

Immunoglobulin E (IgE)-mediated cross-reactivity between mesquite pollen proteins and lima bean, an edible legume

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Summary

Immunoglobulin E (IgE)-mediated food allergy often develops as a consequence of allergic sensitization to pollen proteins. Mesquite (*Prosopis juliflora*) tree pollen is reported to be cross-reactive with other pollen species, but little has been reported on its cross-reactivity with plant-derived foods belonging to the same/different families. The present study investigates the *in vitro* cross-reactivity of mesquite pollen and lima bean (*Phaseolus lunatus*), an edible seed belonging to the Leguminosae family. Of 110 patients (asthma, rhinitis or both) tested intradermally, 20 showed marked positive reactions with *Prosopis* pollen extract. Of these, 12 patients showed elevated specific IgE to *Prosopis* pollen extract alone and four to both *Phaseolus* and pollen extract. *In vitro* cross-reactivity was investigated using inhibition assays [enzyme-linked immunosorbent assay (ELISA) inhibition, immunoblot inhibition], histamine release and lymphoproliferation. *P. lunatus* extract could inhibit IgE binding to *P. juliflora* in a dose-dependent manner, requiring 400 ng of protein for 50% inhibition in ELISA assay. Immunoblot and immunoblot inhibition demonstrated the presence of 20, 26, 35, 66 and 72 kDa as shared IgE binding components between the two extracts. Histamine release, peripheral blood mononuclear cells proliferation and interleukin (IL)-4 levels also suggested allergenic cross-reactivity. In conclusion, there is humoral and cellular cross-reactivity between *Prosopis* pollen and *Phaseolus* seed allergens.

Keywords: cross-reactivity, food allergy, lima bean, mesquite, stripped basophil histamine release assay

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Introduction

Studies have shown that food allergy in adolescent and adult individuals develop because of an allergic sensitization or cross-reaction to inhalant pollen allergens [1,2]. Pollen-associated food allergies are a consequence of the cross-reaction of pollen allergen-specific IgE antibodies with highly homologous proteins contained in foodstuff [3]. Pollen food cross-reactions were first reported in 1942, followed by several such studies by other workers [4–6]. Cross-reactivity between birch pollen (*Betula verrucosa*) and foods have been studied at molecular level [7]. The major birch allergen Bet v 1, that belongs to the pathogenesis-related protein family 10 (PR-10), has been identified in apple (Mal d 1), hazelnut (Cor a 1), pear (Pyr c 1), cherries (Pru av 1) or carrot (Dau c 1) [8,9]. In addition to birch, several other pollen species such as mugwort and ragweeds are shown to

be related allergenically with vegetables such as celery, carrot, mustard and cucumber [6,10].

Mesquite tree (*Prosopis juliflora*) and lima bean (*Phaseolus lunatus*) belong to the family Leguminosae. Previous studies have shown mesquite tree pollen to be an important allergen source in tropical and subtropical countries, with proteins of 14–97 kDa as IgE binding components [11–14]. Further proteins of 14, 41, 52 and 66 kDa were recognized as shared allergens among mesquite and other tree pollens [14]. Lima bean is a common food consumed as pulses and vegetables world-wide [15,16]. In a survey of food allergy among asthma and rhinitis patients in Delhi, lima bean showed a history and skin prick test-positive reactions in 16 of 470 (3.4%) cases. Immunoblot analysis with allergic patients' sera demonstrated 12 IgE binding proteins of 18–96 kDa in lima bean [17]. There are reports suggesting that lima bean cross-reacts with other allergenic legumes, such as soya,

peanut, black gram, etc., based on skin test reactivity [18–20]. The present study was therefore undertaken to investigate cross-reactivity between *P. juliflora* (mesquite tree) pollen and *P. lunatus* (lima bean) food allergens.

Materials and methods

Preparation of extract

P. juliflora pollen powder and grounded *P. lunatus* seeds were defatted using diethyl ether and extracted in ammonium bicarbonate buffer, as described previously. The protein concentration of extracts was determined by modified Lowry's method [14].

