Chapter II

HARACTERIZATION OF THE POLLE POLLEN EXTRACT OF Pennisetum typhoides
INTRODUCTION

Grass pollen is one of the most important etiologic agents to initiate type I allergic reactions in genetically predisposed population [56]. Their aerobiological and clinical importance in Indian sub-continent has been established by various workers in past several decades [3,14-15,38,58,69-70,72-73,75].

A number of pharmacological agents are being used for the treatment of patients suffering from allergic diseases, however, the main drawback of such drugs lies in the fact that they provide only symptomatic relief and do not confer long lasting immunity against offending source of allergens in patients [53,57]. On the other hand, allergen immunotherapy, a well established therapeutic measure, has been found to give long term benefits to patients with type I allergies [35-37]. Allergen immunotherapy involves repeated injections of extract prepared from the offending source in increasing doses, over a long period of time until a maintenance dose is reached. It is also known as desensitization or hyposensitization therapy [7,41]. Introduced by Noon, it is being used successfully all over the world, since the turn of this century [59]. Crude extracts of various grass pollens are in use both for diagnostic and therapeutic purposes in India and elsewhere [69,79]. However, this type of therapy suffers from the lack of well characterized and standardized extracts [53]. Crude pollen extracts are heterogeneous in composition as they contain various allergenic and non-allergenic biomolecules including different proteins and polysaccharides. Besides, crude pollen extract also contains irritants, endotoxins and several other undesirable substances [11-12].

Majority of the pollen components are non-allergenic in nature, while the allergenic components are extremely small in quantity and may vary in different preparations of the extract. Their use in patients during the course of skin test or allergen immunotherapy have raised several controversies. An incidence where non-atopic healthy volunteer became sensitive to grass pollen after few injections of crude pollen extract was reported by Norman (1980) [60]. It is, therefore, essential to characterize these extracts with reference to their allergenic and non-allergenic components. Over a long period of time, several drawbacks of these crude extracts have been realized by various workers. It was observed that allergen extracts are not always stable under normal storage conditions [1,4,78]. Batch to batch variations in
Chapter II Characterization of *Pennisetum typhoides* pollen extract

Extracts prepared by the same manufacturer have been noticed. At the same time, extracts prepared by different manufacturers are also known to differ in their allergenic composition and potency [5, 30-31, 49-50, 52, 54, 81, 92]. Another problem associated with crude allergen extract is that they carry different allergenic proteins which share different sensitivity profile in different patients. Use of poorly characterized crude extracts in allergen immunotherapy has its own inherent risk of causing severe anaphylactic reaction in patients [28, 44]. In a survey, encompassing 42 years of allergen immunotherapy in the USA, 46 fatal systemic reactions after injecting crude extracts were reported [43]. In 1986, the British Committee on Safety of Medicine noted five fatalities during allergen immunotherapy in just 18 months. A series of such incidences resulted in a temporary ban during last decade, in Great Britain, on the practice of allergen immunotherapy [16].

In the light of above mentioned drawbacks, a great deal of attention was focused on the need of standardization of crude allergen extracts to be used in the management of type I allergic diseases [1-2, 10, 13, 42, 92-93]. Standardization of allergen extracts has been proposed for decades, however only in the last 20 years, has there been significant progress in this area. Since then, standardization of allergen extract has taken different forms and has produced considerable confusion about what the units mean and how they should be used in allergy practice. Since patients respond individually and differently to multiple proteins in an extract, the standardization must involve an evaluation of the response to the extract, preferably by skin test [63]. However, it should not be the basis for any long term standardization, since it depends on the selection of patients tested. Although, several in-vivo and in-vitro methods (Table 1) have been used in characterization and standardization of crude allergen extracts, none was found suitable as a single test for the same purpose [2, 11, 47, 77]. Yunginger (1984), therefore, proposed the use of various in-vivo and in-vitro techniques for standardization of crude allergen extracts [92].
### Table 1:

Various *in-vivo* and *in-vitro* methods suggested for standardization of allergen extracts (2,9,25,52,61,91-93).

<table>
<thead>
<tr>
<th>Method</th>
<th>Type</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin Test</td>
<td><em>in-vivo</em></td>
<td>Potential risk of anaphylaxis in volunteers. Difference in immunological response of each individual to various components of allergenic extract.</td>
</tr>
<tr>
<td>Bronchial Provocation Test (BPT)</td>
<td><em>in-vivo</em></td>
<td>Can not be performed on all types of patients from all age groups.</td>
</tr>
<tr>
<td>Passive Cutaneous Transfer (PCA)</td>
<td><em>in-vivo</em></td>
<td>Cumbersome with potential risk of transfer of hepatitis virus in healthy volunteer.</td>
</tr>
<tr>
<td>Immunodiffusion</td>
<td><em>in-vitro</em></td>
<td>May not be useful since high titred rabbit antibodies against allergen extracts are required and only antigenic information can be obtained.</td>
</tr>
<tr>
<td>Cross Immuno-electrophoresis (CIE)</td>
<td><em>in-vitro</em></td>
<td>-do- qualitative.</td>
</tr>
<tr>
<td>Cross Radiimmuno-electrophoresis (CRIE)</td>
<td><em>in-vitro</em></td>
<td>Expenses, time consuming and hazardous.</td>
</tr>
<tr>
<td>Basophil Degranulation Test</td>
<td><em>in-vitro</em></td>
<td>Expenses, time consuming and hazardous.</td>
</tr>
<tr>
<td>Leukocyte Histamine Release</td>
<td><em>in-vitro</em></td>
<td>-do-.</td>
</tr>
<tr>
<td>Lectin Assay</td>
<td><em>in-vitro</em></td>
<td>Secondary standardization measure.</td>
</tr>
<tr>
<td>Isoelectric focusing (IEF)</td>
<td><em>in-vitro</em></td>
<td>Provides only biochemical information on the proteins.</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td><em>in-vitro</em></td>
<td>Provides only biochemical information on the proteins.</td>
</tr>
<tr>
<td>Protein Content [nitrogen content (w/v)]</td>
<td><em>in-vitro</em></td>
<td>Provides only yield information.</td>
</tr>
<tr>
<td>RAST and RAST inhibition</td>
<td><em>in-vitro</em></td>
<td>Less sensitive than skin test. Depends on quality of solid phase allergen extract. Reference serum pool (specific for both major as well as minor allergens) from a large number of patients is required. Hazardous.</td>
</tr>
</tbody>
</table>
Chapter II: Characterization of *Pennisetum typhoides* pollen extract

