Summary and conclusions
Summary and conclusions

Food allergy is an important public health problem worldwide, affecting 6-8% children and 2-4% adults in general population (Roehr et al., 2004; Sampson, 2004; Zuberbier et al., 2004; Osterballe et al., 2005; Han et al., 2012). Most of the allergic reactions in pediatric population are caused by milk, egg, peanut, fish, and tree nuts while peanut, tree nuts, fish, and shellfish are the major offenders in adults (Sampson, 1999; Cucu et al., 2012). Prevalence and sensitization pattern of food allergy vary largely across different countries. This variation may occur partly due to interaction of genetic factors, cultural and dietary habits and/or different methodological designs of the studies (Dalal et al., 2002; Crespo and Rodriguez, 2003). Although any food may provoke a reaction, relatively few foods are responsible for the vast majority of food allergic reactions. Allergic reactions to food occur typically in individuals either previously sensitized to the allergen or in genetically pre-disposed condition (Sicherer, 2000). As legumes are the major source of dietary ingredients throughout the world, the chances of allergic reaction to legumes are more in the atopic population (Sicherer and Sampson, 2009). Food allergy is frequently associated with atopic dermatitis, asthma and allergic rhinitis and is one of the most common triggers of potentially fatal anaphylaxis (Chhabra et al., 1998; Anonymous 2000; Paramesh, 2002; Gaur et al., 2004).

Sensitization to food allergens can occur in the gastrointestinal tract (class I food allergy) or as a consequence of cross-reactivity to structurally homologous inhalant allergens (class II food allergy). Allergenicity of food is largely determined by structural aspects, including cross-reactivity and reduced/enhanced allergenicity with cooking that may convey allergenic characteristics to food (Han et al., 2012). The different legumes have structurally homologous proteins, but they are not all equally allergenic, thus making it difficult to distinguish between their in vitro and in vivo cross-reactivity. Cross-reactivity among lentil, chick-pea, pea and peanut has been demonstrated by various techniques (Ibáñez et al., 2003). However, additional studies are required in different populations which could help in selecting an appropriate panel of allergens for diagnosis.

Presently, the whole mass allergen extracts are used for diagnosis of food allergy which contains several IgE binding components. Several drawbacks such as batch to batch
variability and lack of specificity have been associated with the use of crude extracts for diagnosis. Therefore, the purified allergenic proteins are required for component resolved diagnosis of allergy. Researchers abroad have isolated and characterized allergenic proteins from various food sources such as peanut, milk, soybean, lentil, cowpea, wheat etc (Burks et al., 1991, 1992; Rabjohn et al., 1999) but there is a scarcity of similar studies from India.

India represents about one-sixth of the world population with diverse culture and dietary habits but little is known about the prevalence of food allergy in this subcontinent. The knowledge about food hypersensitivity is limited to a few clinico-immunological studies. Preliminary studies based on SPTs have reported that eggs, milk, cereals (corn, barley) and legumes such as pea and chickpea are major sensitizers (Parihar et al., 1984; Deshmukh 1991; Desai et al., 1991; Kumar et al., 2000). Anecdotal reports on fish allergy are also available. Milk, rice, banana, colocasia, refined flour, radish and citrus fruits are known to induce respiratory symptoms in children upon open (oral) challenges (Sharman et al., 2000). A study from Mumbai reported chickpea as an important allergen in the Indian subcontinent showing prevalence of 2.7% in atopic population (Patil et al., 2001). Most of the studies conducted in the country have included few selected patient populations or lack information on in vitro and in vivo testing of the food allergens. Besides, these may not present as representative food allergens for the entire population of our country with great cultural and dietary diversity. Hence, there is a need to conduct systematic studies on food allergy to identify and characterize the most common offending food allergens in individuals with respiratory allergy.

