Chapter 4
Chapter 4

Evaluation of allergenic potential, identification & characterization of red gram allergens

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4.1. Introduction
Red gram (Cajanus cajan), is a perennial plant commonly known as "Pigeonpea" and belongs to Leguminoseae family. It contains about 22 percent protein, almost 2.5 times that of cereals (http://www.iipr.res.in/pigeonpea.html). Red gram originated in Indian and has spread to East Africa. It is now cultivated throughout the tropical and subtropical areas of the world. India is the largest producer of red gram accounting 73% of total world production (http://faostat.fao.org). Being rich in protein and relatively cheaper, a large section of population especially vegetarians consume red gram for their daily protein requirement. It also contains important amino acids methionine, lysine and tryptophan. Therefore, it is one of the popular pulses along with chickpea, black gram and green gram. In combination with cereals, red gram make a well balanced human food. Due to high consumption of red gram in India, chance of sensitization in prone individuals may increase. In chapter 3, we have reported that red gram causes sensitization in allergic population and was one of the most important allergenic legumes besides chickpea and green gram.

In contrast to soybean and peanut, our knowledge regarding the allergenic potential of red gram is scanty. The red gram proteins responsible for allergic reactions have not been identified. In the present study, we have assessed the allergenic potential of red gram proteins in BALB/c mice as well as in patients of AR and BA. Attempts have also been made to identify allergenic proteins of red gram that show their stability against simulated gastric fluid (SGF) and specific IgE immunoblot after two dimensional-electrophoresis (2DE). The allergenic proteins have been characterized using LC/MS-MS and bioinformatic tools.
4.2. Materials and Methods

4.2.1. Chemicals and reagent kits

All the chemicals were of highest grade purity available. RPMI-1640, antibiotic-antimycotic solution, fetal bovine serum (FBS), pepsin from porcine gastric mucosa (Cat # P-6887), O-phenylenediamine dihydrochloride (OPD), Bovine Serum Albumin (BSA), reagents for Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and goat anti-human IgE peroxidase conjugate (Cat # A9667) were purchased from Sigma Chemical Company, St. Louis, USA. The anti-human IgE antibody preparation is specific for human IgE when tested against purified human IgG, by manufacturer using Ouchterlony Double Diffusion (ODD) and immunoelectrophoresis (IEP). Total human and mouse IgE ELISA kits were purchased from Immunology Consultants Laboratory, Newberg, USA. Histamine assay kit was obtained from SPI-BIO, Montigny le Bretonneux, France. Rat anti-mouse IgG1, IgG2a and IgE peroxidase linked were from Southern Biotech, Birmingham, USA. Hydrogen peroxide (30%) solution was obtained from E. Merck, Darmstadt, Germany. The maxisorp 96-well flat bottom micro titer ELISA plates were of Nunc TM Immunomodule, Roskilde, Denmark. Water was purified by Milli Q Ultrapure water purification systems (Millipore 5A, Molsheim, France) and had a resistivity >18M Ωcm⁻¹. All the other chemicals used were of the highest purity available from other commercial sources. Dry seeds of red gram (Cajanus cajan) were of purchased from local market.

4.2.2. Reagents

Phosphate buffered saline (PBS) - 10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4

Coating buffer - Carbonate-bicarbonate buffer 0.6 M, pH 9.6
Washing buffer - PBS containing 0.05% Tween-20 (v/v) (PBS-T)
Blocking solution - PBS containing 1% BSA (w/v)
Substrate buffer - Phosphate citrate buffer, 0.15 M, pH 5.0
Substrate solution - 5 mg OPD and 10 μl H₂O₂ (30 % v/v) in 25 ml substrate buffer
Stopping solution - 5 N H₂SO₄
Rehydration buffer - 8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer and 0.002% (w/v) bromophenol blue
SDS equilibration buffer - 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% (w/v) bromophenol blue

4.2.3. Preparation of red gram CPE
Protein from red gram was extracted and protein content of CPE was estimated as described previously in chapter 2, section 2.2.4 and 2.2.5.

4.2.4. Study Subjects
Six red gram allergic patients were selected from a population with naso-bronchial allergy during a prospective study performed at the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India. The selection was carried out on the basis of a convincing clinical history of allergic symptoms after consuming red gram, which was confirmed on the basis of positive SPT. SPT and blood collection were carried out with the consent of patients and the study protocol was approved by the human ethics committee of the institute.

4.2.5. SPT and sera collection
SPT and sera collection was performed as described previously in chapter 3, section 3.2.3. Reactions of 2+ and above were considered as marked positive skin reaction. Blood was collected from six SPT-positive patients having history of allergy to red gram. Pooled serum from other 10 patients, allergic
to dander and insects but not to any food or pollen extracts was used as negative control. Sera were separated and stored at -20 °C. Individual patient sera were used for detection of IgE binding proteins by immunoblotting. Pooled sera of these 6 red gram allergic subjects were used in ELISA inhibition assays and IgE immunoblotting of 2D resolved red gram protein.

4.2.6. Total IgE estimation
Patients’ serum total IgE level was determined using commercially available double antibody sandwich ELISA kit following the instructions given by the manufacturer (Immunology Consultants Laboratory, Inc., Newberg, USA). IgE concentration was defined using the standard curve and presented as IU/ml (1 IU/ml = 2.4 ng/ml).

4.2.7. Specific IgE estimation
Specific IgE levels against red gram were estimated by enzyme-linked immunosorbent assay (ELISA) using method of Voller et al. (1980). Briefly, the wells of microtiter plate (Nunc, Denmark) were coated with 1 μg of proteins in carbonate buffer (pH 9.6). After washing with PBS-T, the nonspecific sites were blocked with 1% BSA for 3 h at 37 °C. The plate was washed again and incubated with diluted sera (1:10 v/v) of red gram-hypersensitive patients overnight at 4 °C. The plate was washed and incubated with HRP-conjugated goat-antihuman IgE (1:1000 v/v, Sigma Chemical Co, St Louis, USA) for 3 h at 37 °C. Colour was developed with OPD. The reaction was stopped after 20 min by adding 5 N H₂SO₄ and the absorbance was read at 492 nm.

4.2.8. ELISA inhibition
The allergenic potency of red gram extracts was determined in vitro by ELISA inhibition. One microgram protein of red gram extract was coated in carbonate buffer (1 μg/100 μl per well) overnight at 4 °C in a microtitre plate.
Red gram hypersensitive patients’ pooled sera (1:10 v/v) were pre-incubated with 0.1 ng, 1 ng, 10 ng, 100 ng and 1000 ng red gram CPE at 4 °C and the mixture was then added to the red gram-coated ELISA plate.