<table>
<thead>
<tr>
<th>Method</th>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA-inhibition</td>
<td><em>in-vitro</em></td>
<td>Less sensitive than skin test. Depends on quality of solid phase allergen extract. Reference serum pool (specific for major as well as minor allergens) from a large number of patients is required.</td>
</tr>
<tr>
<td>Major Allergen Content (MAC)</td>
<td><em>in-vitro</em></td>
<td>Not practical since it does not consider minor allergens.</td>
</tr>
<tr>
<td>HPLC</td>
<td><em>in-vitro</em></td>
<td>Provides only biochemical information.</td>
</tr>
</tbody>
</table>

Biological standardization of allergenic extracts was advocated by Turkeltaub in 1989. Two methods of biological standardization of allergen extracts are currently being employed in the USA, to estimate the allergenicity and bioequivalence doses. One of them, the parallel line bioassay method, is used when the extracts are from the same source. For extracts from different sources, ID$_{50}$ EAL method (intradermal dilution for 50 mm sum of erythema diameter response) is used. Both methods utilizes the intracutaneous route for administration of extracts and quantitation of erythema response to define the allergen dose response relationship of each extract assayed. Extracts which produce similar allergic response at similar doses can therefore, be considered as bioequivalent and can be assigned similar units. Because allergen extracts initiate beneficial allergic response in patients, unit assigned to extracts has been termed as *Allergy Unit* or *Allergen Unit* (AU) [85]. On the other hand, in European countries (Nordic countries in particular), biological potency unit is expressed as histamine equivalent prick (HEP) which is equivalent to skin test response to 1 mg/ml concentration of histamine.

In terms of prescribed unit, one HEP is considered equal to 1000 BU/ml [63]. Various units of allergen extracts are given in the following Table 2.

**Table 2:**

Units used for allergen standardization (63).

<table>
<thead>
<tr>
<th>UNIT</th>
<th>BASIS</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noon</td>
<td>Allergens extracted from one µg of pollen</td>
<td>Obsolete</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight in grams per volume in ml.</td>
<td>Used worldwide</td>
</tr>
</tbody>
</table>

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Characterization of Pennisetum typhoides pollen extract

PNU (Protein Nitrogen Unit)

1 PNU = 0.01 μg of protein nitrogen

No information on potency

AU (Allergy/Allergen Unit)

Skin testing to end point

Used worldwide

(As determined by Kjeldahl method or Ninhydrin assay)

BU (Biological Unit)

Skin testing relative to histamine

Used in the United States

IU (International Unit)

in-vitro assay relative to WHO’s international standards (IS)

Used worldwide

Used in Nordic countries and other parts of Europe

Over the years, Allergen Standardization Committee under the auspices of International Union of Immunological Societies (IUIS) proposed several allergenic extracts to WHO, an international agency empowered to designate allergen extracts as international standards (IS) after assigning arbitrary potency unit called international unit (IU) [63,93]. Currently, international reference standards are available from WHO on extracts of cat dander, dog dander, human dander, honey bee venom, mould, mite (Dermatophagoides pteronyssus and Acarus siro), birch pollen, short ragweed pollen, timothy grass pollen, kentucky grass pollen, cocksfoot grass pollen, rye grass pollen and bermuda grass pollen [7-8,23-24,29,40,51,81]. It has been recommended that these IS be used only to calibrate the national or laboratory standard reference for these extracts in various parts of the world. In addition to IS, various countries have their own national standard reference allergen extracts. In India, a limited number of pollen extracts have been standardized. Malik et al. (1991) reported the immunological characteristics of pollen extract of Holoptelea integrifolia (tree pollen) [52]. Similarly, Jaggi et al. (1989) characterized another tree (Cocus nucifera) pollen extract [33]. Sriramaraao et al. (1993) reported the standardization of Parthenium hysterophoria (weed) pollen extract [80]. Another weed pollen Ricinus communis was later standardized by Singh et al. (1992) [71].

Although, a number of grass pollen extracts are being used in Indian allergy clinics, no grass pollen has yet been standardized, despite the fact that it is one of the important sources of aero-allergens in this country. Pollen extract of Pennisetum typhoides is included routinely in the diagnosis and therapeutic battery of allergen extracts in India [79]. Various studies have shown it to be an important sources of
aeroallergen in India and other parts of the world. It is mainly cultivated in arid and semi-arid states around Delhi. It is grown widely in other parts of the country except few hilly states. It has also been observed growing wildly, in and around Delhi metropolitan area [38,58,68,69,79]. In view of its importance in allergy practice, the present study is carried out to characterize the allergen extract of *Pennisetum typhoides* pollen by means of various immunobiochemical techniques and prepare an ‘in-house’ reference standard.

**MATERIAL AND METHODS**

**Collection, Identification and Purity of Pollen**

The pollen of *Pennisetum typhoides* was collected from the inflorescence of the same plants growing in the fields in and around Delhi during their peak flowering season. After drying the floral parts at 37°C, they were passed through different sieves (100, 200, 300 mesh/cm²) to remove the contaminating particles. The purity and the identification of pollen was done according to the method of Cour and Loublier, 1980 [18]. Briefly, 10 mg of pollen was subjected to treatment with 5 ml of glycerol-ethanol (9:1) solution. The suspension was centrifuged at 2000 rpm for 3 minutes. The supernatant was decanted and the remaining ethanol was evaporated by keeping the tube at 30-40°C for 10 minutes. The precipitate was then dissolved in 9.8 : 0.2 (v/v) solution of glycerol-phenol. Approximately 0.05 ml of this solution was mounted on a microscopic slide and examined under the microscope (Zeiss). Minimum of 1000 pollen grains were counted under different fields and the purity of different batches of pollen was calculated. Number of other floral parts, fungal spores, foreign pollens and dust material were also recorded. Reference slide was prepared after staining the pollen grains with safranin.

