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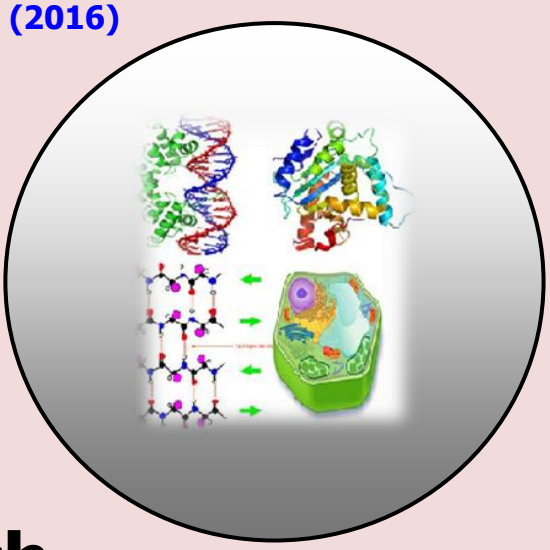
ISSN 2319-3077 Online/Electronic
ISSN 0970-4973 Print

UGC Approved Journal No. 62923
MCI Validated Journal
Index Copernicus International Value
IC Value of Journal 82.43 Poland, Europe (2016)
Journal Impact Factor: 4.275
Global Impact factor of Journal: 0.876
Scientific Journals Impact Factor: 3.285
InfoBase Impact Factor: 3.66

J. Biol. Chem. Research
Volume 35 (2) 2018 Pages No. 574-581



Journal of Biological and Chemical Research

An International Peer Reviewed / Referred Journal of Life Sciences and Chemistry

Indexed, Abstracted and Cited in various International and National Scientific Databases

Published by Society for Advancement of Sciences®

J. Biol. Chem. Research. Vol. 35, No. 2: 574-581, 2018
(An International Peer Reviewed / Refereed Journal of Life Sciences and Chemistry)
Ms 35/02/3010/2018

ISSN 2319-3077 (Online/Electronic)
ISSN 0970-4973 (Print)

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RESEARCH PAPER

Received: 11/08/2018 Revised: 24/08/2018 Accepted: 25/08/2018

Immunoclinical Characterization *of Cassia occidentalis* L. pollen allergens, a Potential Aeroallergen from West Bengal

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ABSTRACT

In the last few years Cassia pollen is reported to be prevalent in the air of West Bengal and considered as an important triggering factor for pollinosis. A continuous 2-year (2014-2015) volumetric aerobiological survey was conducted in Chandernagore, West Bengal to record the occurrence and frequency of airborne Cassia pollen for the first time. The highest frequency of Cassia pollen was recorded in May and July when the temperature was moderately high. In this study we sought to define the immuno-biochemical properties of Cassia occidentalis pollen which was reported to be airborne in different parts of West Bengal but there are no reports regarding its IgE- reactive proteins. Clinical test (skin prick test) showed C. occidentalis pollen to be one of the major causes of respiratory allergy. SDS-PAGE was performed with the total soluble pollen protein which showed a total of 20 distinct protein bands, while 8 of them were detected by western immunoblotting as serore active proteins which are causing respiratory allergy among atopic population. Among them 59.1kDa protein was identified as a major allergen from C. occidentalis pollen.
Key words: Cassia occidentalis Pollen, Seasonal Periodicity, Pollinosis, Clinical Study, IgE- Reactive Proteins and Respiratory Allergy.

INTRODUCTION

The importance of pollens causing pollinosis is well established and documented in India as well as all over the world. Of the various bioaerosols present in the atmosphere, pollens are considered to be offending aeroallergens. Pollen causes respiratory allergy to the sensitive individual when it comes in contact with the upper respiratory tract. The prevalence of respiratory allergy has been reported to be 20-30% across the globe (Acharya 1980, Anonymous 2000, Boral et al. 2004, Ghosal et al. 2015). According to Chanda (1996), 20-30% of the general population in India is atopic and 25-45% of the total IgE in respiratory allergic patients is made up by pollen-specific IgE. A number of tree and shrub pollen grains are considered to be important component of the spectrum of allergy triggering agents from different eco-geographic regions in India (Gupta and Chanda 1991, Banik and Chanda1992, Chakraborty et al. 1998, 1999, 2004, Boral & Bhattacharya 2000, Boral et al. 2004, Mandal et al. 2006, 2008, Hussain et al. 2012, 2013, 2014, Basak et al. 2015,2017), though many places are yet to be surveyed for identification and detection of IgE reactive pollen allergens for their allergenic manifestation on local population.Thus presently it is a very challenging task for aerobiologists and