The present study was therefore undertaken to achieve the following objectives:

Objectives:

1. To assess sensitization with common legumes (foods) in atopic Indian population.

2. To identify IgE binding components in legume extracts using hypersensitive patients’ sera.
3. To investigate effect of heat (food processing) and digestion on stability of allergenic proteins in legumes.

4. To identify allergic cross-reactivity among important legumes and other foods.

5. To purify and characterize a major allergenic protein from most common legume allergen source.

Study on the prevalence of IgE mediated food allergy to legumes in bronchial asthma and allergic rhinitis patients.

A total of 355 patients with mean age of 30.7±13.9 and history of legume allergy from the two centres (Bangalore Allergy Centre, Bangalore (n=198) and US Lavasa Medical and Research Centre, Chandigarh (n=157)) of India, were recruited for the present study. The patients and the control subjects were skin prick tested with common foods and aeroallergens which included 10 selected most common legumes (kidney bean, chick pea, peanut, pigeon pea, black gram, green gram, soybean, pea, lentil and cow pea). Specific IgE and total IgE levels in the sera samples were determined by indirect and sandwich ELISA, respectively.

Highlights:

- Of the total patients recruited, 279 (78.5%) patients were suffering from allergic rhinitis, 11 (3.5%) with asthma and 65 (18.0%) were suffering from both. The patients and controls included in the study were age and sex matched.

- SPT with food and aeroallergens was performed on 355 patients with history of food allergy. Of these, 208 (58.0%) patients exhibited marked positive reactions to one or more legume(s).

- The prevalence of sensitization (SPT +ve) varied from 101 (49.0%) at Bangalore to 107 (68.0%) at Chandigarh.
Summary and conclusions

Kidney bean was found to be the top sensitizer in 78 patients (22.0%) followed by chickpea 65 (18.0%), peanut 53 (15.0%) and <12.0% of patients were sensitive to other legumes.

Blood was collected from 208 patients on the basis of history of food allergy and SPT positivity to the respective legume(s).

The sera samples of these 208 patients were tested against respective legume for specific and total IgE levels.

Most of SPT positive patients showed elevated specific and total IgE levels. The OD values for specific IgE in patients ranged from 0.178-3.166 IU/ml while the total IgE values ranged from 88-2175 IU/ml among different legumes as compared to normal controls.

Specific IgE estimation showed sensitization to kidney bean in maximum 70 (89.7%) patients followed by lentil 3 (42.9%), soybean 14 (41.2%), pigeon pea 16 (39.0%) and black gram 15 (38.4%).

Intensity of SPT reaction correlated significantly with specific IgE (r=0.85, p<0.0001) but not with the total IgE levels (r=-0.16, p=0.3522).

Characterization of major sensitizing legume allergen and its cross-reactivity with different legumes.

Kidney bean was found to be the most prevalent sensitizer in the study population both by SPT and ELISA. The present study was further undertaken to identify important IgE binding components of kidney bean and their characterization by various immunobiochemical methods. The cross-reactivity of kidney bean with other common legumes involved in allergic reactions was also assessed.

Highlights:

- IgE binding components of kidney bean extract were identified by immunoblot
Summary and conclusions

using kidney bean hypersensitive pooled patients’ sera as well as individual patients’ sera. Immunoblotting with pooled patients’ sera exhibited 15 IgE-binding proteins of 120, 95, 70, 58, 55, 50, 45, 42, 40, 37, 34, 26, 24, 18 and 16 kDa with raw kidney bean extract. Further, individual patients’ sera recognized 8 most prevalent (major) allergens of 58, 50, 45, 42, 40, 37, 34 and 18 kDa.

- As the kidney bean is consumed after boiling, the IgE binding of its different boiled forms was also investigated.

- Five IgE binding protein bands corresponding to 58, 55, 37, 24 and 18 kDa were identified using extract prepared by boiling kidney bean for 15 min at 121°C. The kidney bean boiled for 15, 30, 45 and 60 at 100°C showed 7, 6, 5, and 3 allergenic protein bands, respectively.