Skin testing and sera collection

Intradermal tests with *P. juliflora* pollen and other allergen extracts were carried out at the out-patient department, S. P. Medical College, Bikaner for allergy diagnosis. The patients were diagnosed with bronchial asthma (BA) and allergic rhinitis (AR) (Table 1) following American Thoracic Society guidelines and Admopolous *et al.* [21,22]. Skin tests were graded after 20 min [14] as per wheal size of positive control, i.e. histamine diphosphate (100 µg/ml). Phosphate-buffered saline (PBS) was used as negative control. Skin tests were also

carried out on non-allergic volunteers and blood was collected from patients ($n = 20$) and normal controls ($n = 5$). The study protocol was approved by the Institute's ethics committee.

Enzyme-linked immunosorbent assay (ELISA)

Specific IgE binding to both *P. juliflora* and *P. lunatus* extract was determined by ELISA. Briefly, 1 µg protein in 100 µl of carbonate–bicarbonate buffer (pH 9.6) was coated per well in triplicate, incubated overnight at 4°C and blocked with defatted milk. The plate was washed followed by incubation with individual sera (1 : 10 v/v in PBS) from patients showing marked skin reactivity to *P. juliflora*. ELISA was further carried out as described previously [14]. Sera from non-allergic subjects ($n = 5$) were pooled and used as normal human sera (NHS).

ELISA inhibition

For ELISA inhibition, *P. juliflora*-hypersensitive pooled patients' sera (1 : 10 v/v in PBS) was preincubated with different concentrations (1–10 000 ng) of *P. juliflora* or *P. lunatus* extract overnight at 4°C. The preincubated mixture was then added to microtitre plates coated with *P. juliflora* pollen extract (1 µg/100 µl) in triplicate. NHS was used as a

Table 1. Clinical history and specific IgE values in patients with mesquite allergy.

Patient no.	Age (years)/sex	ID to <i>P. juliflora</i> (mm)	Symptoms	Specific IgE to <i>P. juliflora</i> (OD)	Specific IgE to <i>P. lunatus</i> (OD)
1	32 F	14	AR	0.542	0.101
2	34 M	11	AR	0.257	0.080
3	36 M	12	BA	0.471	0.285
4	32 M	12	BA, AR	0.426	0.123
5	42 M	16	BA, AR	0.450	0.288
6	31 M	10	AR	0.301	0.092
7	36 M	15	AR	0.423	0.308
8	32 M	12	BA, AR	0.281	0.077
9	14 M	10	BA, AR	0.250	0.102
10	28 M	12	BA, AR	0.412	0.288
11	38 M	12	BA, AR	0.416	0.156
12	19 M	10	BA, AR	0.201	0.132
13	28 M	11	R	0.163	0.154
14	30 F	12	BA, AR	0.178	0.095
15	29 F	10	BA, AR	0.272	0.178
16	45 M	14	AR	0.325	0.116
17	26 F	12	AR	0.193	0.094
18	25 M	15	AR	0.421	0.114
19	25 F	10	AR	0.382	0.190
20	32 M	12	BA, AR	0.454	0.085
NHS (pool)				0.071	0.075

M = male, F = female; BA = bronchial asthma, AR = allergic rhinitis. ID = intradermal reaction (wheal size in mm), wheal size $\geq$ positive control, i.e. histamine diphosphate (100 µg/ml) was considered ID-positive reaction. Positive control values for patients ranged from 10 to 18 mm. Bold numbers = patients with significant specific IgE levels (≥ 3 times of NHS) against *P. lunatus*. NHS = pool of healthy individual serum ($n = 5$). Sera from 10 patients (patients 1, 3, 4, 5, 7, 10, 11, 18, 19 and 20 with specific IgE ≥ 5 times of NHS against *P. juliflora*) were pooled together or used individually for various assays.

negative control; sera without inhibitor was used as positive control and developed as above. Percentage inhibition was calculated as:

$$1 - \frac{\text{OD of sample with inhibitor}}{\text{OD of sample without inhibitor}} \times 100$$

