide variations in the prevalence of asthma symptoms: the international study of asthma and allergies in childhood (ISSAC). *Eur Resp J*; 1998. 12 (2): 315 - 335.
31. Vishwanathan R. Definition, incidence, etiology and natural history of asthma. *Ind J Chest Dis*; 1964. 6:108 -124.
32. Chhabra S K, Gupta C K, Rajpal S and Chhabra P. Prevalence of asthma in school children in Delhi. *J Asthma*; 1998. 3: 291 - 296.
33. Corsico R, Falagiani P, Ariano R, Berra D, Biale C, Bonifezi F, Campi P, Feliziani V, Frenguelli G, Galimberti M, Gallesio M T, Liccardi G, Loreti A, Marcer G, Marcucci F, Meriggi A, Minelli M, Nardelli R, Nardi G, Nerginni C A, Papa G, Piu G, Pozzan M, D`Ambrosio F P and Riva G. An epidemiological survey on the allergological importance of some emerging pollens in Italy. *J Invest. Allergol Clin Immunol*; 2000. 10 (3): 155 - 161.
34. Corbi A L, Cortés C, Bousquet J, Basomba A, Cistero A, Garcia- Seller J, D` Amato G, Carreira J. Allergenic cross-reactivity among pollens of Urticaceae. *Int Arch Allergy Appl Immunol*; 1985. 77 (4): 377 - 383.
35. Barletta B, Afferni C, Tinghino R, Mari A, Di Felice G, Pini C. Cross reactivity between *Cupressus arizonica* and *Cupressus sempervirens* pollen extract. *J Allergy Clin Immunol*; 1996. 98: 797 - 804.
36. Leiferman K M and Gleich G J. The cross-reactivity of IgE antibodies with pollen antigens. I. Analysis of various species of grass pollens. *J Allergy Clin Immunol*; 1976. 58: 129 - 139.
37. Palsuo T, Panzani R C, Singh A.B, Ariano R, Alenius H, Turjanmaa K. Allergen cross reactivity between proteins of latex from *Hevea brasiliensis*, seed and pollen of *Ricinus communis*, and pollen of *Mercurialis annua*, members of Euphorbiaceae. *Allergy Asthma Proc*; 2002. 23(2):141-147.
38. Acharya P J. Skin test response to some inhalant allergens in patients of naso-bronchial allergy from Andhra Pradesh. *Asp Allergy App Immunol*; 1980. XIII: 14 - 18.
39. Prasad M and Haq H. Allergenic pollen of Aligarh Atmosphere. *Asp. of Allergy Appl Immunol*; 1984. 17: 91 - 98
40. Charpin D, Hughes B, Mallea M, Sutra J P, Balansard and Vervloet D. Seasonal allergic symptoms and their relation to pollen exposure in south-east France. *Clin Exp Allergy*; 1993. 23: 435 - 439.
41. Florvaag E, Elsayed S and Apold J. Comparative studies on tree pollen allergens. II Isolation of alder (*Alnus incana*) pollen allergens: purification and some characteristic of the major allergen pI 4.78. *Int Arch Allergy Appl Immunol*; 1982. 67: 49 - 56.
42. Varela S, Subiza J, Subiza J L, Rodríguez R, García B , Jerez M , Jiménez J A and Panzani R. Platanus pollens as important cause of pollinosis. *J Allergy Clin Immunol*; 1997. 100 (6): 748-754.
43. Singh A.B., Malik, P. Gangal, S.V. and Babu, C.R. Inter-specific variations in the antigenic extracts of *Ricinus communis* (Castor bean). Pollen collected from different source materials. *Grana*, 1992. 31: 229-235.

44. Somville M A, Machiels J, Gilles J G G and Saint Remy J R. Seasonal variation in IgE antibodies of grass-pollen hypersensitive patients depends on the steady state IgE concentration and is not related to clinical symptoms. *J Allergy Clin Immunol*; 1989. 83: 486 - 494.
45. Park J W, Kim C W, Kim K S, Choi S Y, Kang D B, Ko S H, Won J U and Yang J Y and Hong C S. Role of skin prick test and serological measurement of specific IgE in the diagnosis of occupational asthma resulting from exposure to vinyl sulphone reactive dyes. *J. Occup Environ Med* 2001. 58 (6): 411- 416.

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