**Preparation of whole pollen extract of *Pennisetum typhoides***

Pollen (≥ 98% pollen and ≥ 2% other floral parts of the same species) were defatted with peroxide free diethyl ether. The extract (1:20 w/v) was prepared in 0.05 M ammonium bicarbonate buffer (pH 8.0), by continuous stirring for 18 hours on a magnetic stirrer at 4°C. The extract was centrifuged at 10,000 rpm for 30 minutes at 4°C and the supernatant was brought to 90% saturation with ammonium sulfate. The precipitate was dissolved in minimum volume of distilled water and dialyzed.
Characterization of *Pennisetum typhoides* pollen extract

extensively against distilled water at 4°C by using Spectrapore™ dialysis membrane (MW cut off 3000). After passing through 0.22 μm filter (Millipore™), the dialyzed extract was lyophilized in aliquots and stored at -20°C.

**Estimation of protein**

Protein content in the whole pollen extract of *Pennisetum typhoides* was determined by modified method of Lowry et al. (1951) [48] using phosphotungstic (PTA) reagent (15% PTA in 10% HCl) to precipitate the proteins [74]. Bovine serum albumin (BSA) was used as a standard.

**Carbohydrate estimation**

Total carbohydrate content in the whole pollen extract of *Pennisetum typhoides* was determined by phenol-sulphuric acid method as described by Dubois et al. (1956) [21] by using D-glucose as standard.

**Intradermal test (ID) and selection of patients**

Intradermal tests were performed with whole pollen extract of *Pennisetum typhoides* in 303 patients (aged 17-42 years) with history and symptoms of type I allergic diseases, attending the Clinical Research Centre, V.P. Chest Institute, Delhi, according to the method of Shivpuri and Agarwal (1969) [68]. For skin tests, lyophilized extract was reconstituted (1:500 w/v) in phosphate buffered saline (PBS). About 0.01 ml of the extract was injected intradermally in the forearm of the patient, raising a bleb of 2-3 mm at the injection site. Simultaneously, PBS and Histamine diphosphate (0.1 mg/ml) were also injected as a negative and positive control, respectively at different sites. The skin tests were graded after 20 minutes, based on the wheal size produced in comparison to the controls. The ID reactions more than 3 times the negative control were graded as 2+; 3 to 4 times the control with 1 or 2 pseudopodia as 3+; and 3 to 4 times the control with multiple pseudopodia as 4+. Positive control produced skin reaction in the range of 2+. Sera were collected from 26 patients who showed markedly positive (2+ or more) skin reaction to *Pennisetum typhoides* pollen extract. A pool of serum was prepared by mixing equal volume of serum from each of these patient’s and referred to as *Pennisetum* specific pooled patient sera. None of the patient was on immunotherapy or chemotherapy when ID
tests were performed or sera collected. Normal human serum samples (NHS) were also obtained from a group of 5 healthy non-allergic subjects with negative ID tests.

**Pennisetum typhoides** specific rabbit anti-sera

Three rabbits (New Zealand) were immunized subcutaneously, each with 1 mg of protein (*Pennisetum typhoides* whole pollen extract) emulsified with Freund's complete adjuvant (FCA). Booster injections were given at intervals of 4 weeks with the same protein emulsified with Freund's incomplete adjuvant. The rabbits were bled 7 days after the third booster to check for the presence of antibodies by immunodiffusion. The serum samples obtained from each rabbit were mixed in equal volumes to obtain a pool of *Pennisetum typhoides* anti-sera.

**Specific IgE ELISA**

ELISA was performed to measure the specific IgE levels against whole pollen extract of *Pennisetum typhoides*, in individual patient serum by the method of Voller et al. (1976) [86]. Lyophilized extract was reconstituted in carbonate bicarbonate buffer (0.01 mol/L of Na₂CO₃ and 0.01 mol/L NaHCO₃, pH 9.6). To each well of polystyrene microtitre plate (Corning™) 100 µl of the diluted extract (1 µg/100 µl) was added and the plate was incubated overnight at 4°C. After washing three times with PBST (phosphate buffer saline with 0.05% Tween 20), plate was incubated with 2% gelatin (prepared in PBS) at room temperature for 3 hours to block the free sites. The plate was washed with PBST and then incubated overnight at 4°C with 100 µl of 1:10 (v/v) diluted individual patient serum. As a control NHS from subjects with negative ID test to grass pollens including *Pennisetum typhoides* was also tested. After washing with PBST, bound IgE were detected by incubating the plate overnight with alkaline phosphate labelled anti-human IgE (ε-specific, Sigma, USA) at 4°C. After washing the plate, the colour in the wells was developed by incubating with the substrate (1mg/ml p-nitrophenyl phosphate in 0.1 M glycine buffer containing 0.001 M MgCl₂ and 0.001 M ZnCl₂, pH 10.4) at room temperature for 45 minutes. Colour reaction in the plate was stopped with 50 µl of 3N NaOH and absorbance was measured at a wavelength of 405 nm on an ELISA reader (Nunc™). Sera showing ≥ 5 times the ELISA value (OD) obtained with NHS were considered as ELISA positive.
A pool of sera was prepared by mixing equal volume of each ELISA positive serum sample

IgE ELISA inhibition

Inhibition assay was performed by pre-incubating 50 μl of pooled patient sera (1:5 v/v diluted) with 50 μl of inhibitor (whole pollen extract of Pennisetum typhoides) in various concentrations ranging from 0 to 10 μg, for 16 hours at 4°C. The mixture was then added to pollen extract coated wells of a polystyrene micotitre plate. NHS from subjects with negative ELISA and ID response to grass pollen extracts was also used as negative control. A standard ELISA procedure as described above was followed and percent inhibition was calculated by using the following formula.

\[
\text{% inhibition} = \left( \frac{\text{OD of the test sample} - \text{OD of the inhibited sample}}{\text{OD of the test sample}} \right) \times 100
\]

The quantity of allergenic extract required to produce 50% inhibition was calculated from a semi-logarithmic graph plotted with % inhibition versus protein concentration.