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

SDS-PAGE was carried out on 12% gel using a discontinuous buffer system and proteins resolved were visualized by Coomassie brilliant blue staining. For immunoblotting, SDS-PAGE-resolved pollen and food extracts were transferred onto nitrocellulose membrane (NCM) using a transfer buffer (pH 8.3) containing 25 mM Tris, 192 mM glycine and 20% methanol [14]. The unbound sites were blocked by 3% defatted milk for 3 h at 37°C. The NCM strips were washed and incubated with 1:10 v/v *Prosopis* hypersensitive patients' sera in PBS overnight at 4°C. Strips were washed three times with PBS with Tween (PBST), incubated with anti-human IgE peroxidase (1:1000 v/v) for 3 h at 37°C, and detection of IgE binding components was carried out as described previously [14]. Pooled NHS was taken as negative control. An irrelevant allergen control (*Curvularia lunata*, a fungus) was also blotted and probed with *P. juliflora* hypersensitive patients' sera.

Immunoblot inhibition

For blotting inhibition, *P. juliflora*-positive pooled patients' sera was preincubated with inhibitors (200 µg of *P. juliflora* protein extract or 200–600 µg of *P. lunatus* extract) and added to blot strips containing *P. juliflora* proteins. As a positive control, a *P. juliflora* strip was incubated with pooled patients' sera (1:10 v/v) without inhibitor. Another *P. juliflora* strip probed with NHS served as negative control. Strips were washed three times with PBST, incubated with anti-human IgE peroxidase (1:1000 v/v) for 3 h at 37°C and blots developed as above.

Stripped basophil histamine release assay

Heparinized peripheral blood was collected from healthy volunteers ($n = 10$) with no known history of allergy and mixed with a solution containing 0.1 mmol/l ethylenediamine tetraacetic acid (EDTA), 6% dextran, 2% dextrose and 0.9% sodium chloride and incubated for 90 min at room temperature [23]. The supernatant containing plasma, leucocytes and platelets was transferred to another tube and centrifuged at 300 g for 8 min at 4°C. The stripped basophil histamine release bioassay was performed as described previously [24]. The basophils were sensitized passively by incubation with 150 µl of patient plasma in 4 mmol/l EDTA and 10 mg/ml heparin. As a control, basophils were also

sensitized with serum pool from healthy individuals ($n = 5$). Histamine was determined according to the method of Siraganian [25] and expressed as a percentage of the total histamine determined after lysis of the cells with perchloric acid. Histamine release of more than 10% was considered positive, as the spontaneous release was always less than this cut-off point.

Peripheral blood mononuclear cells (PBMC) proliferation and cytokine analysis

PBMCs were obtained from peripheral blood ($n = 10$) by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA). PBMC (1×10^5) were cultured in triplicate in 96-well plates in 200 µl of serum-free RPMI-1640 media containing penicillin (100 U/ml; Sigma) and 10% fetal bovine serum in the presence of 5 µg of *P. juliflora*/*P. lunatus* extract, separately at 37°C with 5% CO₂ in a humidified atmosphere. The culture plates were incubated for 72 h and supernatant collected for cytokine assays. Cells were washed with RPMI-1640 and incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 100 µg/ml). The cells were lysed in acidic isopropanol solution and the absorbance read at 570 nm. Optical density (OD) values more than or equal to three times the negative control were considered with positive stimulation index. Phytohaemagglutinin (5 µg) was used as positive control and cells without stimulant were used as negative control.

Interleukin (IL)-4 levels were estimated by ELISA (OptEIA kits; BD Pharmingen, San Diego, CA, USA) in culture supernatants as described elsewhere [26].

