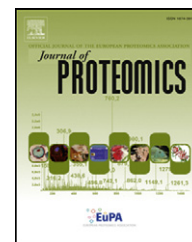


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Phytohemagglutinins augment red kidney bean (*Phaseolus vulgaris* L.) induced allergic manifestations[☆]

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ARTICLE INFO

Keywords:

Food allergy

Lectins

IgE-immunoblotting

Cytokines

Transcription factors

ABSTRACT

Red kidney bean (*Phaseolus vulgaris* L.), a commonly consumed bean has been reported to induce allergic reactions in susceptible individuals. Phytohemagglutinins (PHAs, mainly PHA-P) contribute a major proportion of red kidney bean seeds. However, their roles in red kidney bean induced allergic reactions are still to be explored. This study was carried out to understand the role of PHAs in allergic manifestations using BALB/c mice and cultures of splenocyte, RBL-2H3 cells as well as bone marrow mast cells (BMMCs). Also, the characterization of allergic components from PHA-P was studied by LC-MS/MS. Enhanced levels of specific IgE and IgG1, clinical scores, cytokines and chemokines, β -hexosaminidase, histamine, cysteinyl leukotriene, prostaglandin D₂ and abrupt histological changes in the intestine, lung and spleen indicated a pivotal role of PHA-P in red kidney bean allergy. Further, LC-MS/MS study revealed two IgE binding components of PHA-P as PHA-L and PHA-E. Enhanced specific IgE/IgG1 and β -hexosaminidase level elucidated the possible role of PHA-L and PHA-E in allergic manifestations. Furthermore, in the presence of IgE inhibitor piceatannol, reduced β -hexosaminidase release to some extent was noticed. The up regulated expression of GATA-3 and T-bet expression was observed in PHA-L as well as PHA-E groups. Taken together, this study revealed the fact that allergenicity potential of red kidney bean may get augmented due to the presence of different phytohemagglutinins.

Biological significance

Although food allergy is an immune provocation induced mainly by dietary allergenic protein components of the food, the role of dietary lectins in the food induced allergic manifestations cannot be ruled out. Here we provide the systematic evidences about the allergenic potential of PHAs and further disclosed the culprit components as PHA-L and PHA-E. It is an important finding that the PHA-L and PHA-E can cause allergic manifestations via not only the IgE mediated pathway but also the non-IgE mediated allergic reactions as evident by the Th1/Th2 cytokines and transcription factors. Further, the PHA-L seems to be more allergenic than the PHA-E. This article is part of a Special Issue entitled: Translational plant proteomics.

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<http://dx.doi.org/10.1016/j.jprot.2013.02.003>

1. Introduction

Legume allergy is contributing a major proportion in food allergy prevalence [1]. Most of the legume allergies are mediated through immediate type hypersensitivity and demonstrated a quick onset of allergic symptoms after consuming the allergic foods in susceptible individuals [2]. Allergenicity of several legumes including peanut, soybeans, red gram, chickpea, black gram, green gram, lentils, red kidney beans, fenugreek and green bean has been well studied [3]. Red kidney bean (*Phaseolus vulgaris*) is a commonly consumed bean, which is reported to induce allergic manifestations in susceptible individuals [4,5]. A large fraction of red kidney bean seed protein is represented by its storage protein phaseolin and lectin-related protein family [6–8]. Proteomic studies of common bean seeds by two dimensional electrophoresis and mass spectrometry (MALDI-TOF MS and MALDI-TOF/TOF) suggest that most identified proteins were phaseolin, phytohemagglutinin (PHA-P) and lectin-related α -amylase inhibitor [9]. The lectin-related protein family consists of three major components arcelin, α -amylase inhibitor, and the true lectin, phytohemagglutinins (PHAs, mainly PHA-P). Both PHA-P and α -amylase inhibitor are responsible for the lowering of the nutritional value of beans [10]. Leucoagglutinating phytohemagglutinin (PHA-L) and erythroagglutinating phytohemagglutinin (PHA-E) are two major subunits of PHA-P [7]. Several studies regarding PHAs have been carried out to reveal blood grouping, erythrocyte polyagglutination activity, mitogenic stimulation of lymphocytes, lymphocyte subpopulation studies, fractionation of cells and other particles, histochemical studies of normal and pathological conditions [10,11]. Initially, it was reported that low-dose intragastric administration of PHA does not induce IgE production in Sprague–Dawley rats [12]. Recently, based on a case of severe anaphylaxis to kidney bean it was found that phaseolin and PHA can act as putative allergens [13]. But, still the role of PHAs (PHA-P, PHA-L and PHA-E) in red kidney bean allergy has not been resolved completely.

Therefore, in this study an attempt has been made to explore the possible involvement of PHAs in red kidney bean induced allergenicity. Female BALB/c mice were used to evaluate immunoglobulin levels, anaphylactic scores, histopathological changes and immunohistochemical analysis. Splenocyte culture was carried out to elucidate cytokine and chemokine levels. RBL-2H3 cells were used to study mediator release assay. Characterization of IgE binding components of PHA-P was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. This study was further extended to evaluate the comparative allergenicity of purified PHA-L and PHA-E using BALB/c mice, splenocytes, RBL-2H3 cells, and bone marrow mast cells (BMMCs).