allergologist working together to detect the origin of pollen, identification of them and characterization of allergy causing IgE reactive proteins (Cresti and Tiezzi 1992).For immunotherapy with specific hyposensitization vaccine, proper identification and characterization of pollen allergens is an essential. *Cassia* sp. belongs to the family Caesalpineaceae, are common and wildly growing plant in West Bengal. Some species of *Cassia* are trees, some are shrubs and some are herbs. The different species of *Cassia* sp. plant is well adapted to the hot and humid tropical climate and also grows commonly in different parts of West Bengal in different season depending on their eco climatic condition. Some species of *Cassia* have medicinal values which is used in Unani, Ayurvedic and Allopathic systems of medicines for curing different diseases but the pollen grains of *Cassia* sp. have also been found in the air of various cities of India and cause of IgE-mediated type I hypersensitivity in atopic patients(Banik and Chanda 1992, Majumdar et al. 1988, Boral and Bhattacharya 1999, Boral et al. 2004, Mandal et al. 2006, Singh and Dahiya 2008, Hussain et al. 2013).They are known to initiate 28.5% of respiratory allergy as revealed by previous skin reaction tests (Hussein et al. 2013). Although there are previous reports describing their atmospheric presence and atopic sensitization in West Bengal (Hussein et al. 2013) but there are no reports regarding its IgE- reactive proteins/components so far which may be responsible for immediate hypersensitivity reactions in respiratory allergic patients. This investigation seeks to enhance the knowledge immunoclinical investigation regarding *Cassia occidentalis* (CO) pollen allergy among atopic patients. The objective of the present study is 1) to determine the concentration, seasonal periodicity of *Cassia* pollen grains in the present atmosphere of Chandernagore, Hooghly, WB, 2) to observe the prevalence of sensitization to CO pollen among the respiratory allergic patients in the city, 3) to identify the IgE binding proteins present in it.

MATERIALS AND METHODS

Sampling site

Chandernagore is a small township (20 sq. km) of Hooghly district, situated about 35 km far to the north of Kolkata metropolis. It stands on the banks of the River Hooghly at 22° 52' 9" N and 88° 22' 37" E, and has a population of 166,867 in the 2011 Census. Before 15 August 1947, France reigned over Chandernagore, which was in contrast with the rest of India that was ruled by the British. The French stepped on the soil of Chandernagore back in 1673. The long forgotten French colonial aura can still be witnessed in the nook and corners of Chandernagore.

Aerobiological studies

To record the seasonal periodicity of airborne *Cassia* pollen in the atmosphere monitoring of airborne pollen flora was done with the help of "Burkard portable volumetric sampler" [Burkard Manufacturing Co.,UK] (suction rate = 10L/min) for two consecutive years (January 2014 to December 2015) at three different sites (Bagbazar, Palpara and Mankundu) of Chandernagore to get the actual scenario of pollen spectra of this old colonial French city. Air sampling was done at three different time intervals: morning (09:30hr-10:30hr), afternoon (12:30hr- 13:30hr) and evening (19:30hr-20:30hr) at weekly intervals. The sampler was placed at a height of 0.5m above the ground level. The sampler was operated for 5 min. The hourly counts were then averaged to obtain the mean concentration which in turn gave the monthly concentration. The exposed slides were mounted, scanned thoroughly and counted according to the guidelines of the British Aerobiology Federation (1995) to study the seasonal variation of airborne *Cassia* pollens. The identification of air borne pollen was done mainly with the help of reference slides and also by consulting published literatures (Erdtman 1969, Huang 1972, Gupta et al. 1985, Nayar 1990, Bhattacharya et al. 2006). The hourly counts were then averaged to obtain the mean concentration which in turn gave the monthly concentration. The monthly pollen concentration was expressed as number of pollen grains per cubic meter of air.

