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EUCALYPTUS CITRIODORA POLLEN: AN AEROBIOLOGICAL, CLINICAL AND IMMUNOLOGICAL APPROACH

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ABSTRACT

Background: The pollen of *Eucalyptus citriodora* tree has been known as one of the important pollinosis causing airborne bioparticles. To evaluate aerobiological contribution of *E. citriodora* pollen vis-à-vis clinical and immunological characterization of an important IgE- reactive allergenic component present in this pollen. **Materials & Methods:** The seasonal periodicities of airborne *Eucalyptus* pollen were recorded. The allergising potential of *Eucalyptus* pollen antigen was investigated by skin prick tests[1,2,3], IgE- enzyme linked immuno sorbant assay (ELISA) and IgE specific dot immunoblotting. Demographic characterization of some selected *Eucalyptus* sensitive patients was done. The total pollen extract was first fractionated and then the maximum IgE-reactive fraction was subjected to gel filtration. Out of the eluted four subfractions, the highly active protein component was isolated and was further studied by activity gel, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Detection of glycoprotein component in the allergenic extract was also performed. **Results:** The occurrence of *Eucalyptus* pollen in air was seasonal with two peak periods in the month of October and March. Among 415 patients, skin test result showed 33.73% positive response. Some patients showed different types of respiratory problems. SDS-PAGE revealed the presence of allergenic components in the molecular weight range between 29-115 kDa. A fraction having remarkable IgE- reactivity was isolated as a 115 KD glycoprotein component. **Conclusion:** The aerobiological, clinical and immunological studies demonstrated that *E. citriodora* pollen is an important aeroallergen to cause respiratory disorders in the area of their occurrence.

KEYWORDS: Aeroallergen, *Eucalyptus citriodora* pollen, Immunoglobulin E reactivity.

INTRODUCTION

Eucalyptus citriodora Hook. of the family Myrtaceae is a lemon-scented quick growing evergreen gum tree. *Eucalyptus* populations originated in Australia, but it now grows in almost all tropical and subtropical areas and is cultivated in many other climates including India. It is cultivated in forests, along the road -side for timber, paper, oil and gum production. The tree flowers seasonally having entomophilous pollination mechanism. The prevalence of *Eucalyptus* pollen in air and its allergenicity were reported earlier based on clinical observation.[3,4,5] But there are no reports regarding the nature of its allergenic components so far. Identification, isolation and characterization of proteins responsible for IgE- mediated allergies have been the main goal of research in the last few years, because type I allergy is being an increasing clinical disorder in the developing countries like India.[6] Some pollen allergens (olive-Ole e 9, birch-Bet v 1, rye –Lol p 1, etc) had been internationally standardized.[7,8,9,10] The present study was undertaken to evaluate the importance of *Eucalyptus citriodora* pollen as a potential aeroallergen and to detect the allergenic components present in this pollen extract for clinical and diagnostic purposes.

MATERIALS AND METHODS

Aerobiological sampling

A continuous air sampling was conducted at Central Calcutta for two consecutive years (from July 2001 to June 2003) using a seven-day Burkard automatic volumetric sampler. The sampler was placed at a height about 12.0 m. on the roof of the Bose Institute main campus, situated at the central part of the Calcutta metropolis. The exposed tapes were mounted and microscopically examined according to the guideline of The British Aerobiology Federation[11] to record seasonal periodicities of the airborne *Eucalyptus* pollen.

Source material

Fresh pollen samples were collected from the anther of *E. citriodora* during the pollination months. The batch contained <1% non-pollen impurities.

Preperation of whole pollen extract: The dried pollen grains were defatted with diethyl ether and extracted in phosphate buffered saline (PBS; 0.1 M Na-phosphate, 150 mM NaCl, pH 7.2, 1:10 w/v) by continuous stirring for 16 h at 4^0C. After centrifugation (12500 g for 40 min), the supernatant was dialyzed in PBS and passed through 0.22 µm Millipore filter (Millipore Corp., Bedford, MA, USA). The filtrate was then lyophilized and stored (-70^0C) in sterile vials.

Determination of soluble protein and carbohydrate: The protein content of crude extract and different fractions were determined according to Lowry *et al*.[12] using bovine serum albumin (BSA) as standard. Total carbohydrate was estimated following Duboise *et al*.[13] using D-glucose standard.