2. Materials and methods

2.1. Preparation of crude protein extract from RKB

Red kidney bean crude protein extract (RKB) was prepared as the earlier described method [14].

2.2. Assay of hemagglutinating activity

Hemagglutinating activities of RKB, autoclaved RKB (RKB-A) and PHA-P (Sigma Aldrich, USA) were studied according to the earlier described protocol [15].

2.3. BALB/c mice

Healthy 6–8 week old female BALB/c mice (22 ± 3 g) were obtained from the animal breeding colony of Gehru campus, CSIR-IITR, Lucknow, India. Animals were maintained under standard laboratory conditions in pathogen free environment and on red kidney bean free diet. Animal studies were carried out after approval (Ref: ITRC/IAEC/07/2008) of the institutional animal ethics committee.

2.4. Sensitization protocol

Female BALB/c mice were sensitized intraperitoneally according to the earlier described protocol [16]. In brief, mice were randomly divided into four groups ($n=15/\text{group}$). The first group of mice (designated as Control group) was injected 100 μL PBS. The second group of mice (designated as PHA-P group) was treated with 100 μg PHA-P in 100 μL PBS. The third group of mice (designated as RKB-A group) was treated with 100 μg RKB-A in 100 μL PBS. The fourth group of mice (designated as RKB group) was treated with 100 μg RKB in 100 μL PBS. Mice from each group received aforesaid doses once a week up to 7 weeks. Blood was withdrawn from the retro-orbital sinus on days 15, 43 and 59 and serum was collected. Mice ($n=10/\text{group}$) from each group were challenged intraperitoneally with 10 mg proteins of their respective group on day 60. Tissue samples from organs like lung, spleen and intestine were collected for histopathological studies.

2.5. Specific IgE and IgG1 level estimation

Specific IgE and IgG1 were estimated with the earlier described protocol with slight modifications (Supplementary material, section 1.1).

2.6. Splenocyte culture and cytokine/chemokine levels

Splenocyte culture was carried out according to the method described earlier (Supplementary material, section 1.2).

2.7. Systemic anaphylaxis score and core body temperature

Mice from PHA-P, RKB-A and RKB groups were challenged on day 60th and after 40 min, systemic anaphylaxis symptoms, rectal temperature and histopathological studies were carried out as the earlier described methods [17].

2.8. Mouse mast cell protease-1 and thymic stromal lymphopoietin levels

Serum mouse mast cell protease-1 (mMCPT-1) and thymic stromal lymphopoietin (TSLP) levels in the sera of control, PHA-P, RKB-A and RKB group were quantified by ELISA (eBioscience, Inc. CA, USA) according to the manufacturers' instructions.

2.9. Eosinophil levels

Detection of eosinophils in the lung and spleen was carried out using immunohistochemistry (IHC) according to methods as described earlier with slight modifications (Supplementary material, section 1.3).

2.10. Mediator release assays in RBL-2H3 cells

The release of β -hexosaminidase following PBS, PHA-P, RKB-A and RKB exposure was studied in RBL-2H3 cells (ATCC, Manassas, VA) according to the earlier described protocol [18,19].

2.11. Two dimensional separation and IgE-immunoblotting

Two-dimensional (2D) gel electrophoresis of RKB-CPE followed by IgE immunoblot with pooled sera of PHA-P treated mice was carried out according to the method described earlier [20]. Briefly, RKB-CPE (100 μ g) in rehydration buffer (containing 7 M Urea, 2 M Thiourea, 2% CHAPS and 50 mM DTT, 0.0002% Bromophenol blue) was resolved over linear pH 4–7 immobilized pH gradient (IPG) strip (GE Healthcare Life Sciences, USA) focused up to 20,000 VhT, max. voltage of 4500 V, at 20 °C using Ettan IPGPhor 3 (GE Healthcare Life Sciences, USA). Further, the strips were run over 12% SDS–PAGE gels using a mini gel system (Amersham Biosciences, St Francisco, USA). Out of two identical gels, the first one was stained with G-250 dye while another one was blotted on PVDF membrane. The pooled sera of PHA-P sensitized mice (dilution 1:10) were used as primary antibody while goat anti-mouse IgE HRP-conjugated antibody (dilution 1:1000; Sigma Chemical Co., St. Louis, USA) was used as secondary antibody. The blot was developed by a chemiluminescence reagent (Thermo Fisher Scientific, USA) in a gel documentation system (Syngene, Cambridge, UK).

2.12. LC-MS/MS of IgE binding proteins

We further intended to characterize the IgE binding protein components using LC-MS/MS as per the earlier described methods [16,20]. The G-250-stained gel and IgE immunoblot images were matched by the 2D Platinum ImageMaster™ 6.0 (GE Healthcare Life Sciences). Two spots showing IgE binding capacity were excised from G-250 stained gel. The LC-MS/MS studies were carried out at The Centre for Genomic Applications (TCGA), New Delhi, India. In brief, the gel slices were cut into small pieces of approximately 1 mm³ and destained. The tryptic in-gel digestion was performed according to the earlier described method [21]. Further, 6 μ l of the supernatant was injected into Thermofinnigan LCQ DECA XP (Thermo Fisher Scientific, Waltham, MA, USA) equipped with capLC pump (Waters). The separation of the tryptic peptides was carried out using a gradient formed between 0.1% (v/v) formic acid in MilliQ water (solvent A) and 0.1% (v/v) formic acid/acetonitrile (20/80) [solvent B] on dionex C18 column (Dionex Corp, Sunnyvale, CA). Individual MS/MS spectra of tryptic fragments were searched against mass spectrometry protein sequence data base (MSDB) using the MASCOT search engine as per the method described earlier [22]. Mass tolerance and monoisotopic values (peptide mass tolerance of ± 1.2 Da and fragment mass tolerance of

± 0.6 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 $-10 \times \log(P)$, where P is the probability and observed match was considered as a random event.

