

ALLERGY TO PERIWINKLE POLLEN (*CATHARANTHUS ROSEUS* G. DON.)Debajyoti Ghosh¹, Indrani Roy², Sunirmal Chanda³, Swati Gupta-Bhattacharya¹¹Department of Botany, Bose Institute, Calcutta, India²Allergy Department, Institute of Child Health, Calcutta, India³Centre for Study of Man and Environment, Calcutta, India

Ghosh D, Roy I, Chanda S, Gupta-Bhattacharya S: Allergy to Periwinkle pollen (*Catharanthus roseus* G. Don.). *Ann Agric Environ Med* 2007, **14**, 39-43.

Abstract: *Catharanthus roseus* G. Don. (CR) or periwinkle plants are widely grown/cultivated as garden plants in the tropics and subtropics. In spite of its predominantly entomophilous nature, CR pollen had been reported to be airborne and allergenic. The objective of this study was to discover the seasonal changes of CR pollen concentration in air, to determine its potential to cause respiratory allergy and to analyze its allergenic components. A 2-year aerobiological survey was conducted with a Burkard 7-day sampler in an agricultural farm in the suburban zone of Calcutta city where CR pollen was found to be almost perennial with 3.6-5.4% contribution to the aeropollen load. Skin prick test was conducted on 282 respiratory allergic individuals living within a 15 km radius of the study area. 29.8% of them were positive to CR pollen. Among them, 80.9% were directly involved in gardening. The whole pollen extract was subjected to gel filtration in a Sephacryl S-200 column. Among 5 eluted fractions, fraction I showed optimum IgE-reactivity in ELISA-inhibition. The fraction I shows 4 protein components in SDS-PAGE, within which 3 (40-66 kD molecular mass) were found to be IgE-reactive in immunoblotting using patient sera. It can be concluded that CR pollen can trigger IgE-mediated respiratory allergy in the people living in close proximity.

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Key words: aeroallergen, allergen characterization, *Catharanthus roseus*, pollen, respiratory allergy.

INTRODUCTION

Catharanthus roseus G. Don. (CR) or periwinkle plants of Apocynaceae family are widely distributed in the tropics and subtropics. Periwinkle plants are cultivated for yielding medicinal alkaloids [22] and as a garden plant for nice flowers. Though this is primarily an entomophilous plant, CR pollen grains are reported to be present in the air round the year with a relatively high concentration from October to January. CR pollen had been reported to show 26.7-31.5% positive response in skin reaction tests on respiratory allergic patients [2, 5, 6]. In the present study, we conducted (1) an aerobiological survey to record the seasonal changes of CR pollen concentration (seasonal periodicity) in the air, (2) clinical survey to determine its allergenic

potential among gardeners, who are subjected to its exposure, (3) biochemical and immunological analyses to know the allergens present in the CR pollen extracts used in diagnostics and (4) partial purification of allergenic fraction containing one of its important allergenic component, which may be useful in therapeutic purpose.

MATERIALS AND METHODS

Aerobiological survey. Continuous air sampling was conducted in the Madhyamgram Agricultural Farm of Bose Institute, situated in suburban outskirts of Calcutta Metropolitan over 2-year period (from July 1, 1998 to June 30, 2000) with Burkard 7-day volumetric sampler. The sampler was placed in the field on a 0.5 m high concrete

base. The exposed slides were mounted and trapped pollen grains were microscopically counted following guidelines of the British Aerobiology Federation [33] to study the periodicity patterns.

Source materials. Fresh pollen samples were collected from the flowering plants. The batch used for the entire study contained not more than 5% non-pollen impurities (in term of the number of counted particles).

Extract Preparation. Pollen samples were defatted with diethyl ether and extracted in phosphate buffered saline (PBS, 0.1 M Na-phosphate containing 0.15 M NaCl, pH 7.2) by continuous stirring at 4°C for 16h in 1:50 w/v ratio. After centrifugation at 12,500 g for 40 min, the clear supernatant was dialyzed and passed through a 0.22 µm Millipore filter (Millipore Corp., Bedford, USA). The filtrate was then lyophilized and stored at -70°C in aliquots of known volume in sterile vials. The protein content of whole extract and fractions were determined following Lowry's method [20].

Study group. 282 respiratory allergic patients were recruited in the patient group when attending the allergy clinic in the Institute of Child Health, Calcutta during 2000-2003. 112 (39.7%) of them had allergic rhinitis, 80 (28.4%) had bronchial asthma and 90 (31.91%) had the combination of both allergic rhinitis and bronchial asthma as per physicians' diagnosis. The age range of this group was 19-64 years (mean age 32.28 ± 2.1 years). The gender ratio of this group was male/female = 110/172 = 0.64. The patients with severe perennial asthma, pregnancy/lactation, malignancy and other systemic diseases were excluded. 76 non-sensitized volunteers (confirmed by negative skin reaction and IgE-ELISA) having no history of previous or current episode of any allergic, systemic or joint diseases in themselves/their families were recruited in control group. The subjects were selected from the suburban area who were residing within 15 km distance of the study field. The demographic data of study group (patient and control group) has been described in Table 1. The recruitment of study group was approved by the Ethics Committee of the hospital.