Collection of pollen

Cassia occidentalis plant is shrubby in appearance. The *C. occidentalis* pollen samples were collected from mature anthers of the fresh flowers of different stages from the plants growing around the city during their peak flowering period. They were dried at 37^{o}C, mildly crushed and passed through different sieves (60, 75, 100 and 120 mesh/cm^2) successively to remove the other floral impurities following the methods as mention by Hussein et al. 2013. The purity of the isolated pollen material was checked under the microscope. The batches used throughout the work contained <5% non-pollen impurities.

Preparation of pollen extracts

To test allergic potency, pollen extracts were prepared from pure pollen samples following the method of Sheldon et al. (1967) with little modification as proposed by Mandal et al. (2009) and Hussain et al. (2012).

Concentrating the pollen proteins for protein profiling

The PBS extracted pollen antigens were concentrated for 1D SDS PAGE using UPPA I and UPPA II (GBioscience, A Geno Technology Inc., USA) using their protocol with slight modification.

Fractionation of pollen antigen

Total pollen protein of five species of *Cassia occidentalis* pollen extracted in PBS (pH 7.2) was precipitated by salting out with 90% ammonium sulphate. Protein pellet was reconstituted in 4 ml of 20 mM Bis-Tris buffer and this pellet fraction was dialyzed in tube-o-dialyser [GBioscience, A Geno Technology Inc. (USA)] extensively against the same buffer overnight at 4°C to remove the traces of ammonium sulphate and stored at -20^{o}C.

Skin prick tests (SPT) and sera collection

Skin prick tests were carried out with crude *C. occidentalis* pollen extracts (1:50 w/v) mixed with 10% glycerine solution on 81 adult subjects with case history of allergic rhinitis or mild intermittent bronchial asthma. All of them were reporting *C. occidentalis* plants growing in their surroundings when attending the Mediland Diagnostics Institute, West Bengal during survey period. The exclusion criteria were perennial or severe asthma, pregnancy or lactation, malignancy or other severe systemic diseases during skin testing or sera collection. The detailed history including age, sex, family history, onset and duration of symptoms were recorded.The reaction was graded from +1 to +4 level according to Stytis *et al*. (1982).

Control sera were collected from 2 non-sensitized healthy volunteers (confirmed by negative SPT and IgE–ELISA) having no history of any previous or current allergic symptoms. The consent of all patients was obtained before SPT and sera collection.The study was approved by the Mediland Diagnostics Institute Ethics Committee.

Specific IgE estimation using Enzyme linked immunosorbent assay (ELISA)

Quantitative estimation of specific IgE in patients' sera were performed by indirect ELISA (Engvall and Pearlman, 1971) with a partial modification of Bodinieret al. (2008). 'Monoclonal Anti-human IgE Clone GE-1-alkaline phosphatase conjugate' (Sigma Chemical Co., USA) in 1:1000 dilution was used as secondary antibody. The ratio between mean OD_{405} values of each patient (P) and healthy subject (N) sera was calculated described as following the method of Sircar et al. (2015). For particular serum, P/N ration greater than 3.5 was considered as *in vitro* "positive" with markedly elevated level of specific IgE.

Sodium dodecyle sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

The SDS PAGE of crude pollen extract, Fr.90 and UPPA treated *C. occidentalis* antigen were carried out on 12% acrylamide gel using discontinuous buffer system according to Laemmli (1970). The gel was documented using Gel Doc 1000 (Bio-Rad, USA) using the MOLECULAR ANALYST software (Bio-Rad, California, USA). The molecular mass of the protein bands were calculated by calibrating with standard marker proteins (Genei, Bangalore, India).

IgE-specific immunoblotting

The proteins separated on SDS-PAGE were electrophoretically transferred to PVDF membrane (Sigma Chemicals, USA) following the method of Towbin et al. (1979) with Genei Mini-Tank blot apparatus at 4^{o} C. The Immunoblotting was performed onto PVDF membrane according to Sambrook et al. (1989). Further works were done following the method of

RESULT AND DISCUSSION

Aerobiological survey

In the two years' aerobiological survey from January 2014 to December 2015 in the sun-urban industrial city Chandernagore, *Cassia* pollen grains were found to be prevalent from March to October (Fig 1). The pollen count attained the peak in July and September during which they contributed, 10.12% and 13.32% respectively to the total monthly aeropollen load on an average (Fig 2). The pollen sample i.e. anthers of collected flowers was acetolysed for clear observations of exine layers. Different parameters of the pollen grains like colpa, exine ornamentation etc. was determined from acetolysed grains. Microphotographs of the acetolysed grains were taken by Light Microscopy (LM) (Fig 3). Microscopic studies revealed that *Cassia* pollen grains are triangular in polar view and spheroidal in equatorial view. The polar diameter and equatorial diameter of the pollen grains of *Cassia occidentalis* ranging from PA 30 µm to 33 µm and ED 27.50 µm with tri-colporate aperture and exine show finely reticulate ornamentation.

