Summary & Conclusion
Food allergy is a problem encountered around the globe and its incidence is on increase, including Asian countries. It is mainly associated with intake of protein containing foods and thus dietary habits play a significant role in developing food allergy. Legumes provide a major source of dietary protein in human and animal nutrition and it is expected that they could induce allergic reactions in majority of population. Peanut and soybean are the two major legumes responsible for most allergic reactions in United States of America, United Kingdom and Japan. However, the scenario is different in Asian and other Mediterranean countries, where consumption of peanut and soybean is comparably less. Chickpea and lentil have been shown to cause allergic reactions in these regions. In India, consumption of red gram is maximum among legumes, followed by green gram and lentil. In this study we endeavored to evaluate the allergenic potential of red gram and green gram in animal model and the allergens present therein were further identified and characterized using allergic patient's sera.

An important feature of allergenic food proteins is stability to digestion against gastric enzymes. Therefore, simulated gastric fluid (SGF) assay was used as a tool to screen the pepsin resistant proteins from commonly consumed leguminous crops like, chickpea, black gram, kidney bean, Bengal gram, red gram and green gram. Soybean and peanut crude protein extract (CPE) were taken as positive control, where characteristic details of allergens including their molecular weights (mol wts) were known. SDS-PAGE of soybean CPE before SGF digestion presented 10 distinct proteins with apparent molecular weights (mol wts) ranging from 6.5 to 97 kDa. Six proteins of approximately 70, 65, 26, 22, 20 and 6.5 kDa persisted up to 2 min. Remaining four proteins of mol wts of 97, 84, 80 and 29 kDa were rapidly digested in SGF in less than 15 sec. SDS-PAGE of peanut CPE showed 11 proteins ranging from 6.5 to 100 kDa. Proteins of 70, 66, 22, 18, 17 and 6.5
kDa were stable to SGF digestion up to 4 min whereas proteins of 100, 97, 35 and 15 kDa got digested in less than 15 sec. SDS-PAGE of chickpea CPE showed 10 proteins with mol wts ranging from 18 to 84 kDa. Seven proteins of 70, 64, 55, 45, 35, 20 and 18 kDa remained undigested till 15 min in SGF, whereas proteins of 84, 38 and 29 kDa were easily digested in SGF in less than 15 sec. A new protein band of approximately 42 kDa in chickpea CPE appeared after 15 sec of digestion and was stable up to 60 min. SDS-PAGE of black gram CPE resulted in 13 proteins with mol wts ranging from 6.5 to 84 kDa. Ten proteins of 47, 30, 29, 28, 26, 24, 22, 16, 14 and 12 kDa was stable up to 2 min after SGF digestion, whereas proteins of 84, 66 and 6.5 kDa were digested rapidly in less than 15 sec. SDS-PAGE of kidney bean CPE showed 8 proteins with mol wt ranging from 6.5-116 kDa. Five proteins of 45, 29, 24, 20 and 6.5 kDa were stable up to 1 h after SGF digestion, remaining three proteins were digested in less than 15 sec. SDS-PAGE of Bengal gram CPE showed eight proteins with mol wt ranging from 20 to 97 kDa. Out of eight, only one protein of approximately 20 kDa remained stable till 2 min. SDS-PAGE of red gram CPE resulted in nine proteins of mol wt 18 to 130 kDa. Out of nine, two proteins of 45 and 30 kDa were stable up to 60 mins, whereas 66 kDa proteins got digested after 2 min. SDS-PAGE of green gram CPE showed eighteen proteins from 18 to 170 kDa, of which three proteins of 52, 35 and 30 kDa were stable up to 60 min. Proteins of 18 and 38 kDa got digested after 2 and 15 min, respectively following SGF digestion. A new protein band of 20 kDa appeared after 15 sec and it was found to be stable up to 60 min in SGF.