- The stripped basophil histamine release assay demonstrated biological activity of kidney bean allergens. Passively sensitized basophils stimulated with kidney bean extract released significant histamine in the range of 16-54% in patients (n=30) as compared to controls (n=5).

- Histamine release correlated significantly with SPT (r=0.83, p<0.0001) and specific IgE (r=0.99, p<0.0001) but did not correlate statistically with total serum IgE levels (r=0.13, p=0.4942).

- To assess the cross-reactivity of kidney bean with other legumes, ELISA and immunoblot inhibition was performed using different legume extracts as inhibitors.

- Kidney bean showed cross-reactivity with peanut, black gram, chick pea and pigeon pea.

- For the identification of major IgE binding proteins of kidney bean mass spectrometric analysis was performed.

- Nano LC-MS/MS identified reactive proteins as Alpha-amylase inhibitor precursor, erythroagglutinating phytohemagglutinin, phaseolin and group 3 late embryogenesis abundant protein.
Effect of heat and γ-irradiation on the allergenicity of legume proteins

Food processing is the transformation of raw ingredients of food into other consumable forms. We aimed to investigate the effect of food processing methods on the allergenicity of kidney bean, black gram and peanut. The extracts were subjected to different processing methods and changes in soluble protein content, specific IgE binding, allergenic potential and digestibility of legume proteins were assessed.

Highlights:

- Three different food processing strategies (boiling, γ-irradiation and combination of boiling + γ-irradiation) were followed and their effects on the legumes were evaluated.

- Boiling (121°C for 15 min) led to a 3 to 4 fold reduction (p<0.05) in the soluble protein content of the three legumes. However, γ-irradiation alone did not produce any significant change in the solubility of proteins.

- Significant reductions (p<0.01) in IgE binding to boiled legumes were observed among individual patients as compared to IgE binding to their respective raw antigens. Reduction of 61-83% IgE binding in kidney bean, 60-90% in black gram and 54-78% in peanut extract was observed.

- The IgE binding was significantly reduced (p<0.05) further when combination of boiling and γ-irradiation treatment was used for processing as compared to boiling alone.

- Aggregated (insoluble) protein formed after boiling was converted into soluble form after re-suspending it in urea/thiourea buffer to assess its allergenicity.

- The IgE binding to insoluble boiled protein fraction was reduced to 27-46% in kidney bean (p>0.05), 41-95% in black gram (p=0.001) and 28-63% in peanut (p<0.01), among the 10 individuals as compared to IgE binding to their respective raw antigens.
Summary and conclusions

• The allergenic potential of kidney bean, black gram and peanut was found to reduce significantly (p<0.001) after boiling alone or in combination with irradiation, in both soluble and insoluble fractions of protein as their IC50 values showed significant decrease when compared to their respective unprocessed antigen extracts.

• Thermal processing of all three legumes showed decrease in number of protein bands on SDS-PAGE as well as in the number of IgE binding proteins on immunoblot. This reduction was marked when combination processing was employed.

• Processing by boiling decreased the digestibility of three selected legumes whereas combination of boiling followed by γ-irradiation increased the digestibility of legume proteins in SGF.

• Significant reduction in allergenic potential of study legumes was observed after thermal processing alone or in combination with γ-irradiation. Hence, it could be concluded that both these methods can be employed for preparing hypoallergenic foods for sensitive individuals.

Production of hydrolysates of legume proteins (kidney bean, black gram and peanut) with reduced antigenic activity.

Enzymatic hydrolysis and further processing are commonly used to produce hypoallergenic products. We demonstrated the effect of hydrolysis on kidney bean, black gram and peanut by sequential action of an endoprotease (alcalase) and an exoprotease (flavourzyme). Immunoreactivity to raw and hydrolyzed legume extracts was evaluated in vivo by SPT, ex vivo by histamine release assay and in vitro by indirect ELISA, IgE immunoblotting and inhibition assay using hypersensitive patients’ sera of kidney bean, black gram and peanut.
Highlights:

- The two enzymes hydrolyzed kidney bean, black gram and peanut extracts and the DH of 48.4, 63.5 and 69.4%, respectively, was observed after 8 h of hydrolysis.

