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Biochemical and Immunological Studies on Eight Pollen Types from South Assam, India

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ABSTRACT

A total of 65 pollen types were identified from two years atmospheric pollen survey in the environmental conditions of South Assam. Out of them, eight pollen types viz., *Acacia auriculiformis*, *Amaranthus spinosus*, *Cassia alata*, *Cleome gynandra*, *Cocos nucifera*, *Imperata cylindrica*, *Ricinus communis* and *Trewia nudiflora*, were selected for biochemical studies on the basis of their dominance in the study sites. Among the sample extract tested, *Ricinus communis* was found to contain the highest amount of soluble protein, free amino acid and total carbohydrate, per gram of dry weight followed by *Imperata cylindrica* and *Cassia alata*. Maximum numbers of protein polypeptide bands were detected in the sample extract of *Cassia alata* by polyacrylamide gel electrophoresis method followed by *Acacia auriculiformis*, *Imperata cylindrica* and *Cocos nucifera*. IgE binding protein fractions were maximum in *Cassia alata* and minimum in *Trewia nudiflora*.

Key words: Aerobiology; Allergy; ELISA; Immunology; Pollen

INTRODUCTION

The allergic diseases are a severe problem for the community as well as for the national economy.¹ It is a local problem, therefore the prerequisite for the diagnosis and treatment of allergy patients is the knowledge of season and prevalence of the airborne allergens to which the patients of a given locality are exposed.² Increasing morbidity and mortality due to

allergic disorders is of major global concern today. About 20% of the world populations are estimated to suffer from one allergic disease or another.^{3,4}

Major causes for these escalating trends in allergic diseases are increased exposure to sensitizing allergens and lesser stimulation of our immune system during the critical periods of its development.⁵ A recent survey carried out in India shows that about 20-30% of the population suffer from allergic rhinitis and that 15% developed asthma.^{6,7} Worldwide, the prevalence of asthma varies from population to population, and is influenced by factors like inbreeding, low altitude, damp climate, urbanization, and industrialization. Asthma is more prevalent, more severe, and more of a

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drain on health care resources in poor families and in inner city than elsewhere.⁸

The importance of biochemical and immunological standardization of antigenic and allergenic components is being emphasized for effective diagnosis and management of allergic diseases all over the world.⁹⁻¹³ From the clinical point of view it is important to determine the details about the occurrence of allergenic bioparticales in the atmosphere, their chemical composition and molecular characterization.¹⁴ The well characterized antigens help in quality control of antigenic extracts and assist in proper clinical diagnosis and specific immunotherapy.¹⁵⁻¹⁷ Though several pollens have been studied in detail for purification and characterization of allergic components¹⁸⁻²⁰, many are yet to be studied for their allergic components.

In the light of the above fact, an attempt has been made to identify the common allergic pollen types of South Assam which is located between 24°5' N latitude and 92°48' E longitude and about 26–27m above the mean sea level, through biochemical and immunological assay. Physiographically the area is plain with some hillocks in and around the tea garden area. It is bounded by N.C.Hills district in the North, Mizoram in the South, Manipur in the East and Bangladesh in the West.

MATERIAL AND METHODS

Preparation of Pollen Extract

For biochemical and immunological analysis, flower of 8 selected pollen types were collected during their flowering period and were air dried. After drying, the flowers were crushed gently and pollens were collected through sieving. Pollen with >90% purity was considered for further process. Preparation of pollen extracts was done by the method described by Singh et al.²¹ The samples were sonicated in ultrasonicator (Labsonic, Sartorius grade) at 100% amplitude by using 30mm probe for 20 minutes. The extract was then lyophilized in small aliquots and stored at -20°C till further use.

Biochemical Estimation

(i) Quantitative Estimation

The lyophilized samples were diluted to 1:10 w/v for the biochemical analysis. Soluble protein content of the samples was examined by Lowry's method²², free amino acid content by using ninhydrin solution and

the total soluble carbohydrate content was examined by Anthrone test method.