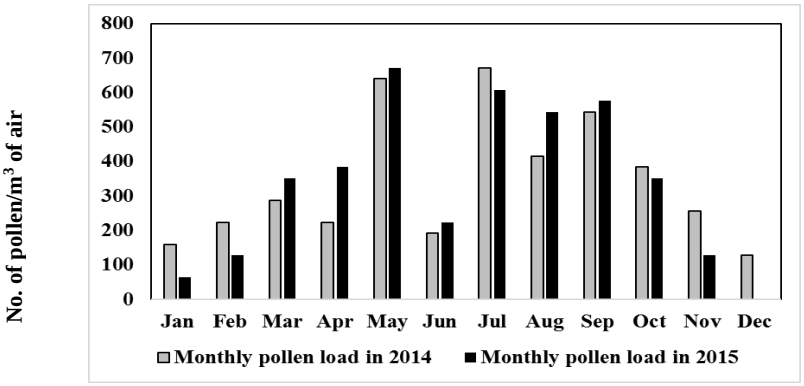

Figure 1. Monthly variation of airborne pollen (No. of pollen/m^3 of air) of *Cassia* pollen in two consecutive years (2014-2015).

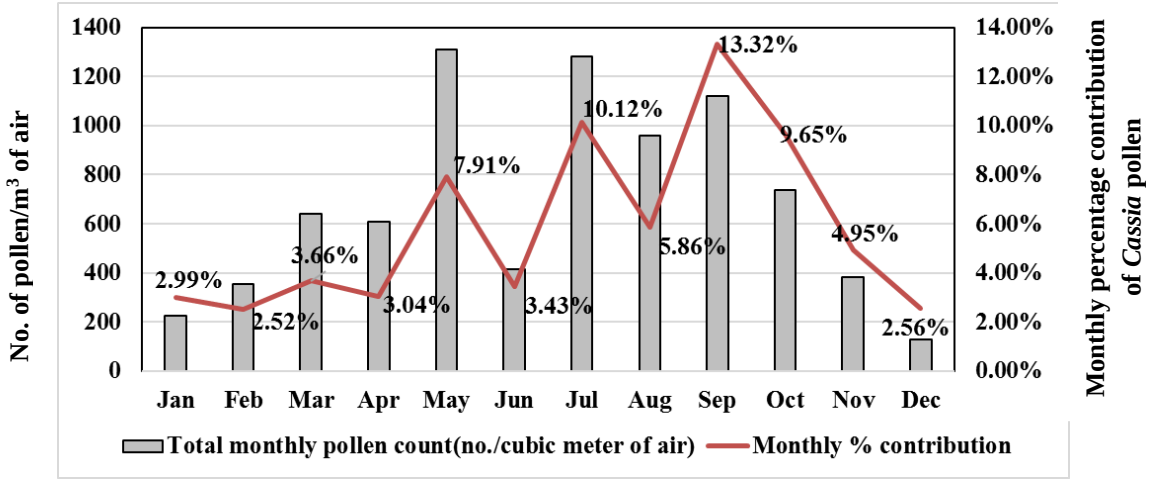

Figure 2. Monthly pollen periodicity of airborne *Cassia* sp. pollen (No. of pollen/m^3 of air) for 2 consecutive years (2014–2015) with respect to total monthly airborne pollen load (percentage contribution of *Cassia* pollen).

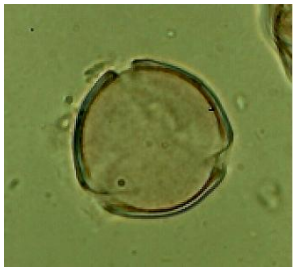

Figure 3. Microscopic picture of CO pollen (polar view)

Clinical test and ELISA

Skin prick tests were performed with *C. occidentalis* pollen extracts in a population of 81 respiratory allergic patients [Age range 20-56; M/F = 34/47] and 40.1% showed positive skin reaction, out of which 51.91% showed ≥ + 2 grade of reaction (Table 2). Among them on the basis of skin reactivity and high *C. occidentalis* pollen specific IgE level, 11 patients with allergic rhinitis or bronchial asthma (or combination of both) were selected for further sera collection (Table 3) and among them8 patients were selected for immunolotting along with control subjects.