To study cross-reactivity, inhibition of IgE binding to red gram was tested with sera of patients pre-incubated with 100 ng, 500 ng, 1000 ng and 5000 ng of soybean and green gram CPE as inhibitor. The mixture was then added to the solid phase red gram extract in the ELISA plate. Red gram antigen incubated with pooled patient’s sera without inhibitors was taken as positive control. The rest of the procedure was similar to that of ELISA. Percentage inhibition was calculated using the formula given below:

\[
\text{Percentage Inhibition} = \left(1 - \frac{\text{OD of sample with Inhibitor}}{\text{OD of sample without Inhibitor}}\right) \times 100
\]

4.2.9. Animal studies

Healthy 6-8 week old female BALB/c mice obtained from the animal breeding facility of IITR, Lucknow, were housed in plastic cages having rice husk as bedding and maintained in controlled atmosphere of a ventilated room at 22 ± 4°C, 12 h light/dark cycle and 50-60% humidity as per rules laid down by animal welfare committee of IITR. Animals were raised on commercial pellet diet (Ashirwad, Chandigarh, India) and water ad libitum. Animal study protocol was approved by the institute’s Ethics Committee.

4.2.9.1. Animal sensitization protocol

Mice were randomly divided into 3 groups (n=15/group). Group 1 received 100 μl of PBS, group 2 and 3 were administered peanut and red gram CPE, respectively. Mice (n=5) were injected intraperitoneally with 100 μg protein in 100 μl PBS once in a week for 7 weeks. Blood samples were collected from retro-orbital sinus to measure serum IgE, IgG1 and IgG2a antibodies on 15, 43 and 59 days. On day 60, mice were challenged intraperitoneally with 1 ml
of 10 mg/ml CPE. Tissue and blood samples were collected as per schedule depicted in Fig. 4.1.

On day 15, a subgroup of mice (n=10) were challenged intraperitoneally with 1 ml of 10 mg CPE/ml and blood and spleen were collected for cytokine study (splenocyte culture and semi-quantitative RT-PCR).

4.2.9.2. Blood samples collection
Blood samples were collected from retro orbital sinus on day 15, 43 and 59 to measure serum total IgE, specific IgE, IgG1 and IgG2a. Serum was separated by centrifugation at 2500g for 20 min and stored at -80 °C for further analysis.

4.2.9.3. Measurement of systemic anaphylactic responses
Anaphylactic signs were evaluated 30 to 40 min after challenge mice (n=5) with 1 ml of 10 mg/ml CPE intraperitoneally on 60th day by 2 separate investigators using the scoring system previously described by Li et al. (1999) as: 0, no signs; 1, scratching and rubbing around the snout and head; 2, puffiness around the eyes and snout, diarrhoea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsions; and 5, death.

4.2.9.4. Determination of plasma histamine levels
Blood was collected 30 minutes after the challenge (n=5) for estimation of histamine level, in the chilled ethylenediamine tetraacetic acid (EDTA)-coated tubes as described previously (Li et al., 1999). Plasma histamine levels were determined by an inhibition assay using the enzyme immunoassay kit (SPI-BIO, Montigny le Bretonneux, France) according to the manufacturer's protocol.
Fig. 4.1. Experiment design to study allergenic responses induced by red gram proteins in BALB/c mice.
4.2.9.5. Histopathological processing
Challenged mice in each group were sacrificed by cervical dislocation on day 60. Lungs, spleen and intestine were taken out and put in 10% buffered formalin and embedded in paraffin after processing. Intestine was cleaned with the help of small spatula to remove the waste material and washed with normal saline. The sections (4-5 \( \mu m \)) were cut, stained with hematoxylin and eosin, and visualized by using light microscopy (125 X) for antigen-induced peribronchial and perivascular inflammation.

4.2.9.6. Total IgE estimation
Determination of total IgE levels in serum was performed by using commercially available sandwich ELISA kit following the instructions given by the manufacturer (Immunology Consultants Laboratory, Inc., North Elliott Road, Newberg, USA).

4.2.9.7. Protein specific IgE, IgG1 and IgG2a antibodies estimation
Serum antibodies specific for red gram and peanut CPE were measured by indirect ELISA (Voller et al. 1980). The microtiter plates (Nunc) were coated with 1\( \mu g/100\mu l \) of proteins in coating buffer (carbonate buffer, pH 9.6) in each well, covered with plastic sealer and left overnight at 4 °C. Subsequently, the plate was washed thoroughly with PBS and blocked with 250 \( \mu l \) of blocking solution (1% BSA in PBS, w/v) and incubated at 37 °C for 2 hours. After blocking, the plate was washed thoroughly with washing buffer (PBS-T) 5 times and finally with PBS. The plates were incubated with mice sera for IgE estimation (1:20), IgG1 and IgG2a (1:1000). Similar dilutions of PBS treated mice sera were also added on separate wells and run along to serve as negative controls. The antigen blanks were used to measure the background absorbance. The plate was sealed again and incubated for two hours at 37 °C and then washed 3 times with washing buffer (PBS-T). After
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washing, HRP conjugated rat anti-mouse IgE, IgG1 and IgG2a antibodies (1:1000; Southern Biotech, Birmingham, USA) were added and incubated for one hour at 37 °C. The plate was washed with washing buffer 5 times. A 50 μl substrate solution was added to each well and the plate was incubated for 30 min at 37 °C in dark. The reaction was stopped by the addition of 50 μl of stopping solution (5N H2SO4) and read at 492 nm in ELISA plate reader (Spectra Max, Molecular Devices, USA).

4.2.9.8. Isolation and culture of splenocytes
BALB/c mice (n=5 per group) were sensitized intraperitoneally with 100 μl of 1 mg/ml peanut and red gram CPE, while control mice received only PBS. On day 15, the animals were euthanized and the spleen was removed aseptically in ice-cold RPMI-1640 media with 25 mmol/l HEPES, 400 μg/ml streptomycin, 400 μg/ml ampicillin and 10% heat-inactivated fetal bovine serum (FBS). Spleens were minced and cell suspensions prepared. The splenocytes were isolated after the removal of the red blood cells using alkaline cell lysing buffer (0.15M NH₄Cl, 1 mM NaHCO₃, 0.1 mM EDTA, pH 7.4) and viable counts were performed by exclusion of trypan blue dye. More than 90% of cells were found viable by trypan blue dye exclusion. Single cell suspension of splenocytes (1x10⁶ cells/ well) were seeded in triplicate into 96-well flat-bottomed tissue culture plates (Corning Costar, Fischer Scientific, Pittsburg, USA) and cultured in the presence of 100 μg of peanut and red gram CPE and maintained at 37° C in a humidified atmosphere of 5% CO₂ in air. Culture was terminated after 24, 48 and 72 hours, the supernatants collected, centrifuged at 100 g for 5 min and stored at -80 °C until analysis.