Prevalence of legume allergy and concomitant sensitization to different allergens was evaluated to measure the extent of legume sensitization in patients of asthma and/or rhinitis. The investigation was conducted in 76 patients of bronchial asthma (BA) and allergic rhinitis (AR) attending the
Department of Pulmonary Medicine, Chhatrapati Shahi Maharaj Medical University at Lucknow, India. In the study population, the patients were between 14-62 years of age, with a mean of 31.7±1.2 years, males numbered 48 while remaining 28 were females. Skin prick test (SPT) was carried out in seventy-six patients with commercially available extracts of commonly consumed foods. Out of 76 patients, 35 (46.1%) had positive SPT (2 or>2) to different food extracts. Out of these 35 patients, thirty (85.7%) were having sensitivity exclusively to legumes and remaining 5 patients (14.3%) were sensitive to other food items. The common food allergens found in this study were chickpea (13.2%); followed by green gram (11.8%), egg white (9.2%), red gram/ bean fresh (7.9% each) and bengal gram/ milk/ mustard leaves (6.6% each). SPT was also found positive towards rice, lentil, banana (ripe), mushroom and chicken only in one patient. Out of 76 subjects, 41 (53.9%) had anamnestic food hypersensitivity due to at least one kind of food item. Only in 6 patients (14.6%), the prick test positivity was exactly compatible with anamnesis. To establish the correlation between IgE binding proteins and pepsin stability in SGF, IgE immunoblot using chickpea and soybean allergic patient’s sera was performed and compared with SGF stable protein profile of respective crops. Immunoblots with individual and pooled sera of chickpea allergic patients demonstrated seven IgE binding proteins of 70, 64, 55, 45, 35, 20 and 18 kDa. Proteins of similar mol wts were found to be stable up to 2 min in SGF. In case of soybean CPE; 70, 65, 26, 22 and 20 kDa proteins were found to have IgE binding potential. Proteins of similar mol wts were stable in SGF. Interestingly, chickpea and soybean proteins that got digested earlier than 30 seconds in SGF did not show any IgE binding, corroborating the hypothesis that stability to SGF digestion may be related to allergenicity. Therefore, immunoblot as well as SGF results when seen together depicted
that pepsin resistant proteins showed IgE binding to known allergens of chickpea and soybean.

Red gram and green gram were selected for further studies on the basis of SGF assay and patients' studies. Six red gram allergic patients were selected having marked positive SPT (with 2+ or more) and elevated specific IgE levels to red gram. All the red gram allergic patients showed elevated specific IgE levels that was more than 2.7 times over negative control. 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, and 17% to lentil, bean fresh and peanut each. 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 inhibitor. Green gram and soybean produced 50% inhibition of specific IgE binding to solid phase red gram extract with 672 and 2200 ng, respectively. The serological responses in BALB/c mice 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 allergic leguminous crop whereas PBS was given as vehicle. On day 15, 43 and 59; similar to peanut, total as well as specific IgE levels in red gram sensitized group was significantly higher ($p < 0.01$) as compared to vehicle treated group. Red gram sensitized group showed significant increase in IgG1 levels ($p < 0.001$) up to 59 days continuously. 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. 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.

Histopathology analysis of lung tissue of red gram sensitized/challenged mice revealed lymphoid infiltration and thickening of alveolar septa throughout the parenchyma. Mild bronchial epithelial hyperplasia was also evident. Spleen from red gram treated group revealed activated macrophages in addition to large sized megakaryocytes and lymphoid hyperplasia. Several infiltrations of inflammatory cells with loss of normal intestine mucosal structure were seen in red gram sensitized/challenged mice in addition to presence of goblet cell hyperplasia in sub mucosal layer.