2.13. Bioinformatic analysis for allergenicity prediction

Allergen online and AlgPred tools were used for the allergenicity prediction of the characterized proteins (Supplementary material, section 1.4).

2.14. Animal sensitization with PHA-L and PHA-E

The mice were randomly divided into four groups (n=15/group) viz, control, PHA-E, PHA-L and PHA-P, sensitized intraperitoneally and immunoglobulin levels and anaphylactic reactions were estimated according to the methods described in Sections 2.4, 2.5 and 2.7.

2.15. Cross reactivities among PHA-E, PHA-L and PHA-P

The Cross reactivities among PHA-E, PHA-L and PHA-P were studied by specific IgE ELISA inhibition following the earlier described method [23].

2.16. IgE-dot blot of PHA-L and PHA-E

To detect IgE binding ability of PHA-L and PHA-E, IgE dot blot was carried out (Supplementary material, section 1.5).

2.17. Bone marrow mast cell (BMMC) culture

BMMCs were cultured according to the previously described methods [24].

2.18. Release assay in the presence of IgE inhibitor

The β -hexosaminidase release assay was studied after exposure of 100 μ g of PHA-L and PHA-E to RBL-2H3 cells (ATCC, Manassas, VA) and BMMCs in the absence and presence of 12.5 μ g IgE inhibitor, piceatannol (Cayman Chemical Company) according to the method described in Section 2.10.

2.19. GATA-3 and T-bet expressions in PHA-L and PHA-E treated BMMCs

The expressions of Th1/Th2 transcription factors GATA-3 and T-bet were observed by immunofluorescence method (Supporting material, section 1.6).

2.20. Statistical analysis

The statistical significance of the data obtained was determined using a software package from InStat version 3.0 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>) using the Bonferroni analysis of variance (ANOVAs) test. Values for all measurements have been expressed as mean $\pm$ SEM. Differences between groups were considered significant when $p < 0.05$.

3. Results and discussion

Food allergy especially legume allergy research has picked up momentum in recent years. The allergic potential of several leguminous foods including peanut, soybean, lupin, lentil, chickpea, red gram and green gram has been explored [3]. The presence of lectins in foodstuffs is an important health concern which may induce acute toxicity and its chronic exposure leads to harmful consequences to human health [25]. These dietary lectins are protein that bind to surface glycoproteins (or glycolipids) on erythrocytes or lymphocyte membranes and may function as allergens as well as hemagglutinating agents [26]. Considering the above facts, this study was initiated to explore the allergenic potential of PHAs using *in vivo*, *ex vivo* and *in vitro* approaches.

3.1. Specific hemagglutination activity

Quantitative estimation of PHAs and its activity in RKB, RKBA-A and PHA-P was the first parameters that were done in this study. Hemagglutination activity is defined as the lowest sample dilution that showed hemagglutination [27]. The specific hemagglutination activity of RKB, RKB-A and PHA-P was 204, 0.2 and 204 titer/mg, respectively (Fig. 1A and B). Many substances attach to molecules present on the surface of RBCs and because of this, at certain concentrations, the substance may bind together or agglutinate the RBCs, which resulted in preventing them from settling out of suspension. The outcome

of this study reveals the fact that PHAs are present in a significant amount in RKB while negligible in the RKB-A. Therefore, while assessing the allergenic potential of RKB, role of PHAs cannot be ignored.

3.2. Immunoglobulin levels in PHA-P sensitized animals

We further extended our study in female BALB/c mice to quantitate specific IgE and IgG1 levels induced by PHA-P treatment. The level of specific IgE was found significantly ($p < 0.001$) enhanced in RKB, RKB-A and PHA-P, respectively when compared to that of control on day 15th, 43rd and 59th (Fig. 1C). The presence of enhanced specific IgE in serum of PHA-P treated mice sera indicated the probability of PHAs induced allergenicity. The level of specific IgE has been reported as one of the most pivotal diagnostic tools to unravel allergy from any food [2,16,28]. The level of specific IgG1 was found significantly ($p < 0.001$) enhanced in RKB, RKB-A and PHA-P treated groups in comparison to control (Fig. 1D). Specific IgE/IgG1 has been reported to be enhanced in food allergic manifestations [29]. The enhanced specific IgE and IgG1 levels in PHA-P treated group suggested the possibility of PHAs induced allergic reactions.