4.2.9.9. Cytokine determination
The cytokine content of culture supernatants derived from splenocytes following 24, 48 and 72 hours in culture was determined using commercially
available capture sandwich ELISA kit (eBioscience, San Diego, USA). Supernatants from the incubated plates were separated and analyzed for levels of IL-2, IL-4, IL-10 and INF-γ. The protocol for cytokine ELISA was followed as per the capture kit following the instructions given by the manufacturer. In brief, microtitre plates supplied with kit were coated with capture antibody (100 µl/well) and incubated overnight at 4 °C. Subsequently, the plates were washed thoroughly with wash buffer and blocked with 200 µl of assay diluent and incubated at RT for 1 hour. Plates were washed; 100 µl/well standard as well as culture supernatant were added, incubated at RT for 2 hours. Detection antibodies was added (100 µl/well) after washing and incubated at RT for 1 hour. Enzyme was added (100 µl/well) and plates were incubated at RT for 30 min. After washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate (100 µl/well) was added. After 15 min, stop solution (2 N H₂SO₄) was added and plates were read at 450 nm.

4.2.9.10. RT-PCR analysis of cytokine mRNA

A semi-quantitative Reverse Transcriptase-PCR analysis of Th1 (IL-2, IL-12 and IFN-γ) and Th2 (IL-4, IL-5 and IL-10) cytokines mRNA in whole blood and spleen of mice sensitized with red gram and peanut CPE was performed taking gene specific primers (Table 4.1). Gene specific Primers for RT-PCR were designed using Primer 3 software (http://seqtool.sdsc.edu/CGI/BW.cgi). During designing the primers, all the sequences were analyzed and validated for self-dimer formation, cross dimerization, hairpin formation and found to be favorable for in vitro amplification reactions. Apart from these, the specificity of the designed primers has been confirmed through the nucleotide BLAST in NCBI (National Centre for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Total RNA from whole blood and
Table 4.1: Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Gene Bank Accession No.</th>
<th>Primer Sequences</th>
<th>Size of PCR amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sense</td>
<td>Anti-Sense</td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>NM_008366</td>
<td>GATGAACCTTGACCTCTCGG</td>
<td>GGGCTTGTGAGATGATGCT</td>
</tr>
<tr>
<td>IL-4</td>
<td>NM_021283</td>
<td>CTCGTCTTGAGGCTTCCAA</td>
<td>TGCTCTTTAGGCTTCCAGG</td>
</tr>
<tr>
<td>IL-5</td>
<td>NM_010558</td>
<td>TGAGGCTTCCTGTCCCTAC</td>
<td>CTTCCATAGCCCACCTCTG</td>
</tr>
<tr>
<td>IL-10</td>
<td>NM_010548</td>
<td>TGCTATGCTGCTGCTCTTA</td>
<td>TCCTGCATTAAGGATCGGT</td>
</tr>
<tr>
<td>IL-12</td>
<td>NM_008352</td>
<td>GCCAGGTGTCTTAGCAGTC</td>
<td>GCTCCCTCTTTGTTGGAAG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>NM_008337</td>
<td>GCATCTGGCTTGGCAGCT</td>
<td>CTTTTTTCGCTTGGCTTGG</td>
</tr>
<tr>
<td>β-actin</td>
<td>NM_007393</td>
<td>GCTACAGCTTCACCACCA</td>
<td>TCTCCAGGGAGGAGGAGG</td>
</tr>
</tbody>
</table>
spleen was isolated with Tri BD and Tri Reagent (Sigma, St. Louis, USA), respectively according to manufacturer’s instructions. Total RNA was treated with RNase free DNase (Fermentas, USA) and the integrity of RNA was determined by running on 1.2% agarose gel prior to further downstream application. The RT-PCR was carried out using One Step RT-PCR kit (QIAGEN, Netherland) and 1 μg total RNA were taken as template for each reaction. β-actin gene was selected as endogenous internal standard. Thermal cycler (G-Storm, Essex, U.K.) was programmed as follows; reverse transcription at 50 °C for 30 minutes and subsequent 40 cycles of 94 °C denaturation for 30 sec, 55 °C annealing for 30 sec and extension at 72 °C for 30 sec, with final extension at 72 °C for 10 min. After completion of PCR cycles, 10 μl of PCR product was resolved on 2% agarose gel electrophoresis and stained with ethidium bromide. The density of each band was estimated by the Genetools software (Syngene, Cambridge, UK).

4.2.10. Identification and characterization of red gram allergens

4.2.10.1. In vitro digestibility of red gram CPE

In vitro SGF was performed as described by Roesler and Rao (2001) with slight modifications as mentioned in Chapter 2, section 2.2.7. SGF digestion stopped after 2 min by addition of stopping solution and digests were resolved over 12% SDS-PAGE according to the method of Laemmli (1970) and stained with CBB (G-250).

4.2.10.2. Immunoblotting of red gram CPE

In order to confirm allergenic potential of pepsin stable proteins, IgE reactivity of red gram CPE was analysed by immunoblotting with sera of red gram hypersensitive patients (1:20). Red gram CPE without SGF digestion was resolved over 12% SDS-PAGE and electrophoretically transferred onto PVDF membrane (Immobilon-P, Millipore Co, USA) according to Towbin et
IgE binding protein bands were detected using enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Amersham, UK) according to the manufacturer's protocol. Images were captured using Syngene gel documentation system equipped with CCD camera (Syngene, Cambridge, UK). Red gram CPE immunoblots were compared with 2 min SGF digests of red gram to see the allergenic potential of pepsin stable proteins.