A significant increase in \( p < 0.001 \) IL-4 and IL-10 (Th2 cytokines) levels was observed in supernatant of cultured splenocytes from red gram primed mice as compared to control mice. There was no difference found in IL-2 levels, a Th1 cytokine, in red gram primed and unprimed splenocyte culture supernatants whereas level of IFN-\( \gamma \) (Th1) was significantly \( p < 0.01 \) decreased in red gram primed splenocyte supernatants. Red gram CPE, also up regulated the levels of Th2 cytokine mRNAs like IL-4, IL-5 and IL-10 \( p < 0.001 \) and did not show any change in IL-2 mRNA level as compared to PBS treated mice both in blood as well as spleen. 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 \( p < 0.001 \) in spleen of red gram treated group. These results suggest that red gram protein has allergenic potential as it up-regulates the expression of Th2 cytokines. The histological changes along with anaphylactic symptoms underpin the modulated cytokines and immunoglobulin levels, proposing towards allergenic potential of red gram.
To detect allergenic proteins in red gram, IgE immunoblotting was carried out using patient’s sera. Pooled patients sera reacted to three red gram proteins of 66, 45 and 30 kDa, that were stable up to 2 min following SGF digestion. These results clearly demonstrate that pepsin stable proteins showed IgE binding. However, IgE specific immunoblotting of two dimensional resolved proteins showed five IgE binding protein spots, one of 66 kDa (approx pl 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). Mass spectrometric analysis of these five IgE binding proteins revealed homology with different subunits of β-conglycinin protein from soybean with significant probability based MOWSE score. The tryptic fragments of 66 kDa protein showed maximum homology with β-conglycinin α chain of soybean. Tryptic fragment of 45a, b and c revealed high sequence similarity with β-conglycinin α prime subunit of soybean. Also, tryptic fragment of 30 kDa protein showed significant homology to β-conglycinin α prime subunit of soybean. Therefore, LC-MS/MS results indicated that the IgE reactive red gram proteins are homologue to different subunits of β-conglycinin of soybean. 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 ordered in silico and subjected to simple FASTA search on allergen online database for sequence homology. Ordered tryptic fragment sequences obtained from CLUSTAL W showed homology with other known allergenic seed storage proteins of different legumes; mainly different subunits of β-conglycinin of soybean, Len c 1.0101 and Len c 1.0102 allergens of lentil, vicillin of garden pea, conglutin β of lupin and Ara h1 of peanut. The scale (low e values) of similarities with known allergens indicates that these immunoreactive red gram proteins may be the allergenic ones as uncovered by immunoblot as well.
In case of green gram, nine patients with marked positive SPT (2+ or more) and elevated specific IgE to green gram extracts were selected. Specific IgE levels in green gram allergic patients were more than 3.02 fold elevated over negative control. All the patients elicited marked positive skin reactions to other legumes as evident by 77% to red gram, 66% to soybean, 55% to chickpea, 33% to Bengal gram, 10% to lentil and bean fresh each. In ELISA inhibition assay; red gram, soybean, chickpea and Bengal gram produced 50% inhibition of specific IgE binding to solid phase green gram extract with 320 ng, 360 ng, 400 ng and 480 ng protein, respectively.

In green sensitized mice, total IgE levels were significantly higher \( (p<0.001) \) as compared to vehicle treated group. Also green gram proteins elicited significantly high \( (p<0.001) \) specific IgE response on day 15, 43 and 59. Pattern of IgE response against green gram and peanut (positive control) was similar and both the groups showed continuous increase in specific IgE levels up to 59 days. Similar to peanut, green gram sensitized group showed significant increase in IgG1 levels \( (p<0.001) \) up to 59 days continuously. In case of IgG2a, a Th1 driven immunoglobulin, green gram sensitized mice showed more than 3 fold decrease on day 15, more than 6 fold decrease on day 43 and more than 5 fold decrease on 59 as compared to vehicle control. Mice sensitized with green gram showed severe anaphylactic symptoms with 60% mortality and marked increase (7 fold) in histamine levels after challenge with CPEs as compared to control animal. Green gram sensitized/challenged mice lung tissue revealed perivascular and peribronchial inflammatory cell infiltrate with milder narrowing of the bronchiolar lumen. Thickening of alveolar septa throughout the parenchyma were also observed. Histopathology of spleen from green gram sensitized/challenged group revealed lymphoid hyperplasia with activated macrophages. Unlike red gram treated mice intestine, where goblet cell
hyperplasia in submucosal layer and mucosal exfoliation were the important features, no histopathological changes were seen in green gram sensitized/challenged group.