Detection of glycoprotein

The presence of glycoprotein in the crude allergenic extract of *E. citriodora* pollen was detected by PAS (Periodic Acid Schiff) technique.[14] Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the crude extracts of *E. citriodora* pollen.[15] Then the gel was first fixed in 12.5% TCA (Trichloroacetic acid). After washing with distilled water the gel was immersed in a solution of 1% periodic acid containing 3% acetic acid. After thorough washing the gel was then transferred to a solution of fuchsin sulphite in dark conditon. Then the gel was placed in 0.5% potassium metabisulphite solution. The glycoprotein bands were detected after washing with distilled water.

Fractionation of *E. citriodora* pollen allergen

The whole pollen extract was fractionated in the range of 0-30%, 30-60%, 60-90% saturation of $(NH_4)_2SO_4$. Each salted out fraction dissolved in PBS was separately dialyzed (molecular cut-off limit 10,000) to remove traces of ammonia and stored at –20^0C. The allergenic activity of each fractions (referred to as EF1, EF2 & EF3) was determined by *in vivo* skin prick test and *in vitro* direct ELISA.[16] The principal allergenic fraction i.e. EF2 was then subjected to sephacryl S-200 column (d = 2.8 cm, h = 48 cm), equilibrated with 10 mM PBS, pH 7.2 for gel filtration. Fractions were collected in 3ml aliquots and the elution was monitored at 280 nm. The eluted protein aliquots respective to the four individual peaks were pooled to get four subfractions. The column was calibrated with a marker protein comprised of an admixture of β amylase (200 KD), Alcohol DH (150 KD) and Bovine Serum Albumin (66 KD) to make a molecular weight calibration curve.

Intradermal skin prick test (SPT) and collection of sera

The *E. citriodora* pollen extracts (1:50 w/v) was subjected to skin prick tests on 525 adult respiratory allergic patients with relevant case history attending the Institute of Child Health, Calcutta. To avoid masking of possible symptoms, corticosteroids and antihistamines were prohibited. The study was approved by the Ethics Committee of the hospital. Histamine diphosphate (10 mg/ml) and PBS were used as positive and negative controls respectively. Tests were performed with 20µl of aliquot of allergen solution placed on the ventral side of the forearm. The reaction was measured after 20 minutes and graded 1+ to 3+ as described by Stytis *et al*.[17] According to international guidelines, the positivity was defined as mean wheal diameter ≥ 3 mm, compared with negative control.[18] Control sera were collected from non-sensitized healthy volunteers [confirmed by negative skin reaction and IgE-ELISA] having no history of previous or current episode of any allergic diseases in them and their families. None of the patients were under immunotherapy, when blood was collected. The consent of every patient was taken prior to skin test and sera collection. Sera showing optical density (OD) > 3.0 times of the normal human sera i.e. negative control were used for various *in vitro* tests.

Case history

Comprehensive case history of each patient was recorded for demographic characterization of *Eucalyptus* sensitive patients by some questionaires. The ventilatory pulmonary function test was performed by a Dry Wedge Bollows Spirometer (Vitalograph) standardized according to the method of Chatterjee *et al*.[19]

Enzyme-linked immunosorbent assay

ELISAs were performed to determine *E. citriodora* pollen- specific IgE levels in individual patients sera against the crude pollen extract or fractions using antihuman IgE horseradish peroxidase conjugate (Sigma-Aldrich, St Louis, MO, USA) in 1:1000 dilution and *o*-phenylene diamine(OPD) substrate.[16] The absorbance was monitored with an ELISA reader (Thermo Labsystems, India, Model No. 352) at 492 nm. The individual serum having P/N value (ratio of optical density of patient sera with respect to control) > 3.0 were selected for further studies. Here, the control is the mean value from the panel of 7 normal (control group) sera.

11% SDS-PAGE

11% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for crude pollen extract and isolated fraction according to Laemmeli[15] in reducing condition. The protein components were observed by coomassic brilliant blue staining.

IgE specific dot immunoblotting

Dot blotting was performed on the pieces (2 X $2cm^2$) of nitrocellulose membrane to observe the IgE specific cross-reaction[20] and a negative control was also maintained. The peroxidase positive spots were detected using diaminobenzine[21] as substrate.

RESULTS

From the two year aerobiological survey of Central Calcutta, the pollen grains of *Eucalyptus* populations are found to be airborne from September to April in 2001-02

and from August to April in 2002-03 "Fig. 1". The pollen grains were tricolporate, oblate, having parasyncolpate exine (± 1.0 µm. thick) and sexine as thick as nexine with obscure pattern "Fig. 2". The measurements of polar axis x equatorial diameter were ± 16.3 x 24.0 µm. The occurrence of pollen in air was seasonal with two peak periods in the months of October 2001-2002 and March 2002-2003. The pollen was found frequently in the air with moderate concentration. During the peak months, the pollen grains contributed 2.50% of total aeropollen load on an average.