Skin prick tests (SPT) and sera collection. Skin prick tests were carried out with CR pollen extracts (1:50 w/v) on 282 adult subjects with case history of allergic rhinitis or mild intermittent allergic asthma. All of them were reporting CR plants growing in their surroundings when attending the Allergy Clinic of the Institute of Child Health, Calcutta, during 2000-2003. The exclusion criteria were perennial or severe asthma, pregnancy or lactation, malignancy or other severe systemic diseases during skin testing or sera collection. To avoid masking of possible symptoms, corticosteroids and antihistamines were prohibited. The study was approved by the Ethics Committee of the hospital.

Table 1. Characteristics of respiratory allergic patients and control subjects selected for study.

| | |
|---|---------------------------------|
| Patient group: | |
| Total no. (n) = 282 | Male/Female = 110/172 = 0.64 |
| Age (Years): | |
| Range = 19-64 | Mean Age (X ± SD) = 32.3 ± 2.10 |
| Symptomatic diagnoses | |
| Allergic rhinitis: n = 112 (39.7%) | |
| Allergic asthma: n = 80 (28.4%) | |
| Combination of allergic rhinitis and asthma: n = 90 (31.9%) | |
| Positive in skin reaction: | |
| Total no. = 84 (29.78%) | Male/Female = 38/46 = 0.83 |
| Direct involvement in gardening (n) = 68 (80.9%) | |
| No. of patients with +2/more level of reaction (n) = 6 (7.1%) | |
| Control group | |
| Total no. = 76 | Male/Female = 30/46 = 0.65 |
| Age (Years): | |
| Range = 23-69 | Mean Age (X ± SD) = 33.5 ± 1.80 |

Histamine diphosphate (10 mg/ml) and PBS were used as positive and negative controls respectively. The reaction measurement was performed after 20 minutes. According to international guidelines, positivity was defined as mean wheal diameter ≥ 3 mm, larger than compared with negative control [9]. The reaction was graded from +1 to +3 level (+1 = erythema, 20 mm in diameter, +2 = erythema >20 mm in diameter, +3 = wheal and erythema) according to Stytiš *et al.* [32]. 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, systemic or joint diseases in them and their families.

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE). SDS-PAGE analysis of whole pollen extract and fractions were performed according to Laemmli [19]. The isolated allergenic fraction of CR pollen was studied in native (untreated) condition according to Hames [17].

Fractionation of CR pollen allergen. The total protein of whole pollen extract was precipitated by 0-90% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitation was dissolved in PBS and dialyzed (mol. cut-off limit 10,000) to remove traces of ammonia and stored at -70°C. The concentrated protein (6 mg/ml) solution was subjected to Sephacryl S-200 column (d = 2.4 cm, h = 55.5 cm) equilibrated with 10 mM PBS, pH 7.2 for gel filtration. The fractions were collected in 3 ml aliquots (void volume 83 ml, flow rate 12 ml/h) at 4°C and the elution was monitored at 280 nm using a spectrophotometer.

Enzyme linked immunosorbent assay (ELISA) and ELISA inhibition. ELISA was performed to determine CR pollen specific IgE levels in individual patient sera using anti-human IgE horseradish peroxidase conjugate (Sigma) in 1: 1200 dilution and o-phenylene diamine substrate [10].

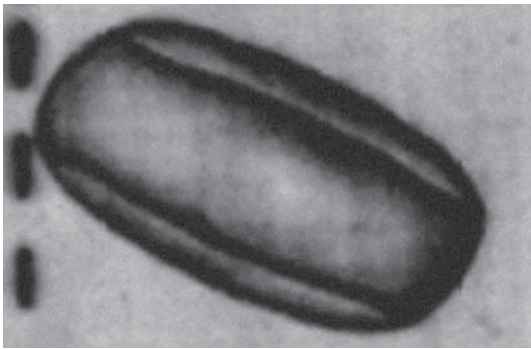


Figure 1. Scanning electron micrograph of *Catharanthus roseus* pollen ($\times 500$).