4.2.10.3. Two-dimensional electrophoresis (2 DE) and immunoblotting of red gram proteins

Red gram CPE (100 μg) in rehydration buffer was resolved over linear pH 3-10 Immobilized pH Gradient (IPG) strip (GE Healthcare Life Sciences, USA) in first dimension. Strips were focused up to 20,000 VhT, with a maximum of 4500 V, at 20 °C using Ettan IPGPhor 3 (GE Healthcare Life Sciences, USA). Focused IPG strips were equilibrated twice in SDS equilibration buffer containing 65 mM DTT for the first equilibration and 135 mM iodoacetamide for the second. Second dimension separation was performed over 12% SDS-PAGE gels. Identical gels were stained with CBB G-250 or blotted on PVDF membrane and incubated with pooled patients sera (1:20 in PBS-T with 3% BSA) followed by HRP-conjugated goat anti-human IgE antibody (Sigma Chemical Co, St. Louis, USA). 2D separated red gram CPE was also immunoblotted with pooled sera of red gram sensitized mice (1:20 in PBS-T with 3% BSA) and detected by HRP conjugated rat anti-mouse IgE antibody (Southern Biotech, Birmingham, USA). Prior to immunoblotting, IgG was removed from patient as well as mice sera using protein G-agarose beads (Pierce Biotechnology, Rockford, USA).
4.2.10.4. Liquid chromatography mass spectrometry (LC-MS/MS) analysis of IgE immunoreactive proteins

CBB G-250 stained gel and immunoblot were matched with the help of 2D platinum software (GE Healthcare Life Sciences, USA). Five proteins showing IgE binding with red gram allergic patient’s sera as well as sensitized BALB/c mice sera were excised carefully from 2D gel and subjected to LC-MS/MS. Mass spectrometric analysis was done at The Centre for Genomic Applications (TCGA), New Delhi. In brief, the gel slices were cut into small pieces of approximately 1 mm$^3$ and after destaining, tryptic in-gel digestion was performed according to Schrimpf et al. (2005). The supernatant of the digested solution was collected and the gel pieces were extracted with 100 μl of 20 mM NH$_4$CO$_3$ for 20 min and this supernatant was recollected. Again gel pieces were extracted twice with 100 μl of 1% TFA in 50% acetonitrile for 20 minutes and supernatants were collected. All the supernatants were pooled and 6 μl of the supernatant was injected into Thermofinnigan LCQ DECA XP (Thermo Fisher Scientific, Waltham, MA, USA) equipped with cap LC pump (Waters). Tryptic peptides were separated using a gradient formed between solvent A [0.1% (v/v) formic acid in MilliQ water] and solvent B [0.1% (v/v) formic acid/acetonitrile (20/80)] on Dionex C18 column (Dionex Corp, Sunnyvale, CA). The gradient used was 10% solvent B for 5 min, followed by a linear gradient from 10% to 60% solvent B during next 50 min. Individual MS/MS spectra of tryptic fragments were searched against Mass Spectrometry protein sequence Data Base (MSDB) using MASCOT search engine (Perkins et al., 1999). Mass tolerance and monoisotopic values (100 ppm for peptide mass fingerprint, peptide mass tolerance of ±2 Da and fragment mass tolerance of ±0.8 Da for MS/MS spectra) were used for searching and carbamidomethyl was considered as fixed modification of tryptic fragments.
with oxidation as variable modification. Probability based MOWSE score was calculated in terms of ion score $-10^a\log(P)$, where $P$ is the probability and observed match was considered as a random event.

4.2.10.5. Sequence analysis
High probability based MOWSE score was used to identify proteins. To decipher the order of tryptic digestion generated peptides in the probable amino acid sequence, alignment of individual peptide sequence was run with the protein hit having maximum probability score using CLUSTAL W program on Biology Workbench 3.2 (www.workbench.sdsc.edu). The resulting ordered peptide sequence was subjected to overall FASTA search on www.allergenonline.com version 8.0 to determine allergenicity of proteins by showing significant homology ($e$ score $<0.001$ was taken as cut-off).

4.2.11. Statistical analysis
Values are presented as means ± SEM. Statistical evaluation was carried with one-way ANOVA (Snedecor and Cochran 1967). In all the cases, $p$ values less than 0.05 were considered significant when compared to controls.

4.3. Results
4.3.1. Patients' study
Specific IgE were estimated in sera of 6 patients with marked positive SPT (with 2+ or more) to red gram. All the red gram allergic patients showed elevated specific IgE levels more than 2.7 times over negative control. Red gram sensitive patients had total IgE levels ranging from 176 to 3054 IU/ml, whereas, patients allergic to dander and insects (negative control) had total IgE level ranging from 225-4305 IU/ml. All the red gram allergic patients elicited positive skin reactions to other legumes also as evident by 83%
concomitant sensitization to green gram, 66% to soybean, 33% to chickpea, 17% to lentil, bean fresh and peanut each (Table 4.2).

4.3.2. Cross-reactivity of red gram with other legumes
Allergenic potency of the red gram extract was determined by ELISA inhibition assay. It was found that 11.11 ng red gram extract required for 50% inhibition (Fig. 4.2 a). To explore the potential cross-reactivity of red gram with other legumes (as shown by SPT) ELISA inhibition was performed using soybean and green gram extracts as inhibitors. Green gram and soybean produced 50% inhibition of specific IgE binding to solid phase red gram extract with 672 and 2200 ng, respectively. ELISA inhibition studies supported the cross-reactivity among legume including soybean and green gram (Fig. 4.2 b).

4.3.3. Immune response to red gram CPE in mice
The serological responses induced by red gram proteins, were analyzed on 15, 43 and 59 days of treatment using indirect ELISA. In the animal experiments, peanut was taken as positive control, as it is a known allergenic leguminous crop, whereas PBS was given as vehicle. On day 15, 43 and 59, total as well as specific IgE levels in both red gram and peanut sensitized groups were significantly higher \( p < 0.01 \) as compared to vehicle treated group (Fig. 4.3 a and b). Pattern of specific IgE responses across the time against red gram and peanut was similar though levels of specific IgE were slightly lower in case of red gram (Fig. 4.3 b). Similar to peanut, red gram sensitized group showed significant increase in IgG1 levels \( p < 0.001 \) up to 59 days continuously (Fig. 4.4 a). Red gram caused more than 2.5 fold decrease on day 15, and four fold decrease on day 43 and 59, whereas peanut caused more than five fold decrease in IgG2a level compared to control from day 15 to 59 (Fig. 4.4 b). These results demonstrated red gram protein's
### Table 4.2: Clinical and immunologic analysis of red gram-sensitive allergic patients