Results

IgE reactivity of extracts

Of 110 patients (asthma, rhinitis or both) tested intradermally, 20 showed a marked positive skin reaction with *P. juliflora* pollen extract. Specific IgE was determined by ELISA against *P. juliflora* and *P. lunatus* in 20 skin test-positive patients (Table 1). Most of the patients (16 of 20) showed elevated IgE levels against *P. juliflora* (OD 0.25–0.54) and of these 16, four patients (patients 3, 5, 7 and 10) also demonstrated elevated specific IgE to *P. lunatus* (OD 0.28–0.30). OD values of NHS against *P. juliflora* and *P. lunatus* were 0.071 and 0.075, respectively. Patient samples (patients 1, 3, 4, 5, 7, 10, 11, 18, 19 and 20; Table 1) showing IgE levels ≥ 5 times of the normal control against *P. juliflora* were used for various assays.

Inhibition of IgE binding to *P. juliflora* using *P. lunatus* extract as inhibitor

ELISA inhibition showed a dose-dependent inhibition of the IgE directed towards *P. juliflora* in patients' sera positive to

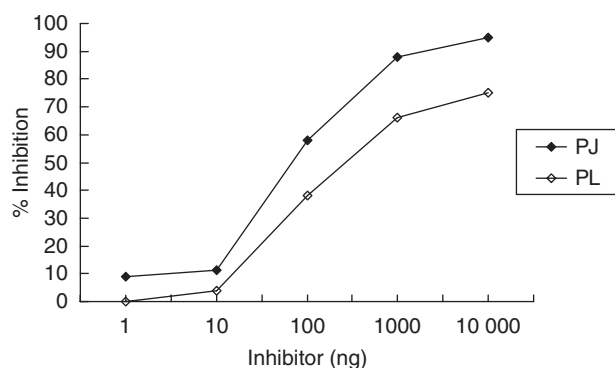


Fig. 1. Immunoglobulin E (IgE) enzyme-linked immunosorbent assay (ELISA) inhibition with *Prosopis juliflora* pollen and *P. lunatus* seeds extract. *Prosopis* extract, 1 µg/well, was coated and incubated with a mixture containing *Prosopis* pooled sera (1 : 10) and 1, 10, 100, 1000 and 10 000 ng of extract as inhibitors (■ PJ; □ PL; *P. lunatus*). The bound IgE was determined using anti-human IgE-horseradish peroxidase (HRP). The values represent the mean of three independent experiments.

Prosopis (Fig. 1). For 50% inhibition of IgE binding, 70 ng of *P. juliflora* extract was required, whereas 400 ng of *P. lunatus* extract (protein) was required to inhibit 50% of the total IgE binding to *P. juliflora*. *P. lunatus* managed to inhibit 80% of the total IgE binding to *P. juliflora* at 10 µg protein. The irrelevant control, *C. lunata*, could not cause 50% inhibition even at 10 µg concentration (data not shown).

Protein profile and shared IgE binding components of extracts

SDS-PAGE resolved pollen and seed extracts into 33 and 22 distinct protein bands, respectively, molecular weight ranging from 14 to >100 kDa (Fig. 2a). Immunoblot analysis of *P. juliflora* pollen extract with pooled sera from hypersensitive patients ($n = 10$) revealed 16 IgE binding components of 14, 16, 20, 24, 26, 29, 31, 35, 41, 45, 52, 58, 66, 72, 95 and 97 kDa. *P. lunatus* extract with the same serum pool detected nine IgE binding components of 14, 20, 24, 26, 29, 35, 52, 66 and 72 kDa. *P. lunatus* proteins separated in a similar molecular weight range to *P. juliflora*, indicating the presence of shared allergens (Fig. 2b).

To demonstrate allergenic cross-reactivity of *P. juliflora* with *P. lunatus*, immunoblot inhibition was performed using *P. juliflora*-positive pooled patients' sera (Fig. 2c). *P. juliflora* extract showed complete inhibition of specific IgE reactivity at 200 µg of self-protein, whereas *P. lunatus* caused partial inhibition of *P. juliflora* extract at 200 µg concentration. Complete inhibition could not be achieved even on increasing the concentration of *P. lunatus* to 600 µg, suggesting the presence of some unique allergens. IgE binding components of 20, 26, 35, 66 and 72 kDa of *P. juliflora* were inhibited by *P. lunatus* extract, indicating the presence of cross-reactive allergens.