The absorbance was measured with an ELISA reader at 492 nm. The individual serum having P/N value (ratio of optical density of patient sera with respect to control) > 3.0 were used for immunoblotting [4]. In immunoblotting the control value was the mean derived from the panel of 12 normal (selected from control group) sera. ELISA inhibition was performed by overnight pre-incubation of patient sera with equal volume of (a) whole extract (100 μg protein/ml) and (b) fractions of CR pollen extract with varying protein concentrations at 4°C. The inhibited serum was then used in ELISA with anti-IgE horseradish peroxidase conjugate. Percentage inhibition was calculated as:

$$\% \text{ Inhibition} = \frac{\text{O.D. of test sample} - \text{O.D. of inhibited sample}}{\text{O.D. of test sample}} \times 100$$

The 0% inhibition was obtained by incubating serum samples with PBS. A dose-response curve was obtained by plotting the percentage of inhibition against the log of protein concentrations.

IgE specific immunoblotting. IgE-immunoblotting was performed onto PVDF membrane according to Sambrook *et al.* [24] for whole pollen extract and isolated fractions. Serum was diluted in 1:5 (v/v) ratio in PBS. The IgE-reactive bands were detected with anti human IgE (Sigma) coupled with horseradish peroxidase enzyme (1:500 v/v dilution and diaminobenzidine as substrate.

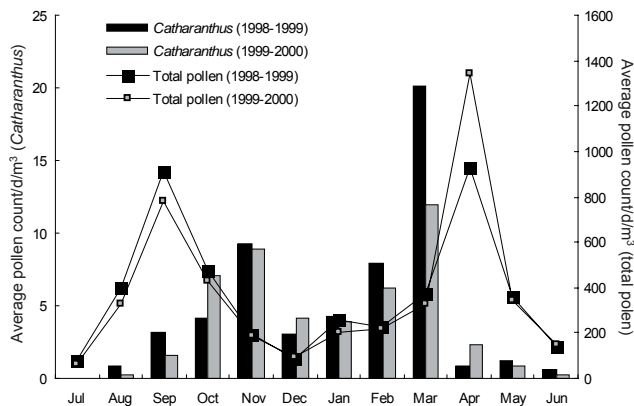


Figure 2. Seasonal mean periodicity of airborne *Catharanthus roseus* pollen (1998-2000) with respect to total airborne pollen load.

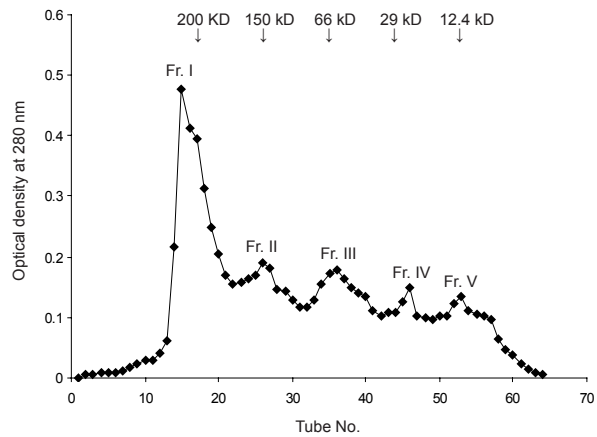


Figure 3. Gel filtration of whole extract of *Catharanthus roseus* pollen in Sephacryl S-200 column.

RESULTS

Aerobiological study. In the volumetric air sampling of the agricultural farm, CR pollen (Fig. 1) was found to be almost perennial (except in July of both 1998 and 1999). This pollen concentration showed the peak in March, when it contributed 5.4% (1999) and 3.6% (2000) of total aero-pollen load respectively (Fig. 2).

Clinical study. We selected 282 adult patients (M/F = 110/172, Mean age 32.3 ± 2.1 years, $FEV_1 < 80\%$) with allergic rhinitis, allergic asthma and combination of both (Tab. 1) for skin reaction study. 84 (29.8%) of the study population showed positive skin reaction towards CR pollen extract in SPT. Among the positive subjects, 6 (7.1%) showed +2/more level of reaction. 68 (80.9%) of the susceptible patients were found to be directly involved in gardening and reported CR plant exposure.

Fractionation of CR pollen extract. The whole protein extract of CR pollen gave rise of five fractions (Fr. I – Fr. V) corresponding to five peaks in Sephacryl S-200 column (Fig. 3).

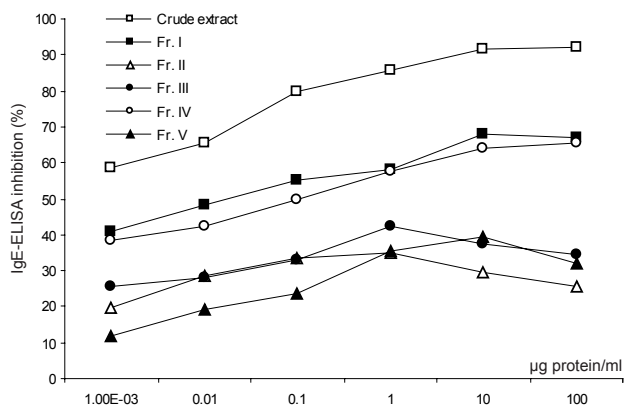


Figure 4. IgE-ELISA inhibition with pooled patient sera using different fractions of *Catharanthus roseus* pollen in comparison with crude extract.