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Age (years)/Sex</th>
<th>Symptoms*</th>
<th>SPT with red gram CPE</th>
<th>Total IgE (IU/ml)</th>
<th>Specific IgE (OD)</th>
<th>Sensitization to other legumes**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>40/M AR/BA</td>
<td>2+</td>
<td>3054</td>
<td>1.30</td>
<td>Gg, Sb</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>44/M AR/URT</td>
<td>2+</td>
<td>176</td>
<td>1.47</td>
<td>Gg, Bn</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>18/M AR/BA</td>
<td>2+</td>
<td>1245</td>
<td>1.29</td>
<td>Gg, Sb, Ch</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>34/F BA/GIS</td>
<td>2+</td>
<td>590</td>
<td>1.01</td>
<td>Sb, Pn, Ch</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>45/M BA</td>
<td>2+</td>
<td>1142</td>
<td>1.59</td>
<td>Gg, Sb</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>26/F AR/BA</td>
<td>2+</td>
<td>2028</td>
<td>1.00</td>
<td>Gg, Ln</td>
<td></td>
</tr>
</tbody>
</table>

*AR, Allergic rhinitis; BA, Bronchial asthma; URT, Urticaria; GIS, Gastrointestinal symptoms.

M: male; F: female; IgE: immunoglobulin E; SPT: skin prick test.

Negative control for Specific IgE: OD 0.36.

Negative control for total IgE: 225-4305 IU/ml

**Abbreviated names of different legumes used in table:

Gg, Green gram; Sb, Soybean; Bn, Bean fresh; Ln, Lentil; Ch, Chickpea; Pn, Peanut.
Fig. 4.2. (a) ELISA inhibition of red gram CPE.
(b) ELISA inhibition of red gram with soybean (−−−) and green gram (−−−) CPE.

Means (n=3) and SEs (bars) are represented.
Serum immunoglobulin responses.
(a) Total IgE, and
(b) Specific IgE in sera of peanut and red gram-sensitized BALB/c mice.

Data are reported as means ± SEM for mice (n=5 per group).
** represent $p$ values <0.01 and *** represent $p$ values <0.001 as compared to control group.
Fig. 4.4. Serum immunoglobulin responses.
(a) Specific IgG1, and
(b) Specific IgG2a in sera of peanut and red gram-sensitized BALB/c mice.

Data are reported as means ± SEM for mice (n=5 per group).
*** represent p values <0.001 as compared to control group.
#The IgG2a levels have been presented in terms of fold decrease as compared to control.
ability to induce IgE and IgG1 levels in mice that peaked on day 43, indicating their allergenic potential.

4.3.4. Antigen challenge of sensitized mice and histamine level
Increase in histamine level is one of the markers of anaphylaxis and allergic reactions. Therefore, allergic symptoms were scored and histamine content was measured after challenge of red gram sensitized mice. Hypersensitive symptoms became evident in mice within 15 to 30 min after challenge with CPE. The most severe symptoms were observed in positive control group of peanut sensitized/challenged mice with 80% mortality due to anaphylaxis, whereas mice sensitized with red gram showed slightly less but still significantly strong reaction with 40% mortality upon challenge (Fig. 4.5 a). However, as expected PBS treated control mice when challenged with either peanut or red gram CPE, showed no symptoms of hypersensitivity. In continuation of this, histamine levels were increased markedly in peanut (9 fold) and red gram (6 fold) sensitized/challenged mice as compared to control (Fig. 4.5 b).

4.3.5. Histology of lungs, spleen and intestine
To study the allergenic effect of red gram at tissue levels, histopathology studies were undertaken for lungs, spleen and intestine. Analysis of hematoxylin and eosin stained lung tissue of red gram sensitized/challenged mice revealed lymphoid infiltration and thickening of alveolar septa throughout the parenchyma (Fig. 4.6 c). Lung section of peanut treated group showed perivascular and peribronchial inflammatory cell infiltrate, with narrowing of the bronchiolar lumen of milder nature (Fig. 4.6 b). Mild bronchial epithelial hyperplasia was also evident in both the red gram and peanut treated groups. However, lung from control group showed normal histological structure (Fig. 4.6 a).
Fig. 4.5. (a) Anaphylactic symptom scores in sensitized mice after peanut or red gram CPE challenge, and (b) Histamine level in plasma.

Histamine is depicted means ± SEM for n=5 mice per group.

***P < 0.001 values are significant as compared to control.
Fig. 4.6. Lung histology of sensitized/challenged mice. (a) PBS (control), (b) Peanut, (c) Red gram.

Perivascular and peribronchial inflammatory cell infiltrate is indicated with black arrow. Mild bronchial epithelial hyperplasia was evident in both the red gram and peanut treated groups as indicated with red arrows.
Spleen from peanut sensitized/challenged group revealed lymphoid hyperplasia with large megakaryocytes (Fig. 4.7 b), while spleen from red gram treated group revealed activated macrophages in addition to large sized megakaryocyte and lymphoid hyperplasia (Fig. 4.7 c). Spleen from control mice had normal histology of white pulp and red pulp (Fig. 4.7 a).

Several infiltrations of inflammatory cells with loss of normal intestine mucosal structure were seen both in peanut as well as red gram sensitized/challenged mice (Fig. 4.8 b and c). Mucosal exfoliation was the prominent feature of the intestine from peanut sensitized/challenged mice (Fig. 4.8 b). Intestine of red gram treated group showed goblet cell hyperplasia in submucosal layer. Like peanut, mucosal exfoliation was also one of the important features (Fig. 4.8 c). Intestine presented normal histological structure from control group mice (Fig. 4.8 a).

4.3.6. Th1 and Th2 cytokine responses to red gram proteins

To determine the effect of red gram CPE on cytokine profiles, we measured IL-4, IL-10 (Th2) and IL-2, IFN-γ (Th1) cytokines in supernatant of splenocytes culture. A significant increase in IL-4 and IL-10 \( (p < 0.001) \) levels was observed in supernatant of cultured splenocytes from peanut/red gram primed mice as compared to control mice (Fig. 4.9 a, b). There was no difference found in IL-2 levels in both red gram and peanut primed and unprimed splenocyte culture supernatants (Fig. 4.10 a). The level of IFN-γ was significantly \( (p < 0.01) \) decreased in peanut and red gram primed splenocyte supernatants (Fig. 4.10 b). Thus, red gram behaved in a similar manner as peanut for cytokine response as it caused significant increase in Th2 cytokines and decrease in IFN-γ production.
Fig. 4.7. Spleen histology of sensitized/challenged mice. (a) PBS (control), (b) Peanut, (c) Red gram.