Demographic characterization of some selected *Eucalyptus* sensitive patients was done mentioning their age, sex, occupation, past history, present symptoms, allergenic sensitivity to other pollen and the ratio of FEV_1 (forced expiratory volume in the first second) and FVC (forced vital capacity) presented in table 1.

Skin prick tests were performed with *E. ciriodora* pollen extract since 1996. In a population of 525 respiratory allergic patients [mean age 33 years (age range: 13-53), M /F = 1.75 / 1.0], 33.73% showed positive response [Table 2]. Again, among the patients having positive skin reaction, 21.54% showed +1, 8.49% showed +2 and 3.70% showed +3 level of reaction. When skin test was performed with the three fractions (i.e. EF1, EF2 and EF3) on 45 *Eucalyptus* - sensitive patients, EF2 elicited maximum percentage of response (75.50%) [Table -2].

Further *in vitro* investigation by direct ELISA with pooled sera of 15 *Eucalyptus* sensitive patients showed that EF2 was the most active among the three fractions "Fig. 3". That's why this fraction (EF2) was selected for further study on the basis of both skin test and ELISA.

On the basis of positive SPT and high titre of *E. citriodora* pollen IgE specific ELISA results "Fig.3", 25 patients (M / F = 17 / 8, mean age 31 years) with allergic rhinitis or bronchial asthma or combination of both were selected for sera collection. Among them, 11 patients showed IgE reactivity to the isolated EF2 protein fraction.

The most reactive FE2 protein fraction was further subjected to gel filtration on sephacryl S-200 column. The eluted protein aliquots respective to the four individual peaks were pooled to get four sub-fractions, i.e. EF2A, EF2B, EF2C and EF2D "Fig. 4".

From skin test of these four sub-fractions on eleven sensitive patients, it was found that EF2A is the most allergenically potent [Table-2]. The potentiality of EF2A was also confirmed by direct ELISA using pooled sera of 9 *Eucalyptus* - sensitive patients "Fig. 5". A negative control was maintained all through.

So, from *in vivo* skin prick test and *in vitro* ELISA, it was found that EF2A showed the highest allergic activity. Molecular weight of the most active fraction was calculated from the molecular weight calibration curve "Fig.6" of the gel filtration column. It was found to be around 115 KD.

The PAGE analysis of crude pollen extract of *E. citriodora* demonstrated the protein profiles and it revealed eleven major protein bands of molecular weights 200, 115, 100, 94, 84, 68, 54, 42, 40, 36 and 30 KD "Fig. 7".

From Periodic Acid Schiff test of crude pollen extract of *E. citriodora*, it was found that three bands of molecular weight 89KD, 66KD and 55 KD contain glycoprotein "Fig. 8".

The dot immunoblotting assay was performed to test the cross reactivity of *E. citriodora* pollen extract to the serum pool prepared from the *Eucalyptus* sensitive patients. It was found that the allergic patient sera gave specific spot while negative control sera did not show any spot "Fig. 9". So, a strong binding of serum IgE was observed with the homologous antigen.

Table – 1: Demographic characterization of some *Eucalyptus* sensitive patients.

Patient No.	Age (Yr)	Sex	Occupation	History of present illness	Present symptoms	Adverse reaction to other pollen	FEV_1\FVC
1	24	M	Stays at home	Suffering for 4-5 years	Asthma, Skin rash, Repeated cold	*Azadirachta, Cocos, Phoenix*	85.04
2	33	F	House wife	Suffering for 10 years	Do	*Azadirachta*	61.48
3	25	M	Student	Since the age of 5 years	Cough, skin rash, sneezing	*Azadirachta, Cocos, Delonix*	65.13
4	34	M	Business	Since last 15-16 years	Do	*Cocos, Peltoforum, Borassus*	68.13
5	13	F	worker	Since the age of 5 years	Cough, asthma, sneezing	*Areca, Cocos, Borassus*	57.05
6	53	M	Business	Since last 4 years	Asthma, rhinitis, skin rash	*Delonix, Cocos*	59.56
7	21	M	Agriculturer	Since last 2 years	Cold, cough, Nose block	*Phoenix, Areca*	72.37
8	47	M	Service	Last 18-20 years	Repeated cold, sneezing	*Cocos, Areca*	75.5
9	18	F	Student	Last 10-11 years	Repeated cold, cough, sneezing	*Areca, Cocos, Delonix*	68.3
10	33	M	Service	Last 17-18 years	Cold, cough, sneezing, eye problem	*Cocos, Areca, Carica*	59.94
11	34	F	Service	Since 5 years	Repeated cold, sneezing	*Delonix, Areca*	65.00%

Table – 2: Results of Skin Prick Tests (SPT) of crude extract and different fractions of *E. citriodora* pollen.