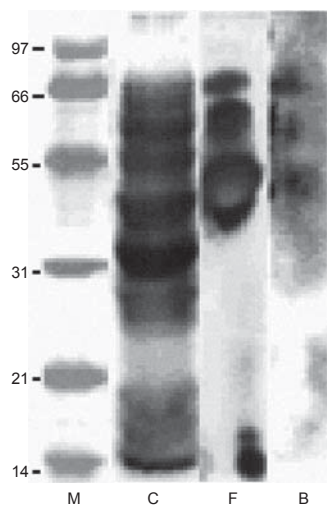


Figure 5. The whole extract (C) & Fraction I (F) of *Catharanthus roseus* pollen and IgE-immunoblot of Fraction I (B) using PVDF membrane, M > Molecular weight marker.

IgE ELISA inhibition. The allergenic potential of each fraction was then studied by IgE ELISA inhibition with pooled sera of seven patients [with P/N value (the ratio of o.d. of patients with normal/control serum) > 3.5 in IgE ELISA]. The Fr. I showed 50% inhibition with < 0.1 µg of protein (Fig. 4), whereas Fr. IV attained 50% inhibition with 0.1 µg of protein. Fractions II and III failed to reach that value within the concentration range studied.

SDS-PAGE and immunoblotting. In the 11% SDS-PAGE, whole extract of CR pollen showed 20-25 protein components within the molecular weight range of 14 to 66 kD (Fig. 5). The Fr. I showed the presence of four protein components. In immunoblotting with pooled CR sensitive patient sera, three bands of 47, 58 and 66 kD molecular mass were found to be IgE reactive.

DISCUSSION

Airborne pollen grains are the earliest known allergen to cause human respiratory allergy. Generally it was considered that the entomophilous plants are allergy-safe as their pollen grains have less chance to be dispersed in air in larger quantities. In spite of that, there are a number of aeropalynological studies [1, 5, 21, 34] from different parts of India in which entomophilous pollen grains had been reported to be airborne. A number of important allergenic pollen types, e.g., the pollen grains of *Cassia* sp. [26], *Anacardium occidentale* (cashew) [12], *Brassica campestris* [29], *Brassica napus* (oilseed rape) [13], *Helianthus annuus* (sunflower) [18], *Ligustrum* sp. (privet) [3], *Olea europea* (olive) [14], etc., are primarily entomophilous.

In the present study the two-year continuous aerobiological survey in an agricultural farm showed a consistent presence of *Catharanthus* pollen in the air. After recruitment of patient group residing in the suburban zone near

sampling site, it was found that all of them resided within 15 km distance from the site. The selected respiratory allergic patients had a fair chance of exposure to CR pollen. Among the sensitive (29.8%) patients, 80.9% were involved in gardening and could have possible exposure to airborne CR pollen. There are a number of reports describing cases of occupational allergy by flowers [8, 11, 15]. Studies on work-related allergic symptoms had revealed prevalence of sensitization to relevant pollen for *Chrysanthemum* [15] and bell pepper [16]. In this respect, CR pollen clearly showed a putative role of this pollen as causal agent of occupational respiratory allergy among gardeners of the study area. In the next step, a detail case study on the CR sensitized gardeners is required to confirm their disorder as occupational disease.

In addition, environmental factors are often reported to play a role to make a pollen allergenic. Interaction with atmospheric dust [23], microbial flora [30], gaseous pollutants [25] and bacterial endotoxin [31] evidently strengthen the allergenic potential of airborne pollen grains.

In the immunochemical analyses the Fr. I of CR pollen whole extract had been found to contain three IgE-reactive components of 47, 48 & 66 kD molecular weight. The role of characterized allergens are very important in the diagnoses and therapy of allergic disorders [7, 28]. This partially purified allergenic fraction may be useful [28] in therapeutic purposes in sensitive patients after detail and in depth analyses.

Hence, in this study, it is evident that the CR pollen is airborne. In spite of its entomophilous nature, CR pollen contributes 3.6-5.4% of total aeropollen load of the study area. In the clinical survey, CR pollen showed 29.8% positive skin reaction among the population of 282 respiratory allergic patients, most of which are involved in gardening. In immunochemical analyses a number of allergenic protein fractions had been detected from the whole protein extract of CR pollen and partial purification of its major allergenic fraction Fr. I will be helpful in allergy diagnostics and therapy of patients susceptible to CR pollen. Finally, CR pollen can be listed as an important occupational aeroallergen (where people are involved in gardening) from India.

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