Lymphoid hyperplasia (indicated by black arrow) and large sized megakaryocytes are indicated by red arrows in peanut and red gram treated groups.
Fig. 4.8. Intestine histology of sensitized/challenged mice. 
(a) PBS (control), (b) Peanut, (c) Red gram.

Heavy infiltrations of inflammatory cells and mucosal exfoliation are indicated by black arrows in peanut and red gram. Globlet cell hyperplasia in sub-mucosal layer is indicated by red arrow in red gram treated group.
Fig. 4.9. Effect of red gram CPE on Th2 cytokine levels in splenocyte culture supernatants. 
(a) IL-4, and 
(b) IL-10 levels were measured by ELISA. 
Data are presented as means ± SEM. 
*** $p<0.001$ values are significant as compared to control
Fig. 4.10. Effect of red gram CPE on Th1 cytokine levels in splenocyte culture supernatants.
(a) IL-2, and
(b) IFN-γ levels were measured by ELISA.

Data are presented as means ± SEM. ** p<0.01 and *** p<0.001 values are significant as compared to control
4.3.7. Red gram allergens induced changes in cytokines mRNA expression

Effects of red gram CPE administration on cytokine production were also evaluated at mRNA levels. Red gram CPE similar to peanut CPE, upregulated the levels of Th2 cytokine mRNAs like IL-4, IL-5 and IL-10 ($p < 0.001$) in whole blood and spleen of sensitized and challenged mice as compared to PBS treated mice (Fig. 4.11 a and 4.11 b). Red gram and peanut CPE treated groups did not show any change in IL-2 mRNA levels as compared to PBS treated mice both in blood as well as spleen. However, significant down regulation ($p < 0.001$) was observed in the IFN-$\gamma$ mRNA level in blood and spleen from peanut treated mice in comparison to control (Fig. 4.12 a, b). Interestingly, IFN-$\gamma$ levels were significantly decreased ($p < 0.01$) only in case of spleen but not in blood of red gram treated mice. Similarly, IL-12 mRNA levels were decreased significantly ($p < 0.001$) only in spleen of both peanut and red gram treated group (Fig. 4.12 b). These results suggest that red gram protein has allergenic potential as it up-regulates the expression of Th2 cytokines and down-regulates Th1 cytokines.

4.3.8. SGF digestibility and IgE binding potential of red gram CPE

SDS-PAGE profile of red gram CPE showed proteins ranging from mol wt 20 to 130 kDa (Fig 4.13 a, lane 2). Three proteins of 66, 45 and 30 kDa were found to be stable up to 2 min (Fig. 4.13 a, lane 4) following SGF digestion. In order to demonstrate the allergenic potential of these pepsin stable proteins, specific IgE reactivity of red gram CPE was analyzed by IgE immunoblotting with sera of red gram hypersensitive patients. Pooled patients sera reacted to three red gram proteins of 66, 45 and 30 kDa (Fig. 4.13 b). Immunoblots with individual sera of all the 6 red gram allergic patients demonstrated two IgE binding proteins of 66 and 45 kDa; whereas IgE binding to 30 kDa protein demonstrated by 50% patients only. Immunoblot of red gram CPE with PBS
Fig. 4.11. Effect of red gram treatment on Th2 cytokines mRNA expression in whole blood and spleen of sensitized mice.
Th2 cytokines included IL-4, IL-5 and IL-10 in (a) blood and (b) spleen.
β-actin was taken as endogenous control.
Data are means ± SEM for 5 mice in each group.
Graph was plotted taking the value of relative intensity of cytokine versus β-actin.
Fig. 4.12. Effect of red gram treatment on Th1 cytokines mRNA expression in whole blood and spleen of sensitized mice. Th1 cytokines included IL-2, IL-12 and IFN-γ in (a) blood and (b) spleen. β-actin was taken as endogenous control. Data are means ± SEM for 5 mice in each group. Graph was plotted taking the value of relative intensity of cytokine versus β-actin.
Fig. 4.13. SGF digestion and immunoblot of red gram CPE.

(a) Lane 1: Pepsin, Lane 2: red gram CPE; Lane 3: SGF digestion pattern of red gram at 0 minutes; Lane 4: SGF digestion pattern of red gram at 2 minutes; Lane 5: Molecular weight markers.

(b) Lane PS: Immunoblot of red gram CPE with pooled sera (1:20) of red gram sensitive patients; Lane 1-6: Immunoblots of red gram CPE with individual serum of red gram CPE sensitive patients. Lane NHS: Immunoblot of red gram CPE with pooled serum from 10 patients, allergic to dander and insects but not to any food or pollen extracts was used as negative control human sera; Lane PBS: Red gram CPE incubated with PBS and HRP linked anti-human IgE (1:1000).
and negative control human sera did not show any binding. These results clearly demonstrate that pepsin sensitive proteins did not show any IgE binding, whereas pepsin stable proteins showed IgE binding.

4.3.9. Identification, characterization and comparison of IgE binding proteins of red gram in mice and human subjects

Next, we identified the IgE binding proteins of red gram after 2D electrophoresis and IgE immunoblotting. IgE specific immunoblot with pooled patients sera showed five IgE binding protein spots, one of 66 kDa (approx pI 5.9), three proteins of 45kDa (with different pl of approximately 5.2 (45a), 5.6 (45b) and 6.1 (45c)) and one of 30 kDa (approx pl 5.3) (Fig. 4.14 c). Sera from red gram sensitized mice recognized the same five IgE binding proteins as sera of allergic patients, one additional protein spot of 30 kDa also showed specific IgE binding with mice sera (Fig. 4.14 b).

4.3.10. Mass spectrometric analysis of the five IgE E binding proteins

Mass spectrometric analysis of IgE binding proteins revealed homology with different subunits of β-conglycinin protein from soybean with significant probability based MOWSE score. β-conglycinin is a known allergen that belongs to cupin-2 superfamily of legume seed storage proteins (Mills et al., 2002). The tryptic fragments RKTISSEDKPFNLRS and KFFEITPEKNPQLRD of 66 kDa protein showed maximum homology with β-conglycinin α chain of soybean. Tryptic fragment KTISSEDKPFNLRS of 45a, revealed high sequence similarity with β-conglycinin α prime subunit of soybean. The tryptic fragments KLFEITPEKN, RKTISSEDKPFNLRS and KNILEASYDKFEEINKV of 45 b revealed sequence similarity with β-conglycinin α prime subunit of soybean. The tryptic fragments KLFEITPEKN and RKTISSEDKPFNLRS of 45c proteins also showed homology with β-conglycinin α prime subunit of soybean. Tryptic fragment KTISSED
Fig. 4.14. (a) Two-dimensional (2D) SDS-PAGE profile of red gram CPE; (b) IgE binding proteins detected after immunoblotting of 2D resolved red gram CPE with pooled serum of red gram sensitized mice; (c) IgE binding proteins detected after immunoblotting of 2D resolved red gram CPE with pooled serum of patients allergic to red gram.
KPFNFRS of 30 kDa protein showed significant homology to \( \beta \)-conglycinin \( \alpha \) prime subunit of soybean (Table 4.3, Fig. 4.15 i-v).