3.3. Cytokine and chemokine levels in splenocyte culture supernatants

Cytokines are key players in the determination of fate of allergic reactions [30]. We have quantitated various interleukins in the

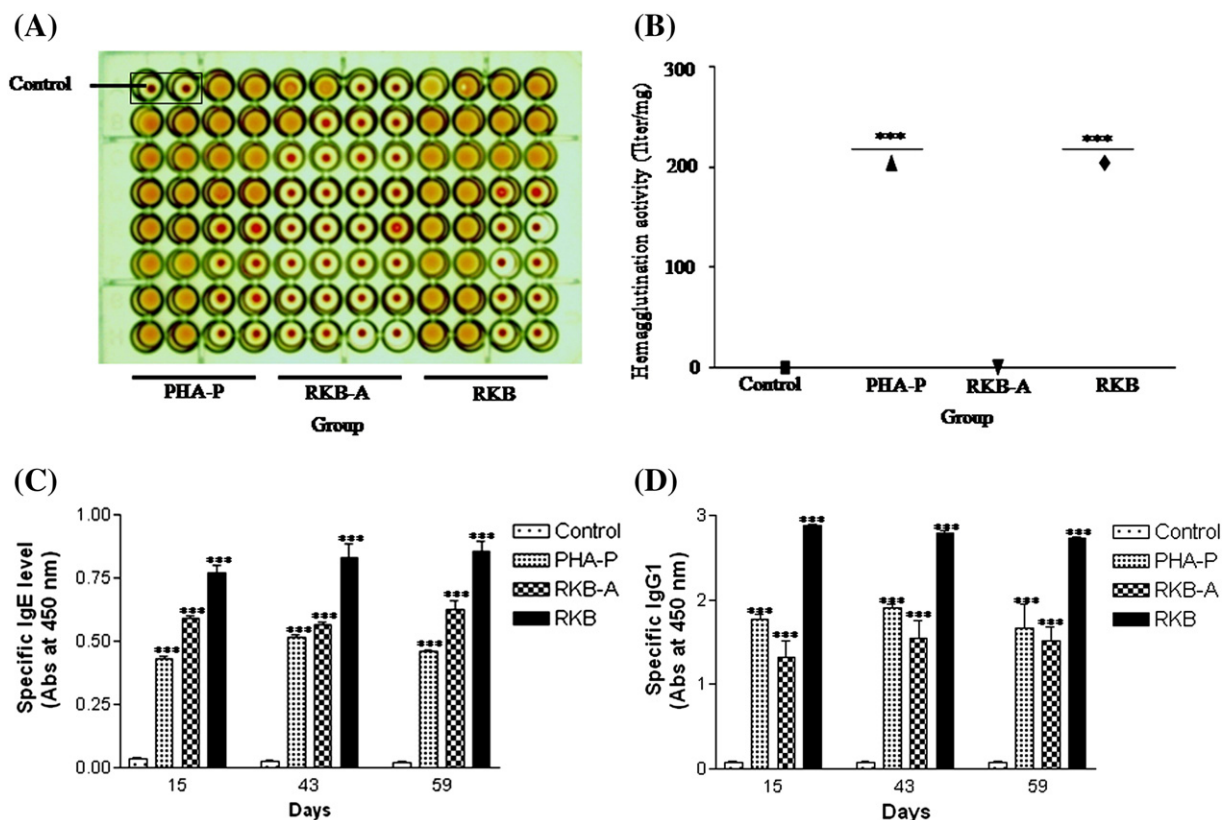


Fig. 1 – Hemagglutination activity and specific immunoglobulins (A) Hemagglutinin titer and (B) hemagglutination activity of PHA-P, RKB-A and RKB using rabbit blood. (C) Specific IgE and (D) specific IgG1 levels on day 15th, 43rd and 59th in control, PHA-P, RKB-A and RKB treated mice. Data represented in mean $\pm$ SE; $n = 3$; (*) $p < 0.001$) when compared with control.**

Please cite this article as: Kumar S, et al, Phytohemagglutinins augment red kidney bean (*Phaseolus vulgaris* L.) induced allergic manifestations, J Prot (2013), <http://dx.doi.org/10.1016/j.jprot.2013.02.003>

supernatants of splenocytes culture to analyze their possible role in allergic manifestations. The PHA-P groups (PHA-P U and PHA-P T) showed significant increase in IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF- α , IFN- γ , RANTES, and CCL-2 levels over the control group (Fig. 2A–M). Pattern of Th1 as well as Th2 cytokine increments indicated the prevalence of mixed allergic reactions as reported in several leguminous food allergies [31]. Further, along with Th1 and Th2 cytokines, a higher level of IL-10, IL-12 and IL-17 was also documented in PHA-P treated group. The levels of chemokines, CCL-2 and RANTES were enhanced as well. The CCL-2 and RANTES have been reported to be enhanced during allergic reactions [32,33]. It has been reported that dietary lectins can induce *in vitro* release of IL-4 and IL-13 from human basophils and also activate T-cells in different cell lines [34–36]. The mixed expression of Th1/Th2 and other cytokines suggested an IgE as well as non IgE mediated or mixed allergic reactions by PHA-P treatment.