- The IgE binding in ELISA decreased with the increase in DH of the three extracts after hydrolysis and a significant inverse correlation was observed between DH and IgE binding in ELISA (p<0.05).

- ELISA with individual patient's sera showed a significant reduction of 58-79%, 61-97% and 72-93% IgE binding in hydrolyzed extracts (8 h) of kidney bean, black gram and peanut or compared to the raw legume extracts.

- No IgE binding proteins were observed on immunoblot after 8 h of hydrolysis in the three legumes.

- Significant reduction in the release of histamine from basophils was observed with legume extracts hydrolyzed for 8 h as compared to the raw extracts.

- There was complete inhibition of allergenic potency of the three extracts after 8 h of hydrolysis since none of the hydrolysate (10 μg) after 8 h could even inhibit 50% IgE binding to respective raw legume as determined by ELISA.

- Significant reduction in the SPT positivity was observed with hydrolysates of legumes when SPT results were compared to their raw extracts.

- Results indicated that sequential hydrolysis of legumes resulted in significant proteolytic destruction of IgE-binding epitopes as shown by in vitro, ex vivo and in vivo experiments.

- In conclusion, enzymatic hydrolysis could be a promising method for preparing easily digestible hypoallergenic foods to reduce the risk of food allergy to legumes in sensitive patients.
Purification and partial characterization of a major allergen from kidney bean: an important legume allergen.

Purified major allergens are required for component based diagnosis and therapy of allergy or may be used for standardization of extracts. In the present study, an allergenic protein from kidney bean was isolated using a combination of anion exchange, gel filtration and reverse-phase hydrophobic chromatography.

Highlights:

- The purified protein resolved as a single band at 31 kDa on SDS-PAGE and showed IgE binding (OD 0.390 - 1.137) to 88% patients' sera by ELISA and immunoblotting indicating it to be a major allergen.

- The potency of the purified protein was assessed by ELISA inhibition. A dose dependent inhibition was observed and its IC$_{50}$ was found to be 102 ng. A maximum inhibition of 98% was achieved with 1 µg of purified protein as inhibitor.

- A maximum inhibition of 61% was observed in IgE binding to crude kidney bean extract (solid phase) when 1 µg of 31 kDa protein was used as an inhibitor with IC$_{50}$ of 976 ng.

- Clinical relevance of purified allergenic protein was evaluated by SPT. Twenty five respiratory allergy patients with history of food allergy were skin prick tested and 14 showed marked positive skin reactions with kidney bean extract. Purified protein recognized 11/14 (78.5%) of kidney bean hypersensitive patients by SPTs.

- Significant release of histamine in the range of 17-48% from sensitized basophils was observed after challenge with purified protein.

- Digestibility of the purified protein was assessed by simulated gastric fluid (SGF). The 31 kDa protein remained stable upto 1 h after incubation with pepsin.

- The purified protein is a glycoprotein as detected by PAS staining. No change in the IgE-binding property was observed on meta-periodate treatment thus ruling
out any possibility of non specific binding due to presence of a carbohydrate moiety.

- The 31 kDa protein was identified as phytohemagglutinin after mass spectrometric analysis. The hemagglutination activity of the purified protein was found to be comparable to the commercially available PHA.

- The 31 kDa protein showed cross-reactivity with peanut and black gram with IC₅₀ of 185 and 228 ng, respectively, when used as inhibitor for inhibition of IgE binding to solid phase 31 kDa protein in ELISA.

- The 31 kDa protein is a potent major cross-reactive allergen that may have implication in better diagnosis and immunotherapy of allergic disorders.