The tryptic fragments of 66 kDa protein corresponded to amino acid 414-428 and 439-453 of \( \alpha \) subunit of \( \beta \)-conglycinin (Fig. 4.16). Peptide fragments of 45 kDa b corresponded to amino acid 355-372, 412-426 and 437-446 position of \( \alpha \) prime subunit of \( \beta \)-conglycinin (Fig. 4.17), whereas 45 kDa c tryptic digested peptide fragments corresponded to amino acid 412-426 and 437-446 position of \( \alpha \) prime subunit of \( \beta \)-conglycinin when alignment was done (Fig. 4.18). According to the positions of tryptic digested peptide sequences of respective red gram proteins on the amino acid sequence of protein with maximum ion score, the order of tryptic fragments were speculated and resultant amino acid sequence were used for further bioinformatics analysis.

4.3.11. Sequence match analysis of tryptic fragments of IgE binding proteins for allergenicity determination

To assess whether 66, 45 and 30 kDa immunoreactive proteins are having similarity to other allergens, the amino acid sequence of fragments obtained through LC-MS/MS were subjected to simple FASTA search on allergenonline database. Ordered tryptic fragment sequences obtained from CLUSTAL W showed homology with other known allergenic seed storage proteins of different legumes mainly different subunits of \( \beta \)-conglycinin of soybean, Len cl 1.0101 and Len cl 1.0102 allergens of lentil, vicillin of garden pea, conglutin \( \beta \) of lupin and Ara h1 of peanut (Table 4.4). The scale (low e values) of similarities with known allergens indicates that these immunoreactive red gram proteins may also be allergenic as uncovered by immunoblot as well.
Table 4.3: Mass spectroscopy analysis of IgE binding proteins of red gram

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Presumed Mol wt by SDS-PAGE (kDa)</th>
<th>Tryptic Peptide Fragments</th>
<th>Sequence Similarity</th>
<th>#Probability based MOWSE Score (Ion Score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>RKTISSEDKPFNLRS</td>
<td>β-conglycinin α prime subunit - Soybean</td>
<td>78 /</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KNILEASYDTKFEEINKV</td>
<td>β-conglycinin α prime subunit - Soybean</td>
<td>78 /</td>
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<td>5.</td>
<td>30 kDa</td>
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<td>51</td>
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</tbody>
</table>

# Ions score is \(-10^\ast\log(P)\), where P is the probability that the observed match is a random event. Individual ions scores > 44 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.
Fig. 4.15. Bar diagrams showing LC/MS-MS results of different red gram IgE binding proteins. LC/MS-MS mass spectra and probability plot of (i) 66 kDa, (ii) 45 kDa (45a), (iii) 45 kDa (45b), (iv) 45kDa (45c) and (v) 30 kDa protein spots of red gram. High probability score were used to identify proteins. Mass tolerance (Peptide mass tolerance of ±2 Da) and monoisotopic values were used for searching.
Fig 4.16. Alignment of the 66 kDa red gram peptide sequences obtained from LC-MS/MS analysis with full length sequence of β-conglycinin α chain of soybean

**Ordered Fragment:** RKTISSEDKPFNLRSKFFEITPEKNPQLRD
Fig 4.17. Alignment of the 45 kDa (b) red gram protein sequences obtained from LC-MS/MS analysis with full length sequence of β-conglycinin α prime chain of soybean

45 kDa [b]  
BWB7553  

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<th>MWARFPDLLLGVVFASVSVPFGYAYWEKQNPISHNKLSCRNSEKDSYRNQACHARCNL</th>
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45 kDa [b]  
BWB7553  

| LRVPAGTYYVYPDENDLAMITLAIVPNVKPRFESFLLSSTQAQQSYLQGFSKNILEA | 360 |

45 kDa [b]  
BWB7553  

| SYDTKFEEINKVLFGREEQPOQGEERLQESVIVESKKQIRELSSRKTISSEDK | 420 |

45 kDa [b]  
BWB7553  

| PFNLRSRDPIYSNLGKLFEITPEKNFKLRDLVFLSVDNEGALFPHBFNSKAVVLV | 480 |

45 kDa [b]  
BWB7553  

| QKEEGNKGRKPLLILRAFY | 621 |

**Ordered Fragment:** KNILEASYDTKFEEINKVRKTISSEDKPFNLRSKLFEEITPEKN
Fig 4.18. Alignment of the 45 kDa (c) red gram protein sequences obtained from LC-MS/MS analysis with full length sequence of β-conglycinin α prime chain of soybean

45_kDa_[c]  
BWB7553  
MGRARFPLLGVVFLASVSFGAYWEKQPShNKLRCNSeKDSYRNQACHARCNL  - 60

45_kDa_[c]  
BWB7553  
SYDKFEISKVLPGREESQQGEEELQESVIEISKKQIRELSKRAKSSSRKTISSEDK  - 420  
********

45_kDa_[c]  
BWB7553  
QKEEGNKGRLFPNLRSKLFEITPEKN  - 480  
******

45_kDa_[c]  
BWB7553  
QKEEGNKGRLPFNLRSKLFEITPEKN  - 621

**Ordered Fragment:** RKTISSEDKPFNLRSKLFEITPEKN
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<th>Protein</th>
<th>Tryptic Peptide Fragments</th>
<th>Ordered tryptic fragments</th>
<th>Sequence similarity using Full FASTS search</th>
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<td></td>
<td>( \beta )-conglycinin storage protein (Glycine max)</td>
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<td></td>
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<td>Allergen Len c 1.0102 (Lens culinaris)</td>
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<td></td>
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<td></td>
<td>Conglutin ( \beta ) (Lupinus angustifolius)</td>
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| 5.    | 30 kDa  | RKTISSEDKPFNLRSG          |                           | \
|       |         |                           |                           | \( \beta \)-conglycinin-\( \alpha \) subunit (Glycine max) |
|       |         |                           |                           | \( \beta \)-conglycinin storage protein (Glycine max) |
|       |         |                           |                           | Allergen Len c 1.0102 (Lens culinaris) |
|       |         |                           |                           | Allergen Len c 1.0101 (Lens culinaris) |
|       |         |                           |                           | Vicilin (Pisum sativum) |
|       |         |                           |                           | Conglutin \( \beta \) (Lupinus angustifolius) |
4.4. Discussion