3.4. Anaphylaxis symptoms in PHA-P sensitized animals

The mice from PHA-P treated group exhibited symptoms of systemic anaphylaxis 40 min after challenge. The systemic anaphylaxis score has been reported as one of the most vital parameters during assessment of food allergy [17]. Mice in the PHA-P treated and challenged group exhibited scratching and rubbing around the head and snout (score 1) in 10% mice; pilar erection, puffiness around the eyes and snout, reduced activity or standing still, increased respiratory rate and diarrhea (score 2) in 20% mice; symptoms of score 1 and score 2 along with labored respiration (score 3) were shown in 30%; near fatal reactions such as loss of consciousness or little activity despite

gentle prodding (score 4) were evident by 30% mice. Mortality (score 5) was noted in 10% mice (Fig. 3A). In this study, 8 out of 10, PHA-P treated and challenged mice showed 3 to 4 °C decrease in core body temperature (Fig. 3B). Enhanced allergic symptoms with decreased core body temperature reflect the severity of food induced systemic anaphylactic reactions [37].

3.5. Histamine, TSLP and mMCPT-1 levels in PHA-P sensitized animals

Most of the food allergic manifestations have been reported to be mediated via release of allergic mediators like histamine, PGD₂ and leukotrienes [2,38]. A 2 fold increase in the plasma histamine level was observed in PHA-P treated mice when compared to control (Fig. 3C). The TSLP has been reported to play a pivotal role in the induction of allergic reactions in several food allergies [39]. Further, TSLP is required for allergic inflammation but not primary sensitization or tolerance to food proteins in the gastrointestinal tract and it was also reported that TSLP amplifies Th2 responses [40]. In our result, PHA-P sensitized mice showed 2.5 fold increases in the concentration of TSLP in comparison to control (Fig. 3D). The level of mMCPT-1 is an indicator of an increase of mucosal mast cell degranulation [31,41]. More than 2 fold enhancement in mMCPT-1 was observed in the serum of mice treated with PHA-P when compared to control (Fig. 3E). Such enhanced levels of mMCPT-1 are very frequently observed in food allergic manifestations [42]. Taken together, the enhanced level of histamine, TSLP, PGD₂ and mMCPT-1 further validated the possibility of PHA-P induced allergic manifestations.

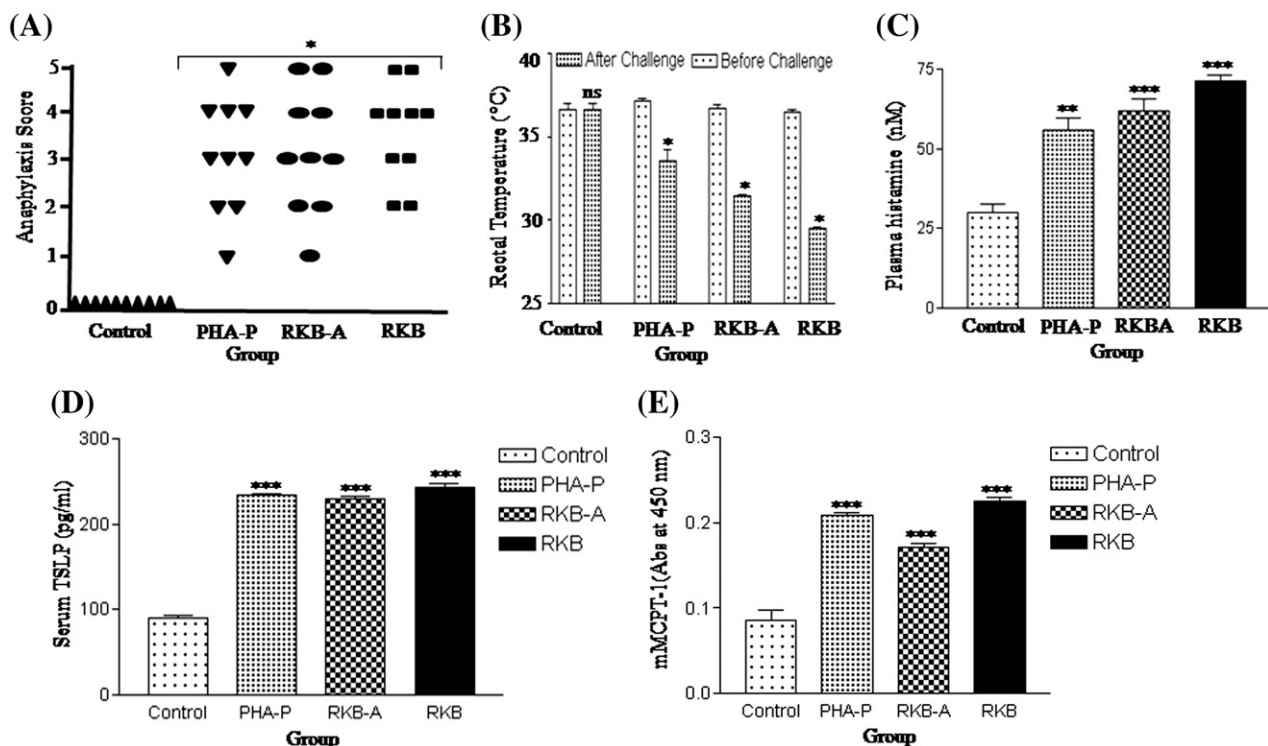


Fig. 3 – Allergic manifestations in PHA-P sensitized BALB/c mice. (A) Systemic anaphylactic score. (B) Core body temperatures before and after 40 min of challenge. (C) Plasma histamine (D) serum TSLP and (E) serum mMCPT-1 levels in challenged PHA-P, RKB-A and RKB and control mice. Data represented in mean $\pm$ SE; n=3; (*p<0.05; **p<0.01; *p<0.001).**

3.6. Histopathology of PHA-P sensitized animals

Histopathological studies for elucidation of allergic severity have been reported as one of the pivotal approaches [16,20]. Histology of the lung, intestine and spleen of PHA-P, RKB-A and RKB treated mice showed the pathological symptoms encountered during allergenic reactions (Fig. 4). The pathological symptoms in PHA-P treated group indicated exfoliations and leukocyte infiltrations in the intestine (Fig. 4A), distension in alveolar cavity and mucus formation in the lung (Fig. 4B) and presence of megakaryocytes in the spleen (Fig. 4C). Such pathological features have been frequently reported during food allergic reactions [16,31].

