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Biological Standardization of Pollen Allergens from India

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SUMMARY **Standardization of allergens are achieved by *in vitro* and *in vivo* methods. Some of the allergens from Western countries are standardized using biological potency of the extracts but no attempt has been made till now to standardize any of the pollen extracts from India based on biological units. Therefore, we have attempted to standardize two important pollen allergens *Ricinus communis* and *Holoptelea integrifolia* by biological methods. Broadly the methods adopted by Dreborg and Grimmer (1983) was followed. Skin prick tests were carried out with the extracts of *R.communis* and *H.integrifolia* on 15 allergic patients in five three fold log dilutions starting with 1:10, in 50% glycerinated buffer. Glycerinated buffer (50%) and histamine dihydrochloride (1 mg/ml) were used as negative and positive controls, respectively. The mean wheal diameter obtained with different concentrations showed a gradual systematic fall with increase in dilution. The mean relative diameter (% of histamine reaction) varied from 124.1 ± 8.9 to 33.7 ± 6.1 and 78.9 ± 5.5 to 21.4 ± 3.8 with the highest and lowest concentrations of *R.communis* and *H. integrifolia* pollen antigens, respectively. The histamine equivalent concentration of antigen 1,000 Biological Units (BU) obtained for crude pollen extracts of *R.communis* and *H.integrifolia* was 1:17 and 1:22 respectively.**

Biological standardization of allergenic extracts using skin prick test was first proposed by Aas *et al*[1] and later recommended by Nordic countries.[2] The aim of biological standardization is to establish standards of allergenic extracts of different species having same biological activity. A unit of 1,000 (Biological Unit) BU/ml is assigned to the concentration of allergenic extracts eliciting a wheal of the same size as that of histamine (1 mg/ml) in the median sensitive patients. Results of biological standardization from Nordic countries have recently been reported[3-5] and also forms the guidelines for registration of allergen extracts for manufacture in Nordic countries.[2]

Pollen allergens of India, a tropical country, are different to those of western flora. The main purpose of this study is to establish reference preparation of indigenous pollen allergens from India. This is best acheived by biological standardization since such antigens are capable of eliciting immunological responses, have little variation in potency from batch to batch and are devoid of non-specific irritants. *Ricinus communis* and *Holoptelea integrifolia* are two important pollen allergens as their pollen are present in high concentration in atmosphere[6] and their extracts produce markedly positive skin reactions in 24% to 42% of the cases.[7-10]

MATERIALS AND METHODS

Pollen extracts

For preparation of antigenic extracts, polliniferous materials from *Ricinus communis* and *Holoptelea integrifolia* were collected during their flowering season, September-March and February-April, respectively. Pollen shed by the natural dehiscence method was collected after sieving and samples were microscopically examined for their purity. Pollen samples having purity of more than 95%, were extracted in phosphate buffered saline (pH 7.4) in 1:10 (w/v) concentration at 4° C by

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Table 1. Results of RAST (positive) performed using sera of 22 patients who showed markedly positive intradermal skin reactions to pollen antigen of *Ricinus communis*

Sera of patients	Counts/min	% binding	RAST class
1	3,148	6	I
2	16,432	32	IV
3	22,242	43	IV
4	6,651	13	II
5	17,197	33	IV
6	2,196	5	I
7	6,977	13	II
8	16,476	31	IV
9	3,513	6	I
10	4,925	9	II
11	24,331	46	IV
12	5,694	11	II
13	5,894	11	II
14	8,619	16	III
15	12,639	24	III
16	7,747	15	III
17	6,388	12	II

Normal human sera showed 0.59% binding
Total counts/min in 50 μl of I^{125} antihuman IgE = 51,973
Sera of other patients showed negative RAST.

continuous stirring for 20 hours. After extraction, suspension was centrifuged at 27,000 × g for 30 minutes and supernatant was dialysed (cut off point 3,500 daltons) against distilled water for 24 hours with several changes. The dialysed extract was then centrifuged, passed through the millipore filter (0.45 μm), lyophilized and stored at -20° C till further use.

Freeze dried allergen preparations were reconstituted in 50% glycerinated phosphate buffered saline for clinical testing to obtain stock solution of 1:10 concentration (w/v). Further three fold log dilutions (1:10, 1:30, 1:100, 1:300, 1:900) from both the extracts were made from stock solution. In addition to different dilutions of allergen, 50% glycerinated buffer and histamine dihydrochloride (1 mg/ml) were also prepared.