Type	Total No. of patient	Level of reaction			Percentage of response
		+1	+2	+3	
Crude extract	525	21.54 %	8.49 %	3.70 %	33.73
EF 1	25	11.50 %	6.0 %	-	17.50
EF 2	25	45.50 %	20.0 %	10.0 %	75.50
EF 3	25	25.0 %	8.25 %	-	33.25
EF 2A	11	32.0 %	10.45 %	3.0 %	45.45
EF 2B	11	21.0%	6.27%	-	27.27%
EF 2C	11	15.18%	3.0%	-	18.18%
EF 2D	11	20.0%	7.27%	-	27.27%

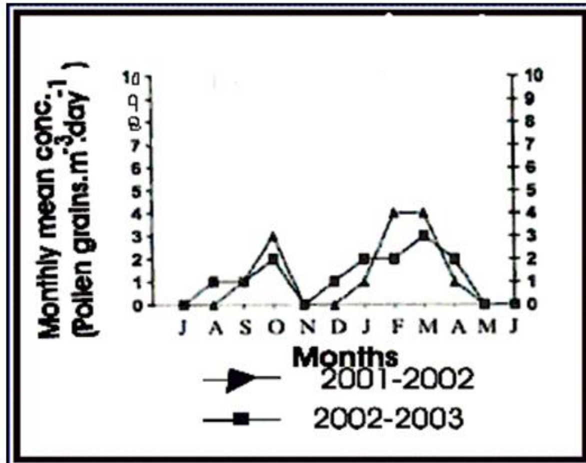


Fig. 1: Seasonal Variation of *Eucalyptus citriodora* pollen.

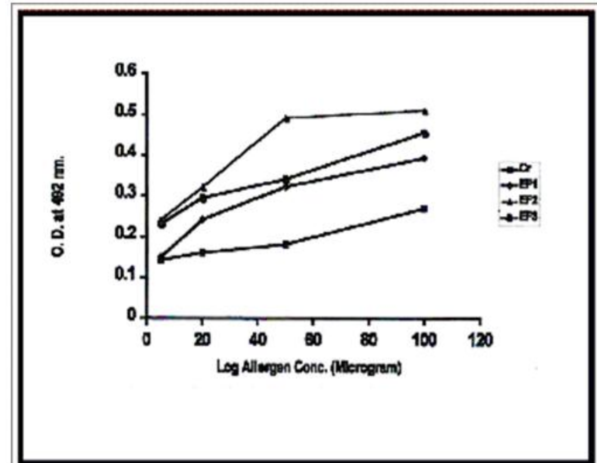


Fig. 3: Comparative ELISA study of a crude antigen and different $(NH_4)_2SO_4$ cut fractions of *E. citriodora.*

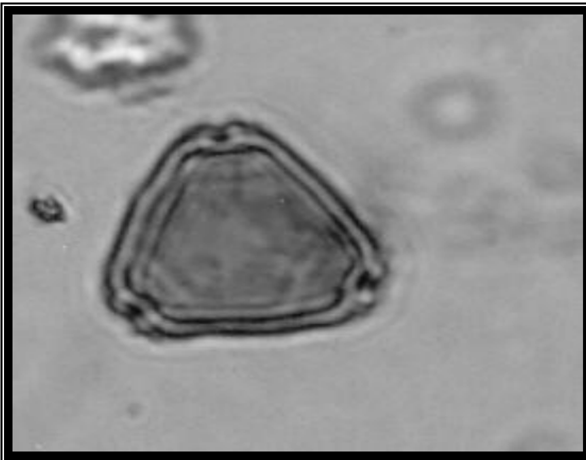


Fig. 2: *Eucalyptus citriodora* pollen grain (40 X).