3.7. Eosinophil levels

We next studied the level of eosinophils in the lung and spleen to correlate the possibility of eosinophils related pathological conditions induced by PHA-P, if any. The IHC studies showed a significantly enhanced eosinophil counts in the lung (Fig. 5A and C) and spleen (Fig. 5B and D) of PHA-P, RKB-A and RKB treated mice in comparison to that of control mice. The eosinophilic inflammation of the airways has been reported in children with asthma and food allergies [43]. Further, the level of eosinophils in spleen has also been reported to be enhanced in chickpea allergy [31]. Taken together, the enhanced level of eosinophils count indicates

that PHA-P can also induce eosinophilic symptoms in the sensitized animals.

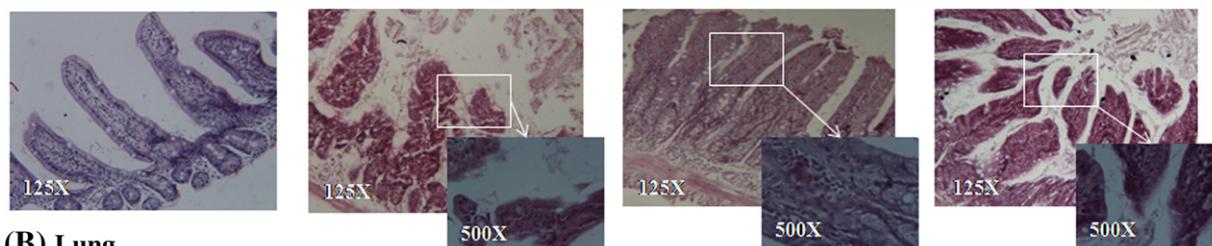
3.8. Mediators release from RBL-2H3 cells

We further extended this study using RBL-2H3 cells as *in vitro* model and assessed mediator release after exposure of RKB, RKB-A and PHA-P. The β -hexosaminidase levels were found elevated ($p < 0.001$) at all concentration of RKB, RKB-A and PHA-P (Fig. 6A). Further, PHA-P treated group indicated up to 3 fold enhanced level of β -hexosaminidase release at 25, 50, 75, 100 and 125 μ g concentrations when compared to control. The β -hexosaminidase release assay from RBL-2H3 cells has been reported as one of the pivotal tools for *in vitro* elucidation of mast cell degranulation [18]. The level of histamine, PGD₂, and CysL was found to be enhanced in RKB, RKB-A and PHA-P treated RBL-2H3 cells (Fig. 6B-D). In the PHA-P treated RBL-2H3 cells, the level of histamine (1.7 fold), PGD₂ (5 fold) and CysL (4 fold) was found enhanced when compared to their respective control. The allergic mediators like histamine, PGD₂ and CysL levels have been reported to get elevated in case of food allergy manifestations [2,38,44,45].

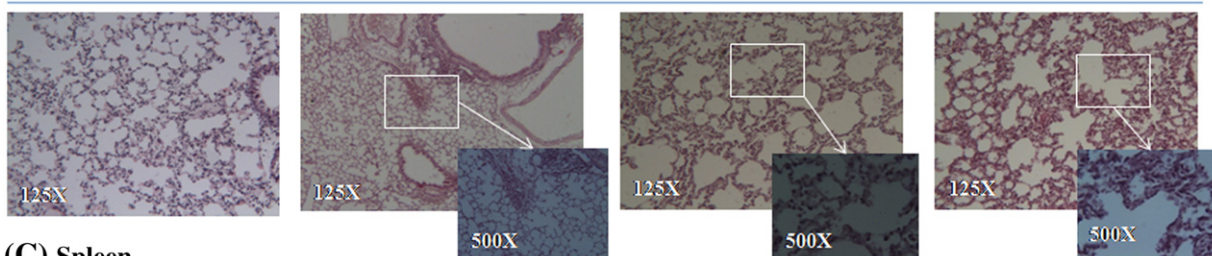
3.9. Characterization of IgE binding components

The aforesaid findings showed that PHA-P may induce allergic responses in the sensitized animals. Our next task was to find