Thin layer iso-electric focusing (TLIEF)

Lyophilized whole pollen extract of Pennisetum typhoides was reconstituted in distilled water for TLIEF. On a readymade polyacrylamide gel of pH range 3.5-9.3 (Pharmacia), extracts from two different batches of Pennisetum typhoides pollen in two different concentrations (1 and 2 μg), were resolved by using Phast™ System (Pharmacia) according to the programme given in the manufacturer’s instruction manual for the system [62]. The resolved protein bands on the gel were stained using silver staining kit (Pharmacia).

Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of whole pollen extract of Pennisetum typhoides was carried out on 12% acrylamide gel using the discontinuous buffer system as described by Laemmli (1970) [39] on midget apparatus (Pharmacia). Recipes for 0.75 mm thick
Characterization of *Pennisetum typhoides* pollen extract

Stacking and separating gels is given in Table 1. Lyophilized extract of *Pennisetum typhoides* was reconstituted in distilled water and 50 µg of it was digested by mixing equal volume of 2X sample buffer (Table 2) followed by boiling for five minutes in a water bath. Digested samples were then loaded in respective lanes and electrophoresis was carried out at 20 mA in electrode buffer (0.025 M Tris, 0.38 M Glycine and 1% w/v SDS) for 60 minutes. After the completion of the run, gel was fixed for 1 hour in fixing solution [methanol: acetic acid: water; 45: 45: 10; (v/v/v), respectively] and stained for one hour in 0.1% Coomassie Brilliant Blue R-250 (CBB) prepared in destaining solution containing ethanol: acetic acid: water; 45: 10: 45 (v/v/v), respectively. Excess of stain was removed by washing the gel in destaining solution and the gel was preserved in 0.7% acetic acid solution.

Simultaneous staining of glycosylated and non-glycosylated proteins of allergen extract of *Pennisetum typhoides* pollen

Proteins of whole pollen extract were separated on 12% SDS-PAGE as described earlier and were electrophoretically transferred to nitrocellulose membrane equilibrated in transfer buffer (39 mM glycine, 48 mM Tris, 0.0345% SDS and 20% methanol) for 3 hours as described by Towbin et al. (1979) using dry blot apparatus (LKB, Pharmacia) [84]. After transfer, the membrane was used for simultaneous detection of glycosylated and non-glycosylated proteins with DIG glycan/protein double staining kit (cat. no.1500783, Boehringer Mannheim, FRG) by following the instructions provided by the manufacturer [32]. A schematic representation of the principle involved in this technique is given in the flow chart 2. Briefly, the transferred nitrocellulose membrane was washed with 50 ml PBS (pH 6.5) and incubated in 10 ml of 0.1 M sodium acetate buffer (pH 5.5) having 0.01 M sodium metaperiodate, for 20 minutes. The nitrocellulose membrane was washed 3 times for 5 minutes each with 50 ml PBS (pH 6.5) and incubated with 2 µl DIG hydrazide (Digoxigenin-3-O-succinyl-L-aminocaproic acid hydrazide hydrochloride) in 10 ml 0.1 M sodium acetate buffer (pH 5.5), for one hour at room temperature. After this step, the nitrocellulose membrane was incubated in a mixture of 5 µl FLUOS (5(6)-carboxy fluorescein-N-hydroxysuccinimide ester) in 10 ml 0.05 M potassium phosphate buffer, pH 8.5 and Nonidet® P40, 0.01 % (v/v) for one hour. The membrane was washed 3 times for 5
minutes each in 50 ml TBS (Tris buffered saline 0.05 M Tris HCl, pH 7.5, 0.15 M NaCl). The membrane was blocked for 30 minutes by keeping in blocking solution supplied by the manufacturer. After washing 3 times for 5 minutes each in 50 ml TBS, the nitrocellulose membrane was incubated with 20 µl each anti-digoxigenin-peroxidase and anti-fluorescein-alkaline phosphatase, in 10 ml TBS, for one hour. The nitrocellulose membrane was washed 3 times for 5 minutes each in 50 ml TBS. Finally, the membrane was kept undisturbed in 10 ml substrate solution [10 ml Tris buffer pH 8.0, 50 µl 2-(-4 iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT), 50 µl X-phosphate (5-Bromo-4-choro-3-indolyl phosphate) [50 mg/ml prepared in dimethyl formamide], 50 µl TETON (4-triethylenetrioxy-1-naphthol) [50 mg/ml in dimethyl formamide], and 6 µl H₂O₂ (30 % w/v)] until clear blue (glycosylated protein) and orange/raddish brown (non-glycosylated protein) bands were visible. The colour reaction was stopped by rinsing the nitrocellulose membrane several times in distilled water.

Table 3:
SDS-PAGE recipe for one 0.75 mm thick gel.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Solution components</th>
<th>12 % Resolving gel (10 ml)</th>
<th>5 % Stacking gel (3 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30 % acrylamide mix [29.1 % (w/v) acrylamide + 0.9 % (w/v) N,N’ methylene bis-acrylamide]</td>
<td>4.0 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Distilled water</td>
<td>3.3 ml</td>
<td>2.1 ml</td>
</tr>
<tr>
<td>3.</td>
<td>1.5 M Tris Cl (pH 8.8)</td>
<td>2.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>1.0 M Tris Cl (pH 6.8)</td>
<td>-</td>
<td>0.38 ml</td>
</tr>
<tr>
<td>5.</td>
<td>10 % (w/v) SDS</td>
<td>0.1 ml</td>
<td>0.03 ml</td>
</tr>
<tr>
<td>6.</td>
<td>TEMED</td>
<td>0.004 ml</td>
<td>0.003 ml</td>
</tr>
<tr>
<td>7.</td>
<td>10% (w/v) ammonium persulfate</td>
<td>0.1 ml</td>
<td>0.03 ml</td>
</tr>
</tbody>
</table>
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Table 4:
Recipe for loading buffer.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Solution components</th>
<th>volume/quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.0 M Tris Cl (pH 6.8)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>2</td>
<td>SDS</td>
<td>2.0 gm</td>
</tr>
<tr>
<td>3</td>
<td>2-mercaptaethanol</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>4</td>
<td>Glycerol (87 %)</td>
<td>5.8 ml</td>
</tr>
<tr>
<td>5</td>
<td>Bromophenol blue</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>