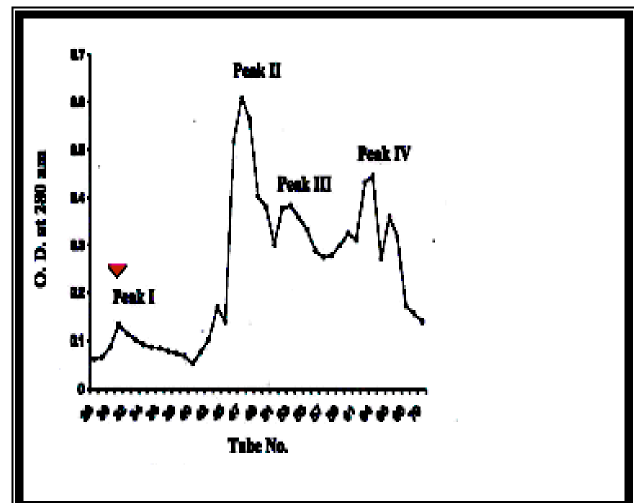


Fig. 4: Elution profile of protein fraction (EF2) of *E. citriodora* pollen by Sephacryl S-200 column.

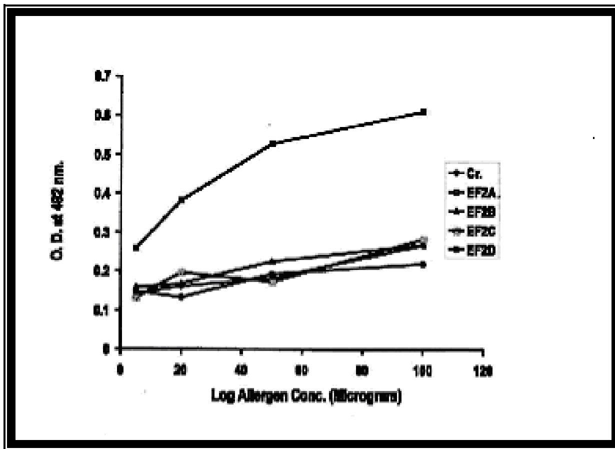


Fig. 5: Comparative ELISA study of a crude antigen and different subfractions of *E. citriodora.*

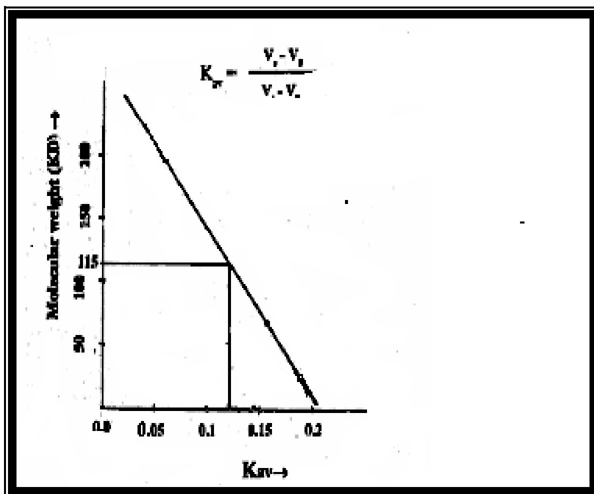


Fig. 6: Molecular weight calibration curve.

Kav = Represents fraction of the stationary gel volume

Vo = Void volume, Ve = Elution volume

Vt = Total bed volume

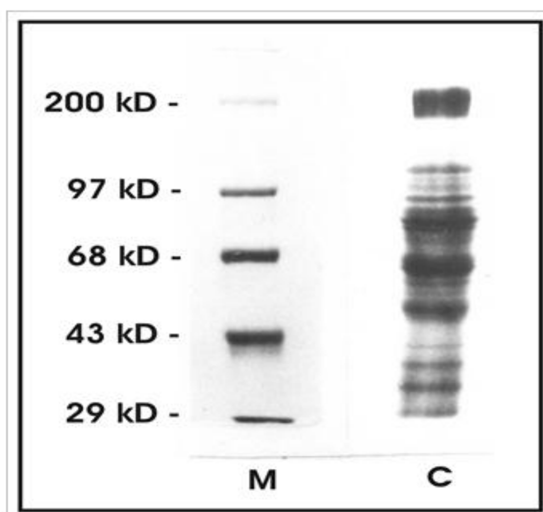


Fig. 7: 11 % SDS-PAGE of crude pollen extract of *E. citriodora* pollen.