Allergy to legumes is well known, with peanut being the most prominent and soybean as the next most important allergenic legume. In this study we endeavored to find out the allergenic potential of another legume; red gram in animal model and the allergens present therein were further identified and characterized using red gram allergic patient's sera. Considering three parameters (history, SPT and specific IgE ELISA), sensitization to red gram was observed in 6 patients. ELISA demonstrated significantly elevated red gram specific IgE levels, indicating that these patients were sensitized to red gram, validating selection of patients on the basis of SPT. In this regard an earlier study has also shown that SPT positive chickpea patients had enhanced IgE levels corroborating our findings (Patil et al., 2001).

Studies in mice showed that red gram caused allergic responses, which are characterized by elevated IgE and IgG1 levels and decrease in IgG2a levels. IgE and IgG1 antibodies are associated with Th2 cells, whereas induction of IgG2a requires Th1 cells (Abbas et al., 1996). Balance between IL-4 and IFN-γ affects levels of these immunoglobulins. IFN-γ promotes IgG2a production and inhibits IgE responses (Coffman and Carty, 1986; Snapper et al., 1988), whereas IL-4 enhances IgE and IgG1 production and inhibits IgG2a production. In our study, red gram administration up regulated Th2 cytokines including IL-4 and did not affect the levels of Th1 cytokines either at mRNA or protein levels except IFN-γ where significant reduction was observed. This alteration in levels of Th2 and Th1 cytokines are consistent with increased IgE and IgG1 levels and decreased IgG2a levels in red gram sensitized mice sera.

Histamine plays a major role in the pathogenesis of allergy process (Galli et al., 1993) and is one of the numerous mediators present in the cytoplasmic granules of basophils and mast cells. On cross linking of mast
cell bound IgE by allergens; histamine is secreted and in association with other mediators contributes to the symptoms of anaphylaxis. Sensitized mice on being challenged with red gram extract showed increased histamine levels that are consistent with observed anaphylactic symptoms.

One of the most striking histological signs of allergic reaction is acute or chronic inflammation and accumulation of inflammatory cells in affected organ (Ryan and Majno, 1977). Allergic airway inflammation is characterized by predominant Th2 responses such as elevated levels of IL-5, a Th2 cytokine, causing infiltration of inflammatory cells (Zhu et al., 2004). In our study, red gram sensitized groups showed the accumulation of inflammatory cells in the perivascular and peribronchial regions of the lung parenchyma like positive control peanut treated group. Upon challenge, diarrhea was observed in all the sensitized mice. In endorsement of this, histology of intestine also showed distorted or exfoliated mucosal structure, a symptom of acute diarrhea. The presence of lymphoid hyperplasia which is a characteristic histological feature of the spleen in response to an allergen should be regarded as reflecting massive proliferation and differentiation of T lymphocytes. These histological changes along with anaphylactic symptoms underpin the modulated cytokines and immunoglobulin levels, proposing towards allergenic potential of red gram which are similar to that of allergic proteins of peanut (Van Wijk et al., 2004).

Stability to digestion against gastric enzymes is thought to be an important feature of allergenic food proteins (Astwood et al., 1996; Besler et al., 2001; Thomas et al., 2004). Our earlier data in chapter 3, also indicated that proteins stable in SGF for more than 2 min showed ability to bind with specific IgE of allergic patient’s sera. In red gram CPE; 66, 45 and 30 kDa proteins were found to be resistant to SGF digestion for 2 min and these proteins also showed IgE binding capacity on immunoblotting with patient’s
sera. However, 2 D-E resolved immunoblot showed five IgE binding proteins of mol wts 66, 45 and 30 kDa when incubated with patient’s sera, where 45 kDa was separated into three spots of different pl. Interestingly, same proteins were recognized by allergic patient’s sera as well as sensitized mice sera. An additional protein of 30 kDa was recognized by mice sera immunoblot. LC/MS-MS analysis of the five IgE binding protein spots identified by immunoblot of 2DE separated CPE with both human as well as mice sera, showed sequence similarity with different subunits of β-conglycinin from soybean. Beta-conglycinin proteins are well characterized and are major allergen of soybean (Ogawa et al., 1995; Fu et al., 2007). The tryptic fragment’s sequences obtained from LC/MS-MS analysis when subjected to FASTA and BLAST search on www.allergenonline.com and www.ncbi.nlm.nih.gov respectively, shows hits on known allergenic seed storage proteins of different legumes, mainly subunits of β-conglycinin of soybean, Len c1.0101 and Len c2.0102 of lentil, vicillin of garden pea and Ara h1 of peanut. All of these proteins belong to cupin-2 superfamily of legume seed storage proteins, indicating that IgE immunoreactive red gram proteins identified in this study may also be the member of this superfamily (Mills et al., 2002). LC/MS-MS results in combination with bioinformatics analysis allowed us to hypothesize specific protein spots as candidate allergens.

The study indicates allergenic potential of red gram CPE as its administration induces Th2 responses and decreases Th1 responses in mice. Also, five IgE binding proteins having mol wts of 66, 45 (three proteins having different pIs) and 30 kDa have been identified as putative clinically relevant allergens (Fig. 4.19). All these are homologous to different isoforms of well-characterized major allergen β-conglycinin of soybean. Since, the identification of new food allergens is the first step towards future diagnostic and therapeutic approaches to deal effectively with food allergy, therefore,
Fig. 4.19. Summary of red gram CPE induced allergenic responses
further studies are needed to explore the full sequences of these allergenic proteins. These sequences then can be used for cloning and recombinant expression of the allergens. This will also go a long way in producing new red gram transgenic varieties.

4.5. Conclusion

Red gram CPE has allergenic potential as it up-regulated the Th2 responses and IgE levels. It contains at least five allergens that induced IgE-mediated reactions in red gram sensitized patients as well as in sensitized BALB/c mice. Bioinformatics analysis of all the five IgE binding proteins revealed sequence similarity with known allergens confirming our clinical findings.