(ii) Qualitative Estimation

a. Enzyme Link Immuno-Sorbant Assay Test (ELISA)

Plate ELISA test of eight selected pollen extracts were performed on 47 suspected allergic patients and 3 normal volunteers. Patients were identified and categorized after verifying their medical reports. Blood sera were collected by expert medical doctors under the medical jurisdiction after informing the patients about the in-vitro tests to be performed. ELISA test was done as per the method outlined by Singh et al.²³ O-phenylenediamine dihydrochloride solution was used as the substrate and optical density was measured at 492nm using an ELISA plate reader (BioRad, USA). Intensity of the reaction was classified into four different grades based on percent binding, as outlined by Kauffman et al.²⁴

b. Protein Profiling by SDS-PAGE

SDS-PAGE was carried out by vertical discontinuous gel electrophoresis method.²⁵ The gel was polymerized with 4% stacking solution containing 10% SDS in Tris-HCL buffer (pH 6.8) and 12% separating gel solution with 10% SDS in Tris-HCL buffer (pH 8.8). The lyophilized samples were diluted 1/10 (w/v µg/µl) before use. About 30µl of the samples containing 80-100µg of protein were loaded in the gel per well. The samples were run at a constant voltage of 50V till the dye crossed the stacking gel and at 60V till the dye reached 0.5 cm above the lower end of the gel. Molecular weight marker protein from SIGMA (USA) was used to calibrate the gels (14.4 to 97.0 KD). After electrophoresis, the gel was stained with Silver stain (2.5% Silver nitrate, 37% formaldehyde, 25% Glutardialdehyde, 5% Sodium thiosulphate). The reaction was stopped by using stop solution (EDTA-Na₂·2H₂O). Washing was done with distilled water.

c. Immunoblot Analysis (Western Blotting)

Immunoblot analysis of IgE binding protein fractions was carried out by the method outlined by Towbin et al.²⁶ The protein samples were electrophoresed in 12% polyacrylamide gel by SDS-PAGE method and the protein in the gel was transferred to PVDF membrane (0.45µ, SIGMA) by immunoblotting method. After immunoblotting, the membrane was immersed in blocking buffer containing

3% BSA and incubated overnight at 4°C. This was followed by washing with wash buffer containing 0.05M phosphate buffer saline (PBS), Tween 20 and Triton X100. After washing, the membrane was incubated at 37°C for 2h with sera (diluted to 1:10 v/v in 0.5% BSA). Non pooled sera positive to plate ELISA test were selected for respective sample. Thereafter, the strips were incubated in antihuman IgE-HRP conjugate (Epsilon chain specific) at 37°C for 2h after washing as mentioned above. For colour development, the strips were treated with diaminobenzidine solution (10mg in 15ml Tris-buffer, pH 7.6, containing 12µl fresh 30% hydrogen peroxide). The reaction was terminated by using distilled water.

Statistical analysis was done to analyze the correlation or variation between or among the estimated data's using the MINITAB 12.2 statistical software.

RESULTS

Altogether 65 pollen types were identified from the atmosphere of Greater South Assam, which include the pollen of trees, weeds/cultigens and grasses. *Acacia auriculiformis*, *Amaranthus spinosus*, *Cocos nucifera* and *Ricinus communis* were found to be the dominant species of the genus *Acacia*, *Amaranthus*, *Cocos* and *Ricinus* respectively. Among the members of Poaceae group, *Imperata cylindrica* was found to be the predominant species in South Assam. Although the pollens of genus *Cleome* and *Trewia* were lower in the atmosphere during the survey period, but *Cleome gynandra* and *Trewia nudiflora* plants were found quite common in the vicinity of South Assam. Therefore all

these species were selected for their biochemical and immunological studies.