M – Molecular weight marker C - Crude pollen extract

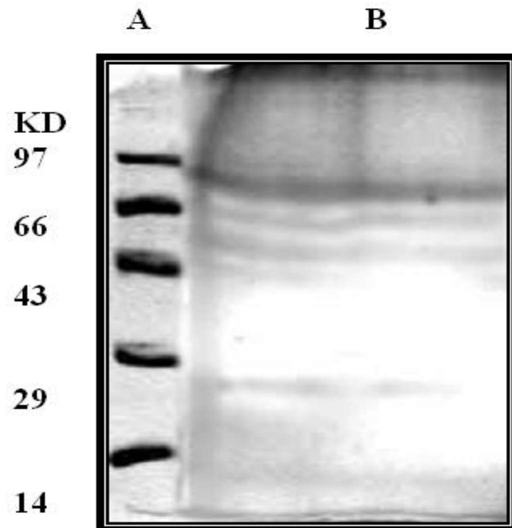
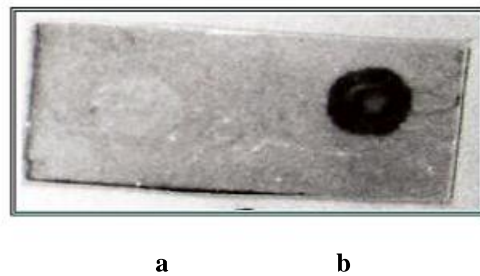


Fig. 8: Detection of glycoprotein of crude pollen extract of *Eucalyptus citriodora.*

A – Molecular weight marker, B –Glycoprotein profile of crude pollen extract



a b

Fig. 9: Detection of allergenic relationship among pollen extracts by IgE specific dot immunoblotting with allergenic patients sera.

The sera used in nitrocellulose paper were collected from:

a. Negative control

b. *Eucalyptus* positive patient

DISCUSSION

In India, the importance of pollen allergy in triggering respiratory allergy has been successfully documented.[1,3,27,29] Pollen allergens are defined as antigens being capable to initiate an allergic reaction.[22] Allergens used for diagnostics in the human medicine as well as allergens being specific for immunotherapy of allergic states are prepared through extraction of allergen substances from materials of biological origin most of the time. A great majority of allergens are proteins, freely soluble in aqueous solutions and have diverse biological functions. The characterization of an allergen is a troublesome and difficult process, as it requires both the precise biochemical characterization of a (glyco) protein molecule and the establishment of its susceptibility to IgE antibodies, as they are the main link to histamine release in some hypersensitivity states (Type I allergies).[23] In the present study, an attempt has been made for the identification, isolation and partial

characterization of the allergenic extract of *E. citriodora* pollen. The role of *E. citriodora* pollen grain as a potential aeroallergen was established with a 115 KD protein component as an IgE- reactive protein.

It was found from the results of aerobiological survey that *Eucalyptus citriodora* trees flower seasonally from end of October to March. A 2-year continuous aerobiological survey at the Central Calcutta demonstrated that the pollen grains are commonly found to be airborne during pollination months.[1,3,24,25] *Eucalyptus* pollen contributed a moderately high concentration in the total aeropollen load during its pollination months. The allergenic potentiality of *E. citriodora* pollen has already been documented.[3] In this study, the results of SPT along with specific IgE-ELISA and dot immunoblotting confirm *E. citriodora* pollen as an important pollinosis causing bioparticle in a large population and also point out that its protein component has the potential to bind IgE with the sera of an allergic patient.

SDS-PAGE elicited the molecular components of allergenic extract. The powerful resolution of SDS-PAGE usually makes it the first choice method for the preliminary screening of potential allergens of a certain source. This molecular level information on the structure of allergen indicates that allergens are heterogeneous protein in nature.

Only a few investigations were carried out in India on the basis of pollen allergen standardization.[26,27,28] In the present study the *E. citriodora* pollen extract was first fractionated by $(NH_2)_4SO_4$ precipitation and then by gel fitration. The EF2 fraction derived from the first step $(NH_2)\ _4SO_4$ fractionation was established as allergenically highly active component both by *in vivo* and *in vitro* studies. Similarly, EF2A fraction (derived from gel filtration column chromatography) was found to be the most active one. The analysis of the activity of EF2 and EF2A by IgE-sensitive ELISA demonstrated the degree of allergenicity. From the standard molecular weight curve of gel filtration, the isolated allergen was found to be a 115 KD protein component.

The determination of soluble protein and sugar in FE2A suggested that the fraction contained a certain level of polysaccharide. From PAS technique it was found that the allergenic extracts contain glycoprotein components which have been found in some cases as a common character of pollen allergen.[29] So, the evidence of an allergenic component of 115 KD which is glycoprotein in nature was isolated for the first time for *E. citriodora* pollen that will improve the ability to handle the allergic disease, both in a diagnosis and a therapeutical sense.

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