Patients

Patients attending the Clinical Research Centre of the V P Chest Institute for treatment of their respiratory ailments were included in the study. The criteria followed for the inclusion of patients for biological standardization is given below :

i) Age between 15 and 50 years.
ii) Positive case history and positive intradermal skin test reaction to *Ricinus communis* and *Holoptelea integrifolia* pollen allergens.
iii) No previous hyposensitization with any pollen allergens.
iv) No treatment with anti-allergic drugs 48-72 hours before the skin test.
v) Presence of specific IgE antibodies against allergen in question (RAST/ELISA positive).

Radio allergo sorbent test (RAST)

Blood samples were collected from *Ricinus communis* and *Holoptelea integrifolia* intradermal test positive patients for specific IgE antibodies. RAST was carried out according to the manufacturer's manual (Pharmacia Diagnostics, Sweden). Allergen discs were prepared with Whatman filter paper (No. 50), activated with cyanogen bromide and then coupled with pollen allergen. In brief, 50 μl of sera were incubated overnight with allergen disc of the two allergens. Discs were then washed and incubated with I^{125} anti-human IgE antibodies. Discs were again washed and bound radioactivity was measured. Discs of birch and sera against birch pollen antigen, provided by the manufacturer, were used as standard. The sera of patients allergic to *Ricinus communis* and *Holoptelea integrifolia* were graded into four classes according to the Pharmacia manual and percent binding was also calculated (Table 1, 2). As is evident from the tables, 77.3% and 63.6% of the intradermal test positive patients showed RAST positivity of different classes to *Ricinus communis* and *Holoptelea integrifolia* antigens, respectively.

Skin prick test

Skin prick tests with both the extracts were performed on 15 patients (14 males, 1 female; 16-44 years of age; median 28 years for *Ricinus*, and 13 males, 2 females; 16-44 years of age; median 27 years for *Holoptelea*). Tests were performed in duplicate on the forearms of the

Table 2. Results of RAST (positive) with sera of 22 patients showing markedly positive skin reactions to pollen antigen of *Holoptelea integrifolia.*

Sera of patients	Counts/min	% binding	RAST class
1	10,140	19	III
2	4,488	8	II
3	5,121	9	II
4	5,816	10	II
5	11,693	22	III
6	8,051	15	III
7	3,161	6	I
8	8,615	16	III
9	6,458	11	II
10	7,728	14	III
11	3,676	6	I
12	4,078	7	I
13	7,498	13	II
14	6,343	11	II

% binding to the sera of normals = 0.57%
Total counts in 50 μl of I^{125} antihuman IgE = 51,973
Sera of other eight patients showed negative RAST.

Table 3. Mean wheal diameter as determined by skin prick test in 15 markedly positive intradermal cases against five tree–fold log dilutions of *Ricinus communis* and *Holoptelea integrifolia* pollen antigens as compared to positive control histamine (1 mg/ml)

Antigen concentration	Mean wheal diameter			
	Ricinus		*Holoptelea*	
	N	$\bar{x} \pm SE\bar{x}$	N	$\bar{x} \pm SE\bar{x}$
1:10	15	6.233 ± 0.433	15	6.500 ± 0.422
1:30	15	4.867 ± 0.464	15	4.933 ± 0.338
1:100	15	3.633 ± 0.446	15	3.700 ± 0.384
1:300	15	2.587 ± 0.386	15	2.700 ± 0.374
1:900	15	1.747 ± 0.318	14*	2.064 ± 0.409
Histamine (1 mg/ml)	15	5.067 ± 0.200	15	4.900 ± 0.148

N = Number of patients studied
$SE\bar{x}$ = Standard error of mean
$\bar{x}$ = Mean wheal diameter
* = Excluding one patient who did not respond to the last concentration

patients. A drop of antigen was placed on the forearm and pricked with the help of a 23 G needle. Immediately after the prick, the excess antigen was wiped off with tissue paper. Beside the antigen, 50% glycerinated phosphate buffered saline and histamine dihydrochloride (1 mg/ml) were also pricked as negative and positive controls, respectively. The wheal diameter was recorded after 15-20 minutes, the wheal area was encircled with a fine filter tip pen and an imprint transferred to patient's record sheet by means of scotch tape.