Biochemical Estimations

The sample extract of *Ricinus communis* was found to contain the highest amount of soluble protein (526.6mg), free amino acids (196.8mg) and total carbohydrate (85.4mg) per gram of dry weight followed by *Cassia alata* (138.4mg soluble protein; 125.0mg free amino acid and 28.2mg total carbohydrate per gram of dry weight, respectively). *Cocos nucifera* and *Imperata cylindrica* showed higher quantity of soluble protein (190.6mg/g & 146.8mg/g) and free amino acid (95.0mg/g & 108.0mg/g) respectively (Table 1).

Statistical analysis of the total soluble protein, carbohydrate and free amino acids content in different pollen extracts shows highly significant variations ($p < 0.01$). Among the different samples tested, maximum variation was recorded in protein content followed by free amino acid and least variation was found in the total carbohydrate content (Table 2).

Enzyme Linked Immuno-Sorbant Assay Test (ELISA)

Out of the 47 patients sera tested by plate ELISA technique against 8 different pollen extracts for the presence of specific IgE, 31 (65.9%) have shown positive binding with the pollen extracts (Table 3). Statistical analysis shows a significant deviation in the OD value of patient's sera from the control value at 1% level of significance (Table 4).

Out of the 31 ELISA positive sera recorded, maximum have shown +1 grade of binding (59.4%), followed by +2 (31.3%) and +3 (21.1%) grade.

Table 1. Estimation of Soluble Protein, Total Carbohydrate and Free Amino acids in some dominant air borne pollen samples collected from South Assam.

Pollen species	Total soluble protein (mg/g)	Total carbohydrate (mg/g)	Total free amino acids (mg/g)
<i>Acacia auriculiformis</i>	88.2	11.8	22.5
<i>Amaranthus spinosus</i>	100.4	14.8	45.0
<i>Cassia alata</i>	138.4	28.2	125.0
<i>Cleome gynandra</i>	156.2	9.6	90.0
<i>Cocos nucifera</i>	190.6	6.8	95.0
<i>Imperata cylindrica</i>	146.8	26.0	108.0
<i>Ricinus communis</i>	526.6	85.4	196.8
<i>Trewia nudiflora</i>	124.8	7.4	94.0

Table 2. Analysis of variance in the soluble protein, total carbohydrate and free amino acid contents between different pollens extracts.

ANOVA test for pollen samples.						
Source	DF	SS	MS	F _{calculated}	P value	F _{tabulated} 1%
Factor	2	102970	51485	6.54	0.006	5.78
Error	21	165310	7872			
Total	23	268280				

Varities	N	Means	St Dev.	SE	Mean and SE
Protein estimation	8	184.0	142.09	45.2	184.0 ± 45.2
Carbohydrate estimation	8	23.75	26.20	9.26	23.75 ± 9.26
Amino acid estimation	8	97.04	52.35	18.5	97.0 ± 18.5

** = Highly significant

Least number of sera showed +4 grade of binding (12.2%). Among the pollen sample tested, protein extract of *Amaranthus spinosus* showed higher grade of IgE binding, followed by *Cleome gynandra*, *Cassia alata* and *Acacia auriculiformis* (all showing +3 and +4 grades of bindings). However, when we compared the level of IgE production by different pollen samples, *Ricinus communis* was found to produce maximum IgE

level in the sera tested followed by *Cleome gynandra*, *Acacia auriculiformis* and *Amaranthus spinosus*. Among the blood sera tested, maximum were positive to the protein extract of *Cassia alata* (46.7%) followed by *Amaranthus spinosus* (23.5%), *Ricinus communis* (22.8%), *Cleome gynandra* (20.9%) and *Trewia nudiflora* (18.6%) respectively.

Table 3. Grading of ELISA test on the basis of percent binding of suspected patients sera with pollen antigen tested.