Specific IgE immunoblot analysis

Three different batches of *Pennisetum typhoides* pollen extract (at equal protein concentration) were separated on 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane as described earlier. Free sites on nitrocellulose membrane were blocked with 2% gelatin (prepared in PBS) solution. After washing three times for five minutes each with PBST, the membrane was incubated with 1:10 (v/v) diluted pooled patients’ sera overnight at 4°C with gentle shaking. The nitrocellulose membrane was then washed with PBST and again incubated overnight at 4°C with 1:500 (v/v) diluted rabbit anti-human IgE peroxidase conjugate (Sigma). After washing the membrane with PBST, the allergenic proteins were detected by keeping the membrane in a substrate solution (12 mg diaminobenzidine and 15 μl 30% (v/v) H₂O₂ in 50 ml sodium acetate buffer, pH 5.0) for few minutes. The colour reaction was stopped by rinsing the membrane several times in distilled water.
FLOW CHART 2:
Schematic representation of the principle involved in the simultaneous detection technique of glycosylated and non-glycosylated proteins.

Step 1: Oxidation of Glycoprotein with Periodate
\[
\text{NH}_2 \xrightarrow{\text{Periodate}} \text{-NH}_2
\]

Step 2: Labeling of Oxidized Sugars with DIG-Hydrazide
\[
\text{-NH}_2 \xrightarrow{-\text{NH}_2} \text{-N-D}
\]

Step 3: Labeling of NH₂-Group with Fluorescein
\[
\text{-NH}_2 \xrightarrow{-\text{-}} \text{-N-D}
\]

Step 4: Antibody Reaction + Anti-Fluorescein-AP
+ Anti-DIG-POD
\[
\text{-N-D} \xrightarrow{\text{red-brown precipitate}} \text{-N-D}
\]

Step 5: Substrate Reactions
\[
\text{-N-D} \xrightarrow{\text{blue precipitate}} \text{-N-D}
\]

- Sugar
- Oxidized Sugar
- DIG-Hydrazide (Digoxigenin-3-O-succinyl-aminocaproic acid hydrazide hydrochloride)
- FLUOS 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester
- Anti-Fluorescein-AP
- Anti-Digoxigenin-POD
- Colour reactions
  - X phosphate (5-bromo 4-chloro 3-indolyl phosphate)
  - TETON (4-Triethylamino)oxy-1-naphthol)
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Specific IgE immunoblot inhibition

Whole pollen extract of *Pennisetum typhoides* was resolved on a 12% SDS-PAGE gel and was electrophoretically transferred onto a nitrocellulose membrane. After blocking the free sites with 2% gelatin solution, the nitrocellulose membrane was cut into 0.5 cm wide strips which were then incubated separately at 4°C overnight with 1:10 (v/v) diluted pooled patient sera containing various concentrations of homologous inhibitory protein (pollen extract of *Pennisetum typhoides*). As a positive control, a strip was incubated with 1:10 (v/v) diluted pooled patient sera with no inhibitory protein. Other steps were the same as described above for specific IgE immunoblotting.

Specific IgG immunoblot analysis

Three different batches of *Pennisetum typhoides* whole pollen extract (at equal protein concentration) were separated on 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane as described earlier. Free sites on nitrocellulose membrane were blocked with 2% gelatin (prepared in PBS) solution. After washing three times for five minutes each with PBST, the membrane was incubated with 1:10^6 (v/v) diluted pooled rabbit anti-*Pennisetum typhoides* sera for 3 hours at room temperature with gentle shaking. The nitrocellulose membrane was then washed with PBST and again incubated for one hour at room temperature with 1:10,000 (v/v) diluted goat anti-rabbit IgG peroxidase conjugate (Sigma). After washing the membrane with PBST, the antigenic proteins were detected by keeping the membrane in the substrate solution (12 mg dianinobenzidine and 15 μl H2O2 in 50 ml sodium acetate buffer, pH 5.0) for few minutes. The colour reaction was stopped by rinsing the membrane several times in distilled water.

RESULTS

Preparation of allergen extract of *Pennisetum typhoides* pollen

Highly pure pollens of *Pennisetum typhoides* were used in the preparation whole pollen extract (fig. 1c-d). A pale yellow coloured extract was obtained after
following the steps shown in the flow chart 1. On an average, extract prepared from one gram dried and defatted pollen was found to contain 71.33 mg protein and 28.07 mg carbohydrate as estimated by the methods of Lowry’s et al. (1951) and Dubois et al. (1956), respectively (Table 5).

Selection of patients and sera collection

A group of 303 patients of type I allergic diseases were screened at V.P.Chest Institute, Delhi. Of these, 66 patients were found to be sensitive only to grass pollen allergens, by means of history, clinical symptoms and skin tests. These patients were then subjected to intradermal skin test with 1:500 diluted Pennisetum typhoides pollen extract. Twenty six patients showed markedly positive (more than 1+) skin test reactivity with allergen extract of Pennisetum typhoides pollen (fig. 2). Besides sensitivity to Pennisetum typhoides pollen, these patients were also found to be allergic to some other grass pollens. None of the patient was found to be monosensitive to Pennisetum typhoides pollen. Serum samples were collected from all 66 patients prior to start of allergen immunotherapy and stored at -20°C.

Specific IgE ELISA

Sixty six patient serum samples were tested for the presence of specific IgE antibodies against whole pollen extract of Pennisetum typhoides pollen. Elevated levels of specific IgE against Pennisetum typhoides pollen extract were observed only in serum samples from patients (n=26) who showed markedly positive ID reaction to allergen extract of Pennisetum typhoides pollen (Table 6). As expected, these serum samples showed IgE binding to other grass pollen extracts as well. However, none of the patient serum showed the presence of specific IgE to either tree pollen (Prosopis juliflora) or weed pollen (Artemisia scoparia) which were used as non-relevant allergen extract in ELISA. In the absence of monosensitized patient, equal volumes of serum samples from each of the 26 Pennisetum typhoides positive patients were mixed to prepare pooled patients’ sera for further immunological investigations.
FIGURE 1a:
A wildly growing flowering plant of *Pennisetum typhoides*
FIGURE 1b:
An inflorescence of *Pennisetum typhoides*. 
FIGURE 1c:
Purity determination by microscopic analysis (at 40x magnification) of *Pennisetum typhoides* pollen.
FIGURE 1d:
Scanning electron micrograph of a pollen grain of Pennisetum typhoides at 2,500 X magnification.
FLOW CHART 1:
Preparation of whole pollen extract of *Pennisetum typhoides*