(A) Intestine



(B) Lung



(C) Spleen

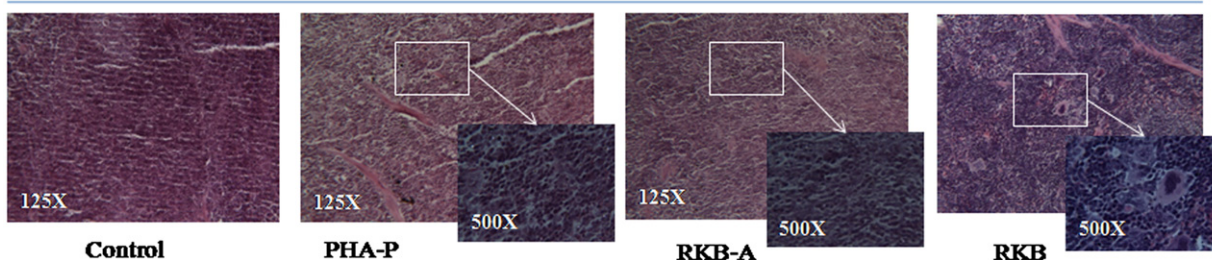
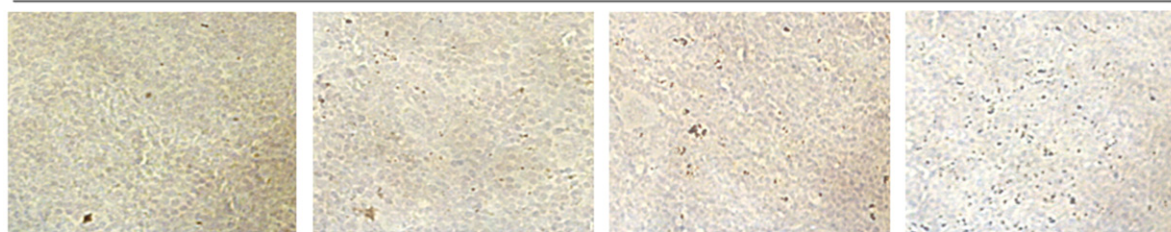
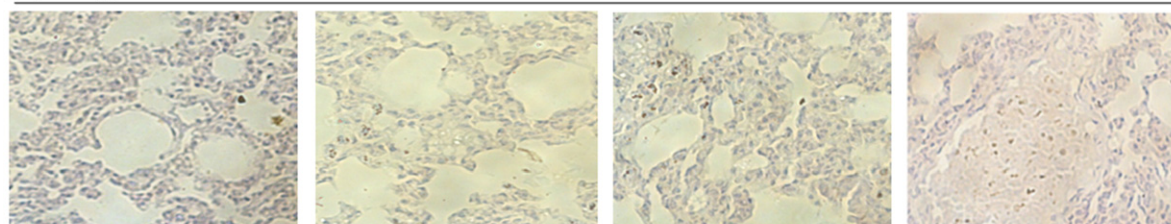


Fig. 4 – Histopathology of lung, jejunum and spleen. Tissues were collected 40 min after the challenge from control, PHA-P, RKB-A and RKB challenged mice for histopathology. (A) Histopathology of the intestine, (B) lung and (C) spleen of control, PHA-P, RKB-A and RKB treated and challenged groups.

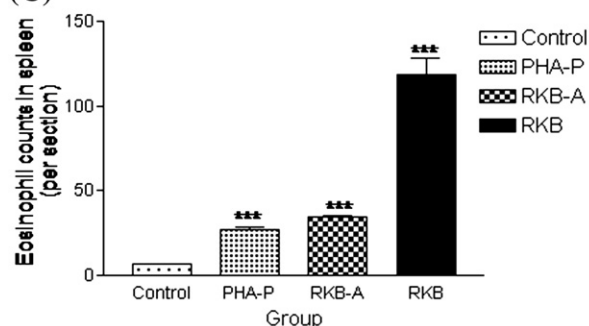
(A) Spleen



(B) Lung



(C)



(D)

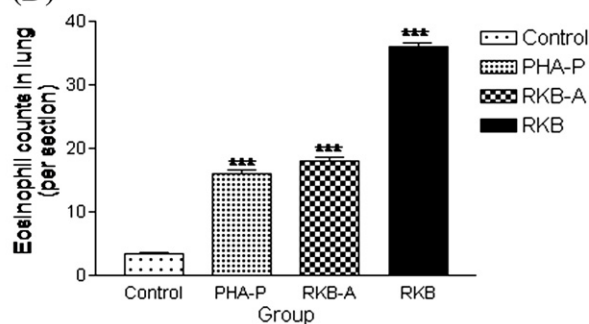


Fig. 5 – Detection of eosinophils by immunohistochemistry. (A) Eosinophil counts in the spleen and (B) lung of control, PHA-P, RKB-A and RKB treated mice. (C–D) Graphical demonstration of eosinophils in the spleen and lung. Data represented in mean $\pm$ SE; n = 3; (*) $p < 0.001$.**

out the culprit subunits of PHA-P responsible for induction of allergic manifestations. The 2D-IgE immunoblot using PHA-P sensitized mice sera recognized two groups of spots (Fig. 7A and B). Further, based on the LC-MS/MS analysis, these two groups of spots were characterized as PHA-L and PHA-E [46–48]. The tryptic peptide fragments, sequence similarity to proteins, their accession number and probability-based MOWSE score (ion score) are given in Table 1. Bioinformatic analyses based on AllergenOnline, indicated similarity of PHA-L with peanut agglutinin precursor (*Arachis hypogaea*), pollen allergen (*Lolium perenne*) and others (Table 2). Another bioinformatic approach based on AlgPred revealed the allergenicity of PHA-L by all the prediction parameters while PHA-E was found allergic only on the basis of SVM module based on amino acid composition (Table 3). The mass spectrometry, proteomic and bioinformatic tools have been found useful in studies of the biochemical, immunological and toxicological aspects of several proteins including legumes and PHAs [49]. Further, the analyses of 2D spots revealed by immunoblot coupled with MS have been used in the identification and characterization of several allergens [50]. Recently, the PHA has been found culprit in a patient suffering from kidney bean allergy [13]. However, the allergenicity of PHA-L and PHA-E has not been explored.