Antigen tested	Grading of ELISA result			
	+1 (30-50%)	+2 (>50-70%)	+3 (>70-90%)	+4 (>90%)
<i>Acacia auriculiformis</i>	5	0	2	0
<i>Amaranthus spinosus</i>	4	1	1	2
<i>Cassia alata</i>	15	4	0	1
<i>Cleome gynandra.</i>	3	3	2	1
<i>Cocos nucifera</i>	1	2	0	0
<i>Imperata cylindrica</i>	2	2	0	0
<i>Ricinus communis</i>	6	2	0	0
<i>Trewia nudiflora.</i>	7	1	0	0

Table 4. Statistical significance of IgE level in the sera of suspected allergic patients tested with the pollen antigens. Results greater than 30% of the negative control were considered positive.

Antigens	No. of observations		Mean OD		Standard deviation	Significance
	Patient	Control	Patient	Control		
<i>Acacia auriculiformis</i>	39	5	0.75	0.07	0.39	<0.01
<i>Amaranthus spinosus</i>	34	5	0.81	0.08	0.36	<0.01
<i>Cassia alata</i>	42	5	0.37	0.03	0.14	<0.01
<i>Cleome gynandra.</i>	43	5	0.68	0.04	0.29	<0.01
<i>Cocos nucifera</i>	42	5	0.40	0.04	0.04	<0.01
<i>Imperata cylindrica</i>	31	5	0.35	0.03	0.18	<0.01
<i>Ricinus communis</i>	35	5	0.79	0.05	0.58	<0.01
<i>Trewia nudiflora.</i>	43	5	0.35	0.02	0.09	<0.01

Table 5. Comparison of IgE binding proteins detected in the sample extracts of eight selected pollens from South Assam.

Pollen	Molecular weight(KD)
<i>Acacia auriculiformis</i>	29, 55, 90, 97
<i>Amaranthus spinosus</i>	63, 80
<i>Cassia alata</i>	27, 37, 40, 50, 55, 67, 85, 90
<i>Cleome gynandra</i> .	18, 20, 35, 60, 90
<i>Cocos nucifera</i>	32, 42, 45, 60, 66
<i>Imperata cylindrica</i>	67, 82, 90
<i>Ricinus communis</i>	33, 50
<i>Trewia nudiflora</i> .	41

Protein Profiling

The proteins of 8 pollen samples were resolved on the basis of molecular sieving. A total of 8-23 protein polypeptide bands were detected in the tested protein extracts (Figure 1).

The sample extract of *Cleome gynandra* resolved highest number of protein bands (27 bands) in the molecular weight range of 97.7-14.4 KD. The sample extract of *Cassia alata* resolved 23 protein bands in the weight range of 90-17KD. *Acacia auriculiformis* and *Trewia nudiflora* showed 19 and 15 protein bands each, in the molecular weight range of 97-15KD and 90-14KD respectively.

Imperata cylindrica and *Amaranthus spinosus* showed equal number of protein bands (14 bands each) in the weight range 19-17 KD and 97-17KD respectively. *Cocos nucifera* produced 13 bands in the weight range of 97-16KD, however, lowest number of distinct protein bands were visible in the sample extract of *Ricinus communis* (8 bands) between 75-33 KD. Nevertheless, a stretch dark band was visible in case of *Cocos nucifera* and *Ricinus communis* in the molecular weight range of 28-20 KD and 30-16 KD respectively.

Western Immunoblotting

Immunoblot analysis was carried out in order to detach the specific IgE (C-chain specific) binding proteins in pollen extracts using ELISA positive serum (Figure 2).

One to eight immunogenic protein fractions were detected in the pollen extracts tested. Maximum immunogenic protein fractions (8 fractions) were detected in the sample extract of *Cassia alata*, in the molecular weight range of 90-27 KD.