Histamine release with pollen and food extract(s) in patients with *Prosopis* allergy

On stimulation of passively sensitized basophils (basophils from non-atopic donors stripped of their IgE and sensitized with *Prosopis*-hypersensitive pooled sera) with increasing concentrations (0.001–1000 ng/ml) of *P. juliflora* and *P. lunatus* extract, a dose-dependent release of histamine was observed with both extracts (Fig. 3a,b). *P. juliflora* induced 76% histamine release at 1 µg/ml allergen concentration compared to 45% by *P. lunatus* at the same concentration. *C. lunata*, the fungus used as irrelevant control, did not induce histamine release. Individual patients' sera with significant IgE against *Prosopis* were tested separately for basophil histamine release with both *P. juliflora* and *P. lunatus* extracts. Dose-dependent histamine release was observed upon stimulation with *P. juliflora* after sensitization with all ($n = 10$) individual sera, whereas in the case of *P. lunatus* extract the same was observed upon sensitization with four (patients 3, 5, 10 and 20) of 10 sera used (Fig. 3a,b). Of these four patient samples, passive sensitization with three sera showed histamine release (30% and more) at a concentration of 1 µg/ml of *P. lunatus* extract compared to all 10 patients inducing $\geq 30\%$ release with *P. juliflora* at the same concentration (Fig. 3c). These three patients (3, 5 and 10) showed elevated IgE levels to *P. lunatus* extract by ELISA (Table 1). Patient 20, with low IgE, also showed a dose-dependent release of 20% at its maximum concentration.

Cell proliferation and IL-4 production on stimulation with *P. lunatus* extract

PBMCs isolated from *Prosopis* allergy patients were stimulated with both *P. juliflora* and *P. lunatus* extract. Stimulation of cells from all 10 individuals with *P. juliflora* extract revealed strong lymphoproliferative responses, whereas significantly lower proliferation was observed upon stimulation with *P. lunatus* extract (Fig. 4; $P \leq 0.001$). PBMCs isolated from non-allergic individuals did not proliferate in response to this allergen.

IL-4 levels were also lower (34–172 pg/ml) in culture supernatants obtained on stimulation with *P. lunatus* allergen compared to *P. juliflora* (198–478 pg/ml, data not shown). However, patients with elevated IgE to *P. lunatus* (patients 3, 5, 7, 10) showed IL-4 levels (85–172 pg/ml) higher than normal controls (15–20 pg/ml).

Discussion

Type I allergic reactions are mediated by allergen-specific IgE. Specific IgE to a particular allergen often cross-react with other allergen(s) sharing sufficient structural and sequential homology [3]. Identification and characterization