Statistical analysis

The concentration of allergen the patient's own histamine wheal diameter was calculated using the formula

$$Q = \frac{DA \times 100}{DH}$$

where
Q = Relative diameter,
DA = Wheal diameter of a specific allergen concentration for a patient,
DH = Wheal diameter of histamine for the same patient.

The concentration of allergen extract eliciting a wheal of the same size as that of histamine was calculated according to the model.

$$\ln Q = a + b \ln (conc)$$

where
ln Q = log of relative wheal diameter percent to the base,
a = intercept,
b = slope,
ln (conc) = natural log of concentration of antigen.

Only mean diameters > 2 mm were considered, as lower diameters of wheals can be provoked by the prick itself.

Two patients however, were considered outliers because in these cases the concentration of the antigen

was not linearly related to relative diameter percent. These were, therefore, excluded from analysis. The histamine equivalent concentration of antigen (Ch value) was calculated for each patient and used for the calculation of median Ch value (1,000 BU/ml).

RESULTS

Positive skin reactions obtained with different concentrations of pollen antigens and histamine dihydrochloride (1 mg/ml) varied considerably among different patients for both the antigens. The results of mean wheal diameter obtained by skin prick test with five different concentrations of both the allergens are given in Table 3. There was a gradual systematic fall in the wheal diameter with increase in the dilution of both the allergens.

The mean relative diameter (% of histamine reaction) of 15 patients against various concentrations of pollen antigens as compared to histamine dihydrochloride (1 mg/ml) are given in Table 4. The mean relative diameter varied from 124.1 ± 8.9 to 33.7 ± 6.1 with the lowest and highest dilutions of *Ricinus communis* pollen allergen. However, for *Holoptelea integrifolia* pollen, the mean relative diameter varied from 78.9 ± 5.5 to 21.4 ± 3.8 with 1:10 and 1:900 concentration. The mean relative diameter also followed the same trend as did the mean wheal diameter.

The results of regression analysis for each patient, for both the pollen allergens are shown in Fig. 1. It was observed that lines were parallel (same regression coefficients, standard deviation from regression and coefficient of multiple regression (R^2). This was expected since we used a proportionate division (histamine wheal diameter of 1 mg/ml) in determining the relative diameter. All the regression lines accounted for more than 90% of relative diameter (sum of R^2) in general thus indicating a good fit to the observed data. On average this was 95.7 and 96.0% for *Ricinus communis* and

Table 4. Relative diameter (Q, percent of histamine reaction) in 15 patients against five concentration of *Ricinus communis* and *Holoptelea integrifolia* pollen antigens as compared to positive control histamine 1 mg/ml

Antigen concentration	Relative diameter (Q)	
	R. communis	*H. integrifolia*
	$\bar{x} \pm SE\bar{x}$	$\bar{x} \pm SE\bar{x}$
1:10	124.1 ± 8.9	78.9 ± 5.5
1:30	96.6 ± 9.2	61.4 ± 5.7
1:100	71.2 ± 8.1	45.3 ± 5.3
1:300	50.5 ± 7.6	32.0 ± 4.7
1'900	33.7 ± 6.1	21.4 ± 3.8

$\bar{x}$ = Mean percent of histamine reaction
$SE\bar{x}$ = Standard error of mean

Table 5. Aggregates over patients of the regression parameters (i.e. constant, regression coefficients, standard deviations from regression and coefficients, of multiple correlation along with histamine equivalent concentration) of the two pollen antitigens *Ricinus communis* and *Holoptelea integrifolia*

Antigen	Histamine concentration	Aggregates of regression parameters					
			Constant intercept	Regression coefficient	SD from Regression	SD from RSQ*	HEC*
		N	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\pm SD$	
R. communis	1 mg/ml	15	2.466 ± 0.161	0.334 ± 0.137	0.062 ± 0.044	0.957 ± 0.032	1:17.15
H. integrifolia	1 mg/ml	15	2.430 ± 0.166	0.293 ± 0.118	0.050 ± 0.030	0.960 ± 0.026	1:22.04

N = Number of patients
$\bar{x}$ = Mean of regression parameters over patients
SD = Standard deviation
RSQ* = Coefficient of multiple correlation
HEC** = Histamine equivalent concentration