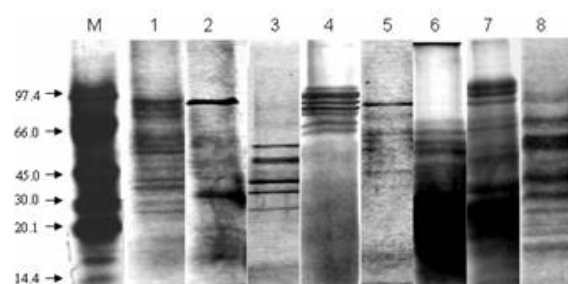


Figure 1. Protein profiling of different pollen types in 12% denaturing gel following SDS-PAGE: 1-*Cassia alata*, 2-*Cleome gynandra*, 3-*Trewia nudiflora*, 4-*Imperata cylindrica*, 5-*Amaranthus spinosus*, 6-*Ricinus communis*, 7-*Cocos nucifera* and 8-*Acacia auriculiformis*.

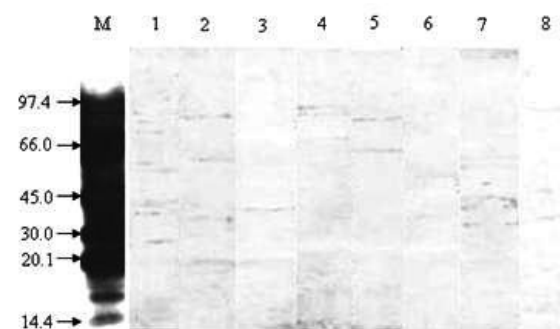


Figure 2 Immunogenic polypeptide bands of different pollen types detected by western blotting method: 1-*Cassia alata*, 2-*Cleome gynandra*, 3-*Trewia nudiflora*, 4-*Imperata cylindrica*, 5-*Amaranthus spinosus*, 6-*Ricinus communis*, 7-*Cocos nucifera* and 8-*Acacia auriculiformis*.

Acacia auriculiformis, *Cleome gynandra* and *Cocos nucifera* were found to contain 5 IgE binding fractions each in the weight range of 97-29KD, 90-18KD and 66-32KD respectively. *Trewia nudiflora* showed single immunogenic band at 41KD. Most of the dark and prominent protein bands visible in SDS gel were found to be immunogenic to the tested sera.

It can be noted that maximum IgE binding fractions were found at high molecular range between 40-97KD. Immunogenic fractions at the weight range of 50,55,60,67 and 90 were common in several pollen samples (Table 5).

DISCUSSION

Variation was recorded in the biochemical and immunological properties among the selected pollen species. Soluble protein content was found maximum in the sample extracts of selected pollen species. There are several reports supporting high protein content in the antigenic pollen extracts of *Ricinus communis*, *Cocos nucifera* and *Cassia alata* (Malik et al., Bhattacharya et al., Mondal et al., Singh et al. and Bist et al.).^{20,27-32} Report by All India coordinated project on aeroallergens and Human health have also stated high protein content in the pollen extract of *Cassia alata* (7.8mg/ml) followed by *Trewia nudiflora* (1.84mg/ml), *Cocos nucifera* (0.49-0.50mg/ml) and *Ricinus communis* (0.36mg/ml) in their studies from different states of India⁶. Malik et al. has quantified high amount of soluble protein (5.5 to 9.1mg/ml) from bud and flower extracts of *Ricinus communis*.²⁰ The pollen samples may show variation in biochemical properties from area to area and time to time due to the environmental variations and/or variation in analytical techniques³².

Out of 47 patients sera tested against 8 different pollen extracts for detection of specific IgE level, 31 have shown positive binding. Maximum sera was found positive to the protein extract of *Cassia alata* (46.7%) followed by *Amaranthus spinosus* (23.5%), *Ricinus communis* (22.8%), *Cleome gynandra* (20.9%) and *Trewia nudiflora* (18.6%).