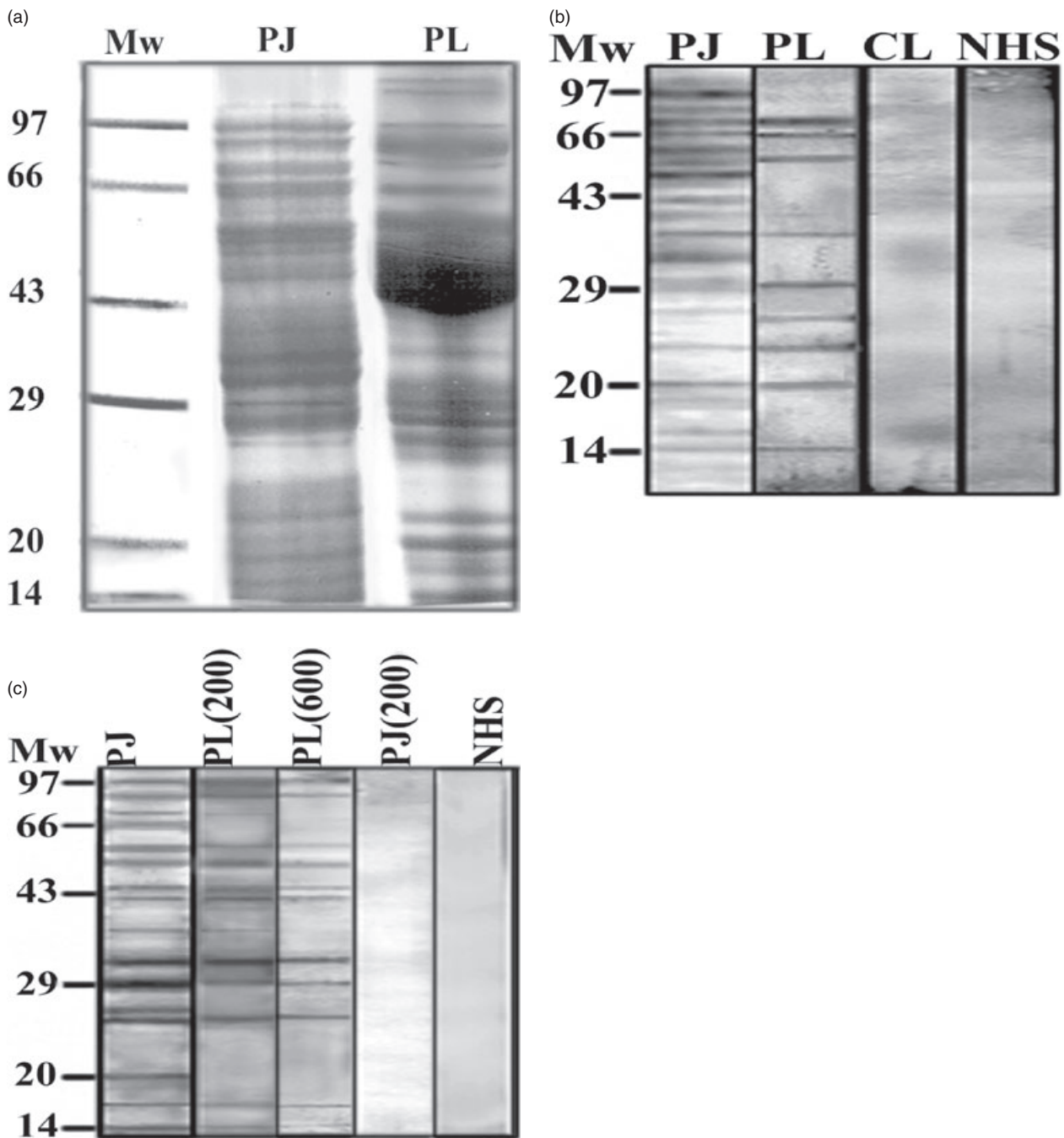


Fig. 2. (a) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) profile of *Prosopis juliflora* (PJ) pollen and *P. lunatus* (PL) seed extract. (b) Immunoblot of *P. juliflora* pollen (PJ) and *P. lunatus* (PL) seeds extract showing immunoglobulin E (IgE)-reactive bands. Extracts were probed with serum pool from *P. juliflora*-sensitive patients. NHS: probed with pooled normal human serum; CL: strip containing *Curvularia lunata* as an irrelevant control. (c) IgE immunoblot inhibition with *P. juliflora* pollen and *P. lunatus* seed extracts. *P. juliflora* protein strips were incubated with serum pool containing 200 µg (PJ 200) of pollen extract and 200 µg (PL 200) and 600 µg (PL 600) of seed extract as inhibitors. PJ: *P. juliflora* strip probed with serum pool without inhibitor NHS: probed with pooled normal human sera; Mw: molecular weight markers.

of cross-reactive allergens can facilitate the study of factors determining clinical relevance of this cross-reactivity and improve the efficacy of immunotherapy [27]. Extensive cross-reactivity has been observed among some legumes (foods) and tree pollen species [2,28]. Recently,

cross-reactivity of *P. juliflora* pollen allergens (family: Leguminosae) with other tree species has also been delineated [14], but efforts are required to investigate cross-allergenicity of foods and pollen belonging to the legume botanical family. Hence, in the present study, IgE cross-reactivity of