More than 98 % pure dried and defatted pollen

Extraction in 0.05 M NH₄HCO₃ buffer (pH 8.0) with 1 mM PMSF for overnight at 4°C

Centrifugation at 10,000 rpm for 30 minutes at 4°C

90% ammonium sulfate precipitation of supernatant for 3 hours at 4°C

Centrifugation at 10,000 rpm for 30 minutes at 4°C

Resuspension of the precipitate in minimum volume of distilled water followed by extensive dialysis against distilled water at 4°C, by using Spectrapor® (MW cut off 3000) dialysis bag

Filteration through 0.22 μm size filter followed by lyophilization in aliquots and storage at -20°C
Table 5:
Protein and total carbohydrate quantitation of whole pollen extracts prepared from three different batches of *Pennisetum typhoides* pollen.

<table>
<thead>
<tr>
<th></th>
<th>Yield (in mg) per one gram dry and defatted pollen of <em>Pennisetum typhoides</em></th>
<th>Protein to carbohydrate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proteins (As determined by modified method of Lowry et al.)</td>
<td>Carbohydrates (As determined by the method of Dubois et al.)</td>
</tr>
<tr>
<td>BATCH I</td>
<td>70.0</td>
<td>28.0</td>
</tr>
<tr>
<td>BATCH II</td>
<td>76.0</td>
<td>30.2</td>
</tr>
<tr>
<td>BATCH III</td>
<td>68.0</td>
<td>27.9</td>
</tr>
<tr>
<td>MEAN + S.D.</td>
<td>71.33 ± 3.36</td>
<td>28.07 ± 1.06</td>
</tr>
</tbody>
</table>

S.D. Standard deviation
FIGURE 2:
Diagrammatic representation of *Pennisetum typhoides* pollen sensitivity (as determined by ID test and ELISA) in a group of 66 grass pollen allergic patients.

60.61% patients sensitive to other grass pollens

39.39% Patients sensitive to *Pennisetum typhoides* pollen
Table 6:
Skin test (ID) and ELISA data of patients selected for the study.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>ID Grade with whole pollen extract of <em>Pennisetum typhoides</em></th>
<th>Specific IgE ELISA with <em>Pennisetum</em> whole pollen extract (OD at 405nm)</th>
<th>Also found ID positive to Specific IgE ELISA with <em>Artemisia</em> whole pollen extract (OD at 405nm)</th>
<th>Specific IgE ELISA with <em>Prosopis</em> whole pollen extract (OD at 405nm)</th>
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<tbody>
<tr>
<td>1</td>
<td>++</td>
<td>0.387</td>
<td>C(++) S(++)</td>
<td>0.004</td>
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<tr>
<td>2</td>
<td>++</td>
<td>1.714</td>
<td>C(++)</td>
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<tr>
<td>3</td>
<td>++</td>
<td>1.943</td>
<td>Y(++)</td>
<td>0.007</td>
</tr>
<tr>
<td>4</td>
<td>++</td>
<td>1.361</td>
<td>Y(++)</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>0.301</td>
<td>I(++)</td>
<td>0.010</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
<td>0.390</td>
<td>Y(++)</td>
<td>0.030</td>
</tr>
<tr>
<td>7</td>
<td>+++</td>
<td>1.614</td>
<td>C(++) Y(++)</td>
<td>0.017</td>
</tr>
<tr>
<td>8</td>
<td>++</td>
<td>1.014</td>
<td>C(++) Y(++)</td>
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<td>++</td>
<td>0.367</td>
<td>C(++) Y(++)</td>
<td>0.017</td>
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<td>10</td>
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<td>0.398</td>
<td>C(++)</td>
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<td>0.783</td>
<td>C(++) Y(++)</td>
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<tr>
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<td>+</td>
<td>0.285</td>
<td>I(++) S(++)</td>
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<tr>
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<td>+</td>
<td>0.677</td>
<td>Y(++) I(++)</td>
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<tr>
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<td>+</td>
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<td>Y(++) I(++)</td>
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<tr>
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<td>Y(++) I(++)</td>
<td>0.047</td>
</tr>
<tr>
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<td>+</td>
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<td>Y(++)</td>
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</tr>
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<td>Y(+)</td>
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<td>C(+) I(+)</td>
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</tr>
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<td>I(+)</td>
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</tr>
<tr>
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<tr>
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<td>0.011</td>
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<td>C(+) Y(+)</td>
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</tr>
<tr>
<td>25</td>
<td>+</td>
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<td>I(+) S(+)</td>
<td>0.041</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>0.513</td>
<td>C(+) S(+)</td>
<td>0.014</td>
</tr>
<tr>
<td>NHS</td>
<td>-</td>
<td>0.037</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. *Cenchrus ciliain; Y. Cynodon dactylon; I. Imperata cylindrica; S. Sorghum vulgare*

OD = Absorbance
ID = Intradermal
* = Weed
** = Tree
Specific IgE ELISA inhibition

Three different batches of *Pennisetum typhoides* whole pollen extract were used as inhibitors of specific IgE (from pooled patients' sera) binding to solid phase homologous extract on ELISA (fig. 3). A concentration (ID$_{50}$) which produced 50% inhibition of specific IgE binding to solid phase extract was determined by plotting inhibitory concentration against percent inhibition on a semi-logarithmic graph. ID$_{50}$ obtained with 3 different batches of *Pennisetum typhoides* pollen were found to be quite similar (i.e. 280, 275 and 285 ng for batch I, II and III, respectively)

TLIEF

Different batches of *Pennisetum typhoides* whole pollen extract, produced similar electrofocusing profile when subjected to TLIEF in the pl range of 3.5-9.3. In all, 37 silver stained bands were observed in each batch. Most of the protein bands were found in the acidic pl (5.8-3.5) region of the gel. Protein bands with pl values 3.7, 4.5 and 5.0 were the most prominently stained bands (fig. 4).