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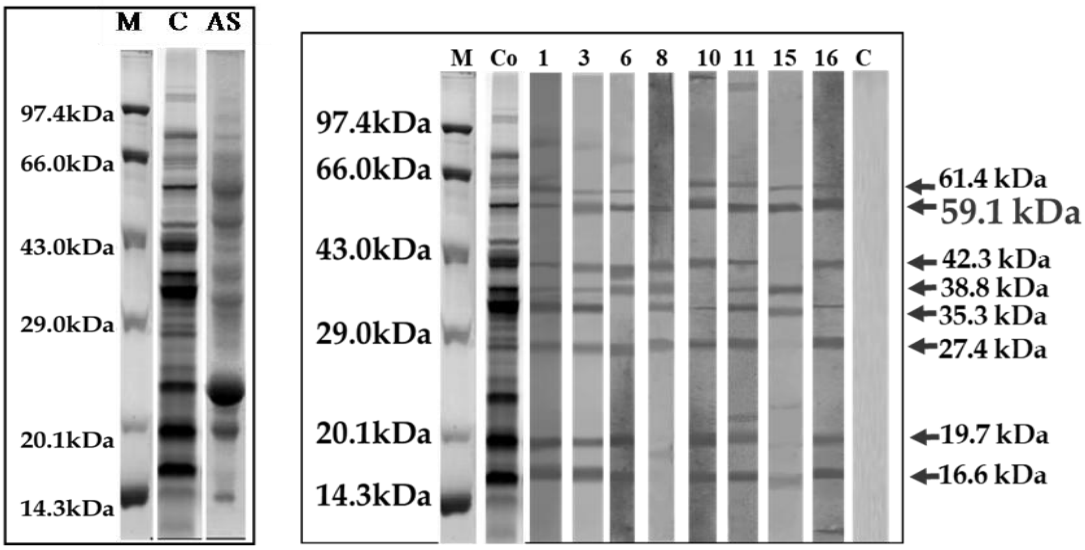
Table 2. Result of Skin Prick Tests with pollen from avenue trees in patients with respiratory allergy.

Pollen Allergen Extract	Patients tested (N)	No. and percentage of sensitized patients (+1 to +4)	No. and percentage of sensitized patients showing response≥ + 2
Cassia occidentalis(CO)	81	33(40.1%)	18(51.91%)

Table 3. Clinical characteristics of the 11 sensitized patients (selected for IgE immunobloting).#AR-Allergic rhinitis, BA-bronchial asthma.

Sl.No.	Gender/Age	SPT grade	Specific IgE Value (P/N)	Symtoms (AR/BA)
1	21/M	++	3.56	AR+BA
2	36/F	++	3.58	BA
3	39/F	+++	4.07	AR+BA
4	55/F	++	3.94	AR+BA
5	31/M	+	3.94	AR+BA
6	35/M	++	4.78	AR+BA
7	33/M	++	3.96	AR+BA
8	41/M	+	3.65	BA
9	29/M	+	3.79	AR
10	21/M	++	3.56	AR+BA
11	36/F	++	3.58	AR+BA



A B

Figure4 A: 12% SDS-PAGE of crude and ammonium sulphate fraction pollen extracts of *Cassia occidentalis* [M= Molecular Marker, Crude= UPPA I and UPPA II treated Crude extract, AS: 90% ammonium sulphate fraction]B: Specific IgE-binding protein fractions of pollen extract of *Cassia occidentalis* when transferred to PVDF membrane and probed with 11 individual serum samples from ELISA-positive patients. Lane M= molecular wt. marker, 1: SDS PAGE of crude extract of *Cassia occidentalis* pollen,Numbered lanes:individual patient serum, C: Negative control sera.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

On reducing SDS-PAGE (12%), the UPPAI and UPPAII treated extract (C) of *Cassia occidentalis* resolved into more than 20 distinct bands between 14.3 kDa and 97.4 kDa while 95% ammonium sulphate precipitated

extract (AS) showed 15 clear bands (Fig 4A) which were most prominent and stained strongly in with Coomassie brilliant blue (CBB) both the lanes. Some of the bands ranging from molecular weight 43 to 66kDa were less distinct but sharp. Other bands were weak and less stained but were clearly visible and distinguishable on the gel (Fig 4A).