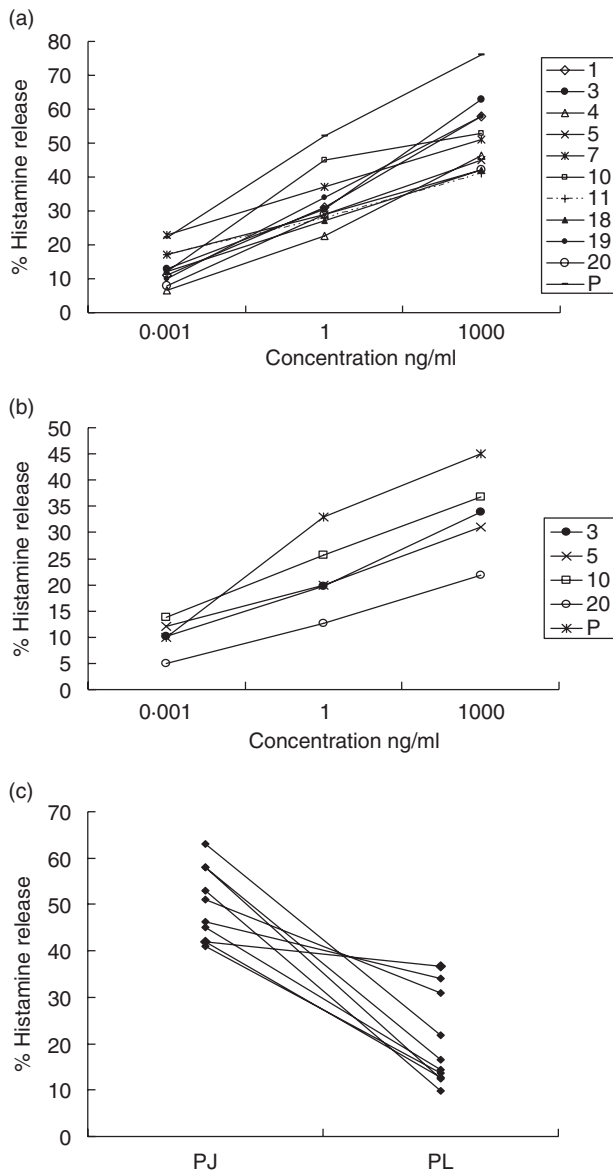


Fig. 3. (a) Stripped basophil histamine release assay. Leucocytes obtained from non-atopic individuals were stripped of their immunoglobulin E (IgE) and sensitized with *Prosopis juliflora* hypersensitive individual patients' serum ($n = 10$: patients 1, 3, 4, 5, 7, 10, 11, 18, 19 and 20). Passively sensitized basophils were then stimulated with three concentrations (0.001, 1.0 and 1000 ng/ml) of *P. juliflora* pollen extract. (b) Stripped basophil histamine release assay. Leucocytes obtained from non-atopic individuals were stripped of their IgE and sensitized with *P. juliflora* hypersensitive individual patients' sera ($n = 4$: patients 3, 5, 10 and 20). Sensitized basophils were then stimulated with three concentrations (0.001, 1.0 and 1000 ng/ml) of *P. lunatus* seed extract. P: Pooled patients' sera. (c) Histamine release on stimulation of passively sensitized (sensitized with individual patient serum from patients 1, 3, 4, 5, 7, 10, 11, 18, 19 and 20, separately) basophils with *P. juliflora* (PJ) and *P. lunatus* (PL) extracts at 1 µg/ml (1000 ng/ml) concentration.