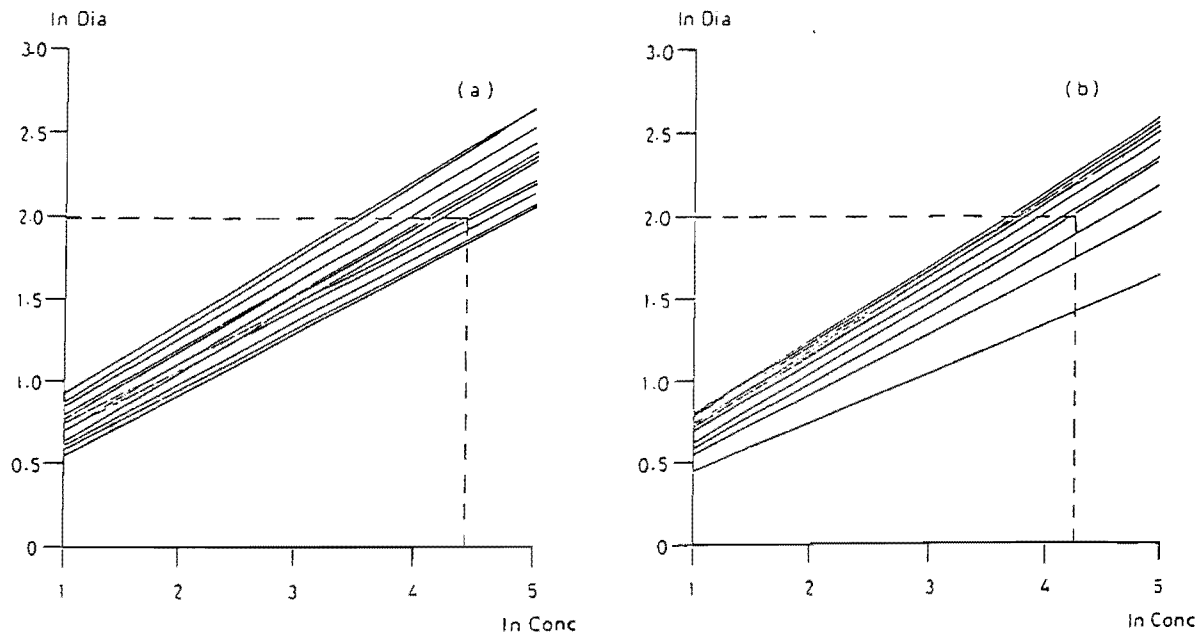

Fig. 1 The calculated regression lines of the allergen dose response relationship with (a) 15 patients positive to *Ricinus communis* pollen antigen and (b) 13 patients positive to *Holoptelea integrifolia*. The X–axis denotes the five three fold log dilution used for skin prick test and the Y–axis depicts their relative percent diameter.