IgE-specific immunoblotting

Proteins separated by SDS-PAGE were subjected to immunoblotting using *Cassia occidentalis* positive pooled patients' sera to identify the IgE-binding components of *C occidentalis* pollen. In ELISA, 11 patients' sera showed P/N ratio greater than 3.5 (Table 3). These sera were used for IgE specific immunoblotting for detection of immunoreactive proteins present in this pollen antigen. For this purpose, sera No. 1, 3, 6, 8, 10, 11, 15 and 16 which were sensitive to CO pollen was used to perform immunoblotting. Of the different protein bands that resolved on SDS-PAGE, 8 of them were identified as specific IgE-binding protein fractions (Fig 4B). Among the higher molecular weight proteins, 61.4 kDa band showed IgE reactivity with individual patient sera, while lower molecular protein band 16.6kDa recognized by specific IgE antibodies from most of the tested patients' sera. Among them, one IgE-reactive components of molecular weight 59.1kDa was found to be present in all the cases, showing 100 % binding frequency. So, 59.1kDa IgE reactive component was identified as the major aero allergen from *Cassia occidentalis* pollen causing immediate hypersensitivity upon inhalation.

CONCLUSION

The incidence and quantity of pollen grains of native plants capable of causing allergy are the main determining factor of allergic respiratory ailments of a population in a topographical region (Puc 2003). Allergy to the pollen of flowering plants considerably impacts on the human health in many parts of the world (Pawankar *et al.* 2013). *Cassia* is a genus of flowering plant belongs to family Fabaceae and sub family Caesalpinoideae shaded their pollen in air and showed their prevalence in May, July, September and October contributing 7.91%, 10.12%, 13.32% and 9.65 % of total monthly pollen load respectively in the in atmosphere of Chandernagore, Hooghly, West Bengal, India. During the peak flowering period, the monthly mean values for *Cassia* can be expressed as 'high', ranging from 1280 to more than 1312 grains/m^3 of air. Such a high concentration of *Cassia* pollen has not been evidenced from the previous studies (Banik and Chanda 1992, Majumdar et al. 1988, Boral and Bhattacharya 1999, Parui et al. 2002, Boral et al. 2004, Mandal et al. 2006, Singh and Dahiya 2008, Hussain et al. 2013). 40.1% of total tested patients have shown SPT sensitization against *C. occidentalis* antigen have history of pollen allergy and showed bronchial asthma combined allergic rhinitis as their major atopic symptoms. The patients' sera were selected for the specific-IgE immunoblotting on the basis of hi titre value (P/N≥3.5) in indirect ELISA which was performed to check and verify the degree of sensitization in SPT. Clinical demographic profile of the 11 sensitized patients against *C. Occidentalis* pollen antigen revealed that most of the patients were suffering from both bronchial asthmas combined with rhinitis. Besides the biomonitoring of airborne pollen allergen, research should be carried out at molecular level for precise detection and identification of allergy-causing components present in their pollen. Though the atmospheric presence of *C. occidentalis* pollen was reported earlier by Hussain et al. (2012) but their sero-reactive component was not identified till date. SDS-PAGE of *C. occidentalis* pollen extract revealed several protein bands with the estimated MWs from 14.3 kDa to 97.4 kDa but 8 IgE reactive protein fractions were identifiedin immunoblotting when probed with 8 individual serum samples. Among them MW of 59.1kDa sero-reactive protein was found to be present in all the cases, showing 100 % binding frequency thus considered as major aeroallergen identified from *C. occidentalis* pollen. This finding will help the allergologist and clinician to treat the *C. occidentalis* allergic patients using IgE-specific immunotherapy.

ACKNOWLEDGEMENTS

We express our sincere thanks to the clinicians of Mediland Diagnostic Centre, Kolkata, for their help in skin prick test and sera collection. Thanks are due to the Head of the Department, DST-DRS and FIST sponsored Department of Botany, Visva-Bharati for providing instrument facilities.

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