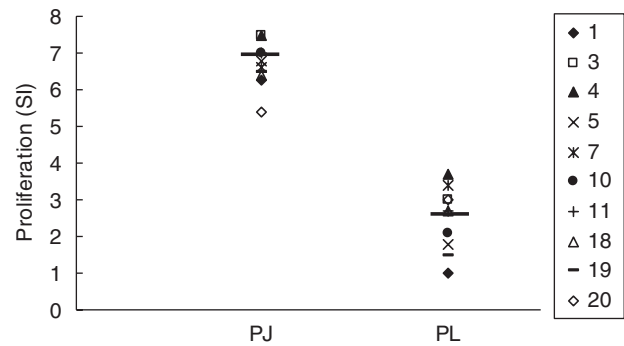


Fig. 4. Lymphoproliferation (SI) of peripheral blood mononuclear cells (PBMC) derived from 10 *Prosopis*-sensitized patients (patients 1, 3, 4, 5, 7, 10, 11, 18, 19 and 20) in response to stimulation with 5 µg of *P. lunatus* (PL) and *P. juliflora* (PJ) extract. Horizontal lines indicate the median value. Proliferative responses induced by PL were significantly lower than PJ (Wilcoxon signed ranks test, $P = 0.001$).

P. juliflora (mesquite) tree pollen and *P. lunatus* (lima bean), a common legume food, was investigated.

Earlier studies show that individuals clinically reactive to one legume often show IgE binding to several other legumes [18,29]. In the present study, of 20 *P. juliflora* skin test-positive patients, none showed marked positive skin reactivity to *P. lunatus* extract. Of these ($n = 20$), 16 patients showed elevated IgE to *P. juliflora*, whereas four showed raised IgE levels to *P. lunatus*. As the patients selected were skin test-negative to lima bean, positive IgE levels may be due to the presence of cross-reactive IgE. Although skin tests and ELISA both detect allergen-specific IgE, the results do not always correlate with each other [30,31].

ELISA inhibition has been used previously to identify cross-reactivity among pollen, fungi and other allergens [32–34], whereas immunoblot inhibition demonstrates a component-based allergenic relationship. Using these methods, allergenic cross-reactivity has been demonstrated previously between protein components of *P. juliflora* pollen and important tree pollens such as *Ailanthus excelsa*, *Cassia siamea* and *Salvadora persica* allergens [14]. In the present study, ELISA inhibition using pooled *P. juliflora* hypersensitive patients' sera suggested the presence of cross-reactive IgE antibodies that bind to *P. lunatus* extract and inhibit their binding to solid-phase *P. juliflora* pollen extract. Immunoblot inhibition demonstrated components of 20, 26, 35, 66 and 72 kDa as shared IgE binding components between *P. juliflora* and *P. lunatus*. *P. juliflora* pollen extract contains four major allergenic proteins of 26, 29, 52 and 66 kDa [14]. Of these, major allergens of 26 and 66 kDa were found to be shared with *P. lunatus* extract.

Stripped basophil histamine release bioassay was proved useful to complement and extend serological detection of allergen-specific IgE [24]. It was also found useful in determining the cross-linking potency (biological activity) of the allergens *in vitro*. In the present study, stripped basophil

histamine release assay was used to determine biological activity of cross-reactive allergens by stimulating *Prosopis*-sensitized basophils with *P. lunatus* extract. The results showed that *Prosopis*-positive patients with raised specific IgE (patients 3, 5 and 10) to *P. lunatus* released histamine almost equivalent to *P. juliflora*. Results of histamine release in these patients correlated well with ELISA, suggesting biological activity of both the extracts. Patient 20, with low IgE to *P. lunatus*, also showed dose-dependent histamine release. Stripped basophil histamine release assay, being highly sensitive, may detect histamine release in patients having IgE against minor allergens [24]. Moreover, release of mediators depends on the valency (no. of cross-reactive epitopes) and affinity of IgE to the IgE receptors [35].

The present study also suggest cellular cross-reactivity between the two allergens as predominantly *Prosopis*-specific PBMCs showed proliferation on stimulation with *P. lunatus*. IL-4 levels were also higher in some patients against *P. lunatus*, although the levels were comparatively lower than those against *P. juliflora*.

The present study suggests allergenic cross-reactivity among mesquite and lima bean, with proteins of 20, 26, 35, 66 and 72 kDa as shared allergens. It also suggests both humoral and cellular cross-reactivity between the two allergens. However, further studies are required to establish risk to patients using food challenge tests with *P. lunatus*.

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