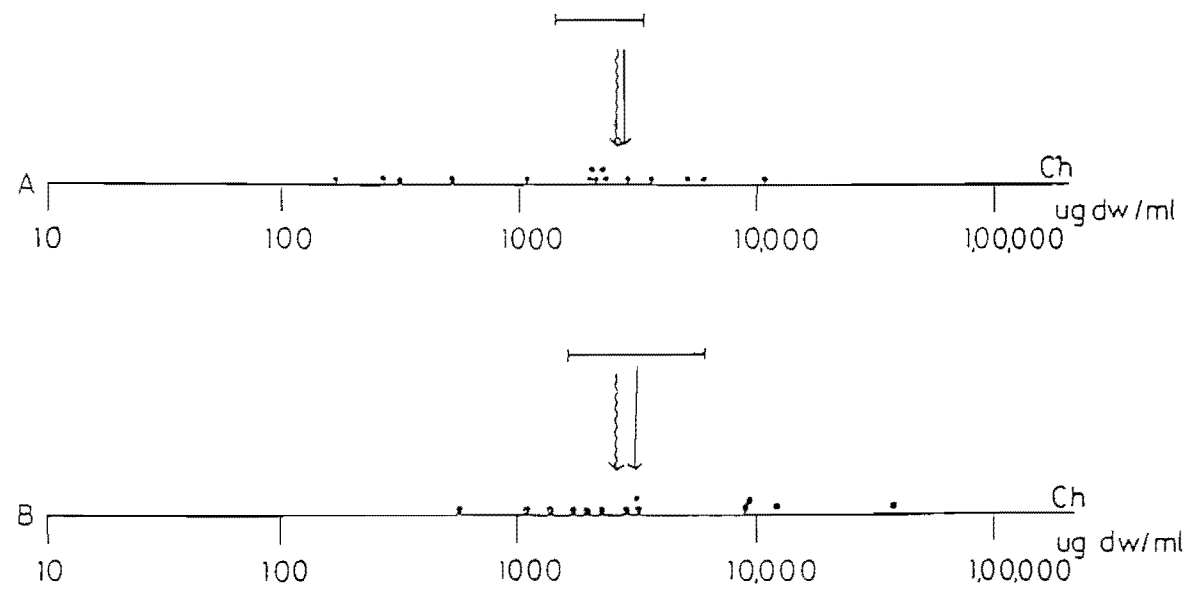

Fig. 2 The histamine equivalent concentration (Ch), mean (⟶), median (⟶) and the 95% confidence intervals (bars) for patients tested with pollen extracts of (a) *Ricinus communis* and (b) *Holoptelea integrifolia*.

Holoptelea integrifolia pollen antigens, respectively (Table 5). Standard deviations from regression were also not very large, again indicating adequate fit of the regression lines.

The median Ch value for crude *Ricinus communis* pollen extract was 1:17 or 4,000 μg dw (dry weight) antigen/ml (range 0.207-10.1 μg dw/ml; with 95% confidence limit of 2.3-5.6). The median Ch value for crude *Holoptelea* pollen extract was 1:22 concentration or 5,500 μg dw antigen/ml (range 0.73-15.28 with 95% confidence intervals 2.99-8.095). The 1:17 and 1:22 dilutions of *Ricinus communis* and *Holoptelea integrifolia,* respectively are equivalent to 1,000 BU.

DISCUSSION

Much attention is being paid nowadays to standardization of allergenic extracts as it is important for proper diagnosis and effective immunotherapy of allergic disorders. Different methods are employed for standardizing extracts so as to have reference preparation of each extract and to avoid batch-to-batch variability. Biological standardization is being carried out using both intracutaneous test [11] and skin prick test. [3] Several pollen allergens of temperate countries such as Rye grass, Timothy, Mugwort, Birch, *Parietaria judiaca* and *P.officinalis* are standardized in terms of biological units. [4,5,12,13] But too little attention has been paid in tropical countries like India to the biological standardization of allergen preparations. As the fauna and flora of tropical countries are different than those of temperate countries, efforts were made to standardize offending pollen allergens of *Ricinus communis* and *Holoptelea integrifolia* indigenously. These pollen allergens were also investigated in detail for their protein content, protein profiles and allergenic determinants using various clinico-immunological studies. [14,15] The pollen samples collected during the middle of the pollen season and giving optimum information on protein profile and allergenic determinants for both the taxa were used for the study.

All the 15 patients selected were included in biological standardization for *Ricinus communis,* where as for *Holoptelea integrifolia* two patients were considered as outliers and therefore excluded from analysis. The Ch value obtained from crude *Ricinus communis* pollen allergen was 4,000 μg dw/ml and for *Holoptelea integrifolia* 5,500 μg dw/ml. Dreborg and Grimmer [3] carried out skin prick test with different batches of crude and purified allergenic fraction of timothy *(Phleum pratense).* The amount of dry weight of purified fraction necessary to obtain an activity of 1,000 BU varied from 0.7 μg dw/ml to 3 μg dw/ml. For Mugwort pollen, 17.4 μg dw/ml corresponded to 1,000 BU/ml [13]. However, Dreborg *et al* [4] found 8.3 μg dw/ml of crude extract and 1.8 μg dw/ml of purified fraction to correspond to 1,000 BU, for the same pollen. The difference in the two studies was explained by Dreborg *et al* [4] as being due to differences in skin prick test technique and statistical analysis or due to clinical sensitivity of the patients tested by them. Besides pollen, some fungal, mite and dog allergens have also been standardized on the basis of their biological activity.

Biological standardization has been used for studying geographical and seasonal variations in the antigenic extracts and to check the allergenic activity of two batches, in addition to various *in vitro* methods used. Dreborg and Grimmer [3] based on their observations concluded that biological standardization has a good precision, gives comparable results in different regions if the skin prick test method, inclusion criteria for patients and statistical methods are the same. Thus, two pollen allergens from tropical India have been standardized for the first time based on their biological activity.

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