A significant increase \((p<0.001)\) in Th2 cytokines IL-4 and IL-10 was observed in splenocytes of green gram primed mice as compared to naive mice, whereas no significant difference was observed in the IL-2 level. IFN-\(\gamma\) levels, a Th1 cytokine was significantly decreased in green gram primed \((p<0.01)\) splenocytes similar to peanut primed \((p<0.001)\) compared to PBS treated mice. Green gram CPE similar to peanut also up regulated the Th2 cytokines like IL-4, IL-5 and IL-10 \((p<0.01)\) at mRNA levels in whole blood and spleen of sensitized and challenged mice as compared to PBS treated mice, however did not show any change in IL-2 mRNA levels as compared to vehicle. Down regulation of Th1 type cytokines IL-12 \((p < 0.001)\) and IFN-\(\gamma\) \((p< 0.01)\) at mRNA level was observed in spleen of green gram treated groups using RT-PCR.

IgE immunoblot of green gram CPE with pooled patient’s sera revealed five IgE binding proteins of 52, 38, 35, 30 and 18 kDa. Interestingly, all of these five proteins were found to be stable up to 2 min following SGF digestion. IgE specific immunoblotting of 2D electrophoresis resolved green gram CPE with pooled patients sera showed six IgE binding protein spots of 52, 50, 38, 35, 30 and 18 kDa. Sera from green gram sensitized mice recognized the four IgE binding proteins of 52 kDa \((\sim \text{pI} 5.65)\), 50 kDa \((\sim \text{pI} 5.79)\), 30 kDa \((\sim \text{pI} 6.58)\) and 18 kDa \((\sim \text{pI} 5.53)\), named as Gg1, Gg2, Gg3 and Gg4, respectively for the sake of convenience and were taken for mass spectrometry analysis. On the basis of significantly high MOWSE score, LC/MS-MS analysis revealed Gg1, Gg2 and Gg3 protein spots as 8S globulin \(\beta\) isoform precursor, 8S globulin \(\alpha\) isoform precursor and seed albumin of green gram, respectively.
Summary and conclusion

To assess whether these IgE binding proteins were having any similarity to other allergens, their ordered amino acid sequences were subjected to over all FASTA search on allergen Online database. Upon full length FASTA search, 8S globulin β isoform precursor (Gg1) and 8S globulin α isoform precursor (Gg2) showed homology to known allergenic proteins like β-conglycinin-α subunit and β-conglycinin storage protein of soybean, allergen Len c 1.0101 and Len c 1.0102 of lentil, vicilin of pea, Ara h1 of peanut and conglutin β of lupin. Ordered fragments of seed albumin (Gg3) showed sequence homology to seed storage protein / 13 S globulin of buckwheat and Bet v 5 of birch pollen allergen. Gg1 and Gg2 allergens belong to cupin super family and contain two cupin conserved domains. These results suggest that red gram as well as green gram contain allergenic proteins that are capable of inducing IgE-mediated reactions in respective legumes sensitized patients as well as in BALB/c mice.

To sum up, all the selected legumes namely; chickpea, green gram, red gram, black gram, kidney bean and Bengal gram have one or more proteins stable to in vitro pepsin digestion. Identification of non-digestible protein may probably be useful in predication of allergenic response and needs further evaluation. Our findings suggest that legumes seem to be the potent sensitizing agent by virtue of being the major source of food protein for majority of population. Concurrent sensitization among different legumes as well as between legumes to other aeroallergens was found in patients. Red gram and green gram were taken for further study as allergens from these two crops are still unidentified unlike chickpea. Red gram contains at least five allergens and green gram having four allergens that may induce IgE-mediated reactions in sensitized patients as well as in BALB/c mice indicating their allergenic potential. Mass spectrometry and
bioinformatics analysis of all the IgE binding proteins of red gram and green gram revealed their sequence similarity with known allergens, confirming our clinical findings. The present effort needs to be followed up by a more extensive study to identify the remaining allergens, if any. Since, the identification of new food allergens is the first step towards future diagnostic and therapeutic approaches to deal effectively with food allergy, therefore, further studies are needed to explore the full sequences of these allergenic proteins. This will also go a long way in producing new transgenic varieties of these legumes.
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