SDS-PAGE

A 12% SDS- polyacrylamide gel resolved the *Pennisetum typhoides* whole pollen extract into 24-26 CBB stained protein bands in the molecular weight range of 10-100 kDa. Bands with molecular weights 80, 70, 57, 50, 40, 30 and 14 kDa were stained prominently, whereas bands with molecular weights 85, 43, 25 and 18 kDa were stained only moderately. Bands in the molecular weight range of 27-19 kDa were not stained adequately. No difference in the protein profiles of three different batches of allergen extract of *Pennisetum typhoides* pollen was observed (fig. 5)

Simultaneous detection of glycosylated and non-glycosylated proteins

Bands with molecular weights 70, 50, 43, 34, and 14 kDa were stained as non-glycosylated proteins, whereas 85, 80, 30, kDa bands were stained as glycosylated proteins (fig. 6). Very high molecular weight glycan moieties from the pollen extract were observed in the stacking region of the gel. No batch to batch
variation was observed with respect to the content of glycosylated and non-glycosylated proteins in the pollen extract of *Pennisetum typhoides*.

**Specific IgE immunoblot and immunoblot inhibition**

Twelve IgE binding proteins in the molecular weight range of 14 - 85 kDa were observed in the whole pollen extract of *Pennisetum typhoides* by immunoblotting using pooled patients' sera. Allergenic bands with molecular weights 85, 80, 70, 57, 50 and 34 kDa appeared very prominently. Other allergenic bands (with molecular weights of 43, 40, 30, 25, 18 and 14 kDa) were stained either moderately on immunoblot. IgE binding profiles of all 3 batches of *Pennisetum typhoides* pollen extract appeared similar thereby indicating the consistency of the allergen composition, both in terms of both the number of allergens and their respective quantity (fig 7). Specificity of IgE in the pooled patient sera, to the components of *Pennisetum typhoides* pollen extract was demonstrated by means of immunoblot inhibition. Inhibition of specific IgE binding to SDS-PAGE separated pollen proteins was achieved by pre-incubating various amounts of homologous pollen extract with pooled sera, prior to its use in the immunoblotting. Hundred µg of pollen extract was found sufficient to inhibit specific IgE binding to low molecular weight allergenic proteins. Upto 500 µg of extract was needed to inhibit specific IgE binding to 70, 57, 50 and 43 kDa allergenic proteins. As high concentration as 1000 µg was required to inhibit specific IgE binding to solid phase allergens with molecular weights of 80 and 85 kDa (fig 8).

**Rabbit antisera and specific IgG immunoblot**

High titered *Pennisetum typhoides* specific anti-sera were produced in all 3 rabbits and pooled after the last bleeding schedule. This pooled serum was used in immunoblotting, for the identification of antigenic proteins in the pollen extract of *Pennisetum typhoides*. Twelve proteins in the molecular weight range of 14-85 kDa were identified as antigenic. Of these, 70, 50, 30 and 14 kDa proteins were found to be highly antigenic as these were stained heavily on immunoblot. Bands with molecular weights 85, 80, 47, 43, 40 and 36 kDa were found to be moderate in their antigenicity. Protein bands with molecular weight 34 and 26 kDa were identified as
FIGURE 3:
ELISA inhibition. Solid phase: Whole pollen extract of *Pennisetum typhoides*. Inhibitory allergens: Three different batches of whole pollen extract of *Pennisetum typhoides*. 
FIGURE 4:
TLIEF analysis of two batches of *Pennisetum typhoides* pollen extract, at two different protein concentrations.

FIGURE 5:
SDS-PAGE profile of three different batches of whole pollen extract of *Pennisetum typhoides*.
FIGURE 6:
Simultaneous detection of glycosylated and non-glycosylated proteins in three different batches of whole pollen extracts of *Pennisetum typhoides*.
FIGURE 7:
FIGURE 8:
IgE immunoblot inhibition analysis of whole pollen extract of *Pennisetum typhoides* by pre-incubating different amounts of homogeneous extract with pooled patients sera.

weak antigens since they did not bind strongly to specific rabbit IgG antibodies in immunoblotting experiments (fig 9). No batch to batch variation in the antigenic composition of pollen extracts of *Pennisetum typhoides* was observed.

**DISCUSSION**

The use of ill-defined and crude allergen extracts causes false diagnosis in allergy and is probably responsible for many therapeutic failures. It is, therefore, essential that clinicians working with allergen extracts must know the quality and the potency of the extract, in order to ensure safety and precision in diagnostic and therapeutic procedures [2].

In the present study, an attempt was made to characterize the pollen extract of *Pennisetum typhoides* by means of a combination of immunobiochemical techniques. It is known that a shift to basic pH in the extraction buffers favours the release of maximum number of proteins from pollen grains [56]. Fabricant (1941) demonstrated that during allergic rhinitis, the pH of the nasal fluid changes from acidic to alkaline (7.2-8.3) [22]. Later, Marsh et al. (1981) suggested that alkaline pH facilitates the rapid release of allergens from pollen grains deposited in the upper respiratory tract, soon after its inhalation [56]. In the light of these facts, highly allergen extract from three different batches of *Pennisetum typhoides* pollen was prepared at basic pH conditions (in 0.05 M ammonium bicarbonate buffer pH 8.0) and in the presence of 1mM phenylmethyl sulfonyl fluoride (PMSF), a known protease inhibitor. A number of studies have been carried out on the kinetics of allergens release from the pollen grains. Generally allergens are described in the molecular weight range of 10-100 kDa. Most of the low molecular weight allergens are released from the pollen within few minutes of extraction whereas high molecular weight allergens are released very slowly (upto 24 hours) [22,45,65,87]. Therefore, in order to prepare an extract having representation of proteins from a wide molecular weight range, extraction was carried out overnight at 4°C. Using the protocol mentioned in the flow chart 1, approximately 7% (w/w) protein was extracted from one gram of dried and defatted pollen of *Pennisetum typhoides*. Very high protein to carbohydrate ratio was estimated in all the batches of the pollen extract (Table 5). High carbohydrate content could be attributed to the presence of a number of glycoproteins and/or to the presence of free structural carbohydrate moieties in the extract. A number of
glycosylated proteins were evident when pollen extract was subjected to DIG based simultaneous detection of glycosylated and non-glycosylated proteins. High molecular weight glycan moieties were visible in the stacking gel (fig. 6). Group 1 and group 4 allergens with molecular weights 28-34 and 57 kDa, respectively, from other grass pollen extracts have been reported as glycoproteins [53]. Group 1 allergen are known to have upto 5 % carbohydrate [27,34]. No significant batch to batch variation was observed in the protein and carbohydrate profiles and their contents in different batches of *Pennisetum typhoides* pollen extract (Table 5 and fig. 6).