All India coordinated project on aeroallergens and Human health however have reported 15.4% of ELISA positive cases of *Amaranthus spinosus*, 18.6% of *Cocos nucifera*, and 53.8% in *Ricinus communis*.⁶ Higher percentage of allergenicity of *Ricinus communis* was also reported by Dhaiya and Singh³³ and Rawat³⁴. Kumar et al. found 7% positive cases of *Imperata cylindrica* pollen extract by allergy skin test however in the present study 8.5% of positive cases were recorded by plate ELISA technique.³⁵ In contrast to the present report of 7% ELISA positive cases of *Cocos nucifera*, Choudhury et al. have reported 45.7% positive response in *Cocos nucifera* when tested by skin prick test method in Calcutta.³⁶ Heterogenicity was recorded in the IgE binding pattern of individual patient's sera, which could be due to the variation in environmental factors, sample extraction technique, sensitivity of patients against the antigens.^{1,37-39}

Protein profile of different pollen samples tested above showed variation except at certain molecular levels (viz., 50, 55, 60, 66 and 90KD), where similarity were observed among few samples. Variation in protein profile of pollen extracts can also be seen in earlier research findings.^{6,40} Variation can also be seen in the protein profile of the same species reported from different geographical area or even from the same geographical area. Bijli et al. resolved 37 polypeptide fractions from freeze dried *Imperata* inflorescence and 23 from air dried *Imperata* inflorescence.⁴⁰ Singh and Kumar have resolved 24 Commassie-Brilliant Blue stained bands in *Imperata cylindrica* in the molecular weight range of 10-100KD.³⁰ Present report however showed only 14 protein bands from air dried *Imperata* pollen. Singh and Dhaiya⁴¹ reported 22 protein bands in the weight range of 14-70KD. Nonetheless, the report by All India coordinated project on aeroallergens and Human health⁶ is similar to our findings and both have resolved 14 protein bands of *Amaranthus spinosus*. Present report also conforms to the findings of Singh et al.³¹ who have reported 50 and 66KD protein fraction of *Amaranthus spinosus* as prominent and thick. Garcia-Gonzalez et al., reported the molecular mass of the IgE binding proteins of *Ricinus communis* in between the molecular weight of 67-15.5 KDa, which is within the range of the present findings.⁴² Kumar et al. have reported 7 major IgE binding fractions of *Imperata cylindrica* at 85, 62, 57, 43, 4, 20 and 16KD but in the present report only 3 immunogenic protein fractions were detected.³⁵ Verma et al. and Bijli et al., however have also supported the present findings and have reported 67 KD protein fractions of *Imperata cylindrica* as highly immunogenic.^{43,44} Jaggi et al. reported similar number of immunogenic bands of *Cocos nucifera* as reported in the present study.⁴⁵ Literatures on the immunogenic protein profile of *Cassia alata*, *Cleome gynandra* and *Trewia nudiflora* could not be collected online, possibly no such work has yet been carried out on these pollen types.

Variation in the binding pattern of the pollen extract has been reported with individual sera. Heterogenicity in sera of 16 patients to grass pollen was reported by Peltre et al., as some sera recognized only one band whereas sera from other patients showed binding to 30 protein fractions.³⁷ Similar variation in binding pattern was also reported by Shen et al. in his findings on *Cyanodon dactylon* pollen extract on 21 patient's sera.³⁸ Present observation also proved heterogenicity

in the sera of 45 patients tested with the pollen extracts. Lesser number of immunogenic bands in the present study could be due the use of non-pooled sera. Variations could also be due to the variation in environmental conditions under which the plant grows, sample extraction procedure and sensitization of patients against antigens.¹

From the above, it can be stated that *Cassia alata*, *Amaranthus spinosus*, *Cocos nucifera*, *Imperata cylindrica*, *Acacia auriculiformis*, *Ricinus communis* and *Cleome gynandra* may contribute significantly to the aerobiology of South Assam in particular with different degree of allergenicity. *Trewia nudiflora* was found to be a weak allergen as compared to other tested species. Detailed atmospheric and clinical studies may probably help in controlling the allergic diseases contributed by the above studied pollen samples.

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