The whole pollen extract produced in this study was tested intradermally on 66 grass pollen sensitive patients. Twenty six patients showed markedly positive skin test reactivity to the *Pennisetum typhoides* pollen extract. Fourteen patients showed no response to *Pennisetum typhoides* pollen extract, whereas 26 patients showed weak response to the extract. Patients who showed weak or no skin test response to *Pennisetum typhoides* pollen were found to be sensitive to other grass pollen (*Cynodon dactylon*) extract. *Cynodon dactylon* belongs to sub-family Chloridoideae whereas *Pennisetum* is a member of sub-family Panicoideae, in the family Gramineae. Weber (1986) suggested a close botanical relationship in the allergenicity of different grass pollens belonging to the same group [89]. These facts could provide an explanation for the non-responsiveness of 14 patients and weak reactivity of 26 patients in a group of 66 grass pollen sensitive patients.

Skin tests have been recommended by many authors for *in-vivo* standardization of allergen extracts. However, it is not always a practically feasible parameter of standardization for commercially prepared allergen extracts [2]. In-vitro tests like RAST and RAST inhibition assays have been used for the standardization purposes [25,88]. A good correlation between skin test and RAST was reported by many studies [19,66]. Enzyme based ELISA and ELISA inhibition assays have been suggested as good alternatives for RAST and RAST inhibition, respectively since no radiohazard is involved in enzyme based assays [19,25,67]. Therefore, ELISA and ELISA inhibition were used in this study to evaluate the specific IgE levels against *Pennisetum typhoides* pollen extract in the patients serum samples and the determination of potency of pollen extract of *Pennisetum typhoides*, by studying it;
FIGURE 9:
Immunoblot analysis of three different batches of whole pollen extracts of *Pennisetum typhoides* using rabbit anti-*Pennisetum* antibodies.

ability to inhibit binding of specific IgE to solid phase homologous extract, respectively.

A good correlation between ID test grades and ELISA results was observed (Table 6). Only 26 patients sera showed raised levels of specific IgE against Pennisetum pollen extract. On ELISA, these sera showed IgE binding to other grass pollen extract as well. At the same time, no IgE binding was observed with non-relevant pollen extract prepared from tree and weed pollens, in all serum samples.

Standardization of an allergen extract required the expression of its activity in terms of its potency. Various in-vivo methods have been proposed for the biological potency estimation (Table 1). However, in-vitro potency in terms of RAST inhibition has been preferred because of its convenient and reproducible nature. In the light of possible hazards associated with the use of radiolabelled reagents, a safe and equally sensitive method in the form of ELISA-inhibition was devised. Three different batches of pollen extract were tested for their ability to inhibit 50% binding of specific IgE (from pooled patients' sera) to solid phase homologous extract. All batches of allergen extract produced the similar inhibition curves (fig. 3). An average ID50 value of 278 ng revealed the highly potent nature of the extract prepared in this study.

TLIEF resolved the extract into 37 silver stained protein bands, most of these bands were found in the acidic pl region. It has been reported that majority of the allergenic proteins are acidic in nature [55]. Only a few reported allergens (most notably, the Lol p 4) are known to have basic pl [76]. Due to the ease of operation and visual clarity, TLIEF is also proposed as a ‘fingerprinting’ technique in the standardization of allergen extract. Various IS have been standardized by IEF alongwith other in-vivo and in-vitro techniques [7-8,23-24,29,40,51,81].

Previously CIE and CRIE were used to detect allergenic/antigenic proteins present in allergen extracts, but these techniques were later on, replaced by more amenable and sensitive techniques such as SDS-PAGE and immunoblotting [82-83]. In this study 24-26 CBB stained bands were detected in the pollen extract of Pennisetum typhoides. No batch to batch variation was observed when three different batches were subjected to SDS-PAGE (fig. 5). Bands in the molecular weight range of 28 - 17 kDa were poorly stained which in turn indicate the relatively low quantity of these proteins in the pollen extract. Tovey and Baldo (1984),
proposed that immunoblotting after SDS-PAGE, should be used as a tool to standardize allergen extracts. They reported the standardization of house dust mite allergen extract by the same technique [83]. Peltre and David (1984), also used immunoblotting for the standardization of allergen extract of cocksfoot grass (Dactylis glomerata) pollen [61]. The present study revealed the presence of 12 IgE binding proteins in the pollen extract of Pennisetum typhoides, when pollen extract was analyzed with pooled patients’ sera in immunoblot. Most prominent allergenic proteins were found in the region above 40 kDa (fig 8). No appreciable change in the allergen profiles of Pennisetum typhoides pollen from 3 different batches was observed. Results from immunoblot inhibition confirmed the specificity of the IgE antibodies to components of allergen extract of Pennisetum typhoides pollen.

One of the major drawbacks of in-vitro techniques involving specific IgE from pooled patients’ sera is the availability of these reference pooled sera to various manufacturers. However, this problem may be circumvented by the use of high titre specific rabbit anti-sera which can be produced in desired amounts. A high titre Pennisetum extract specific rabbit anti-sera was produced in this study. Immunoblotting with this anti-sera produced similar antigenic profile in all the three batches of pollen extract. In all, 12 antigenic proteins were identified in the molecular weight range of 14-85 kDa.

In conclusion, a potent allergen extract (whole pollen extract) of Pennisetum typhoides pollen has been prepared and characterized by means of various immunobiochemical techniques. The ‘in-house’ reference immunobiochemical standards delineated in this study will be useful for the future standardization of allergen extract of Pennisetum typhoides pollen, availability of which will improve the accuracy of diagnosis and maximize the effectiveness of allergen immunotherapy.
Chapter II

Characterization of *Pennisetum typhoides* pollen extract

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