3.10. Specific IgE level, cross-reactivity and IgE-dot blots of PHA-L and PHA-E

Study in female BALB/c mice was extended to explore whether PHA-L and PHA-E can cause allergenicity especially by inducing specific IgE and IgG1. The level of specific IgE and specific IgG1 was found significantly enhanced in PHA-L and PHA-E treated groups in comparison to that of the control (Fig. 8A and C). It has been reported that specific IgE, and IgG1 production, are very common phenomena in food allergy [29]. The enhanced level of IgE and IgG1 supports the possibility of PHA-L and PHA-E induced allergic reactions. In this study, per cent cross reactivity between PHA-P and PHA-E was 25, 40, 54, 73, and 96 at concentrations 1, 10, 100, 1000 and 10000 ng. The per cent cross reactivity between PHA-E and PHA-L was 26, 36, 49, 69, and 92 at concentrations 1, 10, 100, 1000 and 10000 ng (Fig. 8B). Exploration of cross reactivity is a much needed requirement for elucidating allergenic potential of two closer or distant allergens. It has been reported that homologies to major peanut allergens with lupin proteins can elicit clinical cross-reactivity in susceptible individuals [51]. This result suggests that the allergenic potential of PHA-E and PHA-L is very similar and both may possess similar types of epitopes.

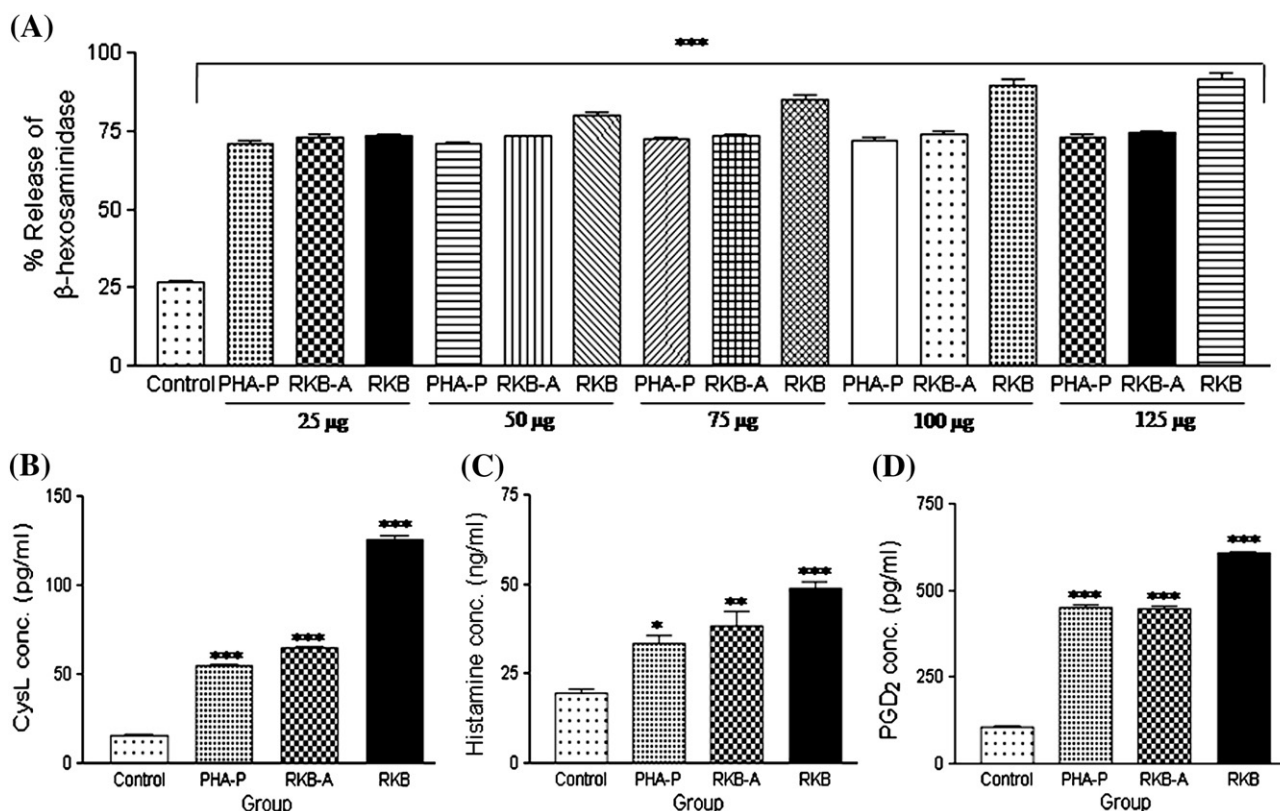


Fig. 6 – Mediators release in RBL-2H3 cells. (A) β -hexosaminidase release in RBL-2H3 cells using different doses of control, PHA-P, RKB-A and RKB. (B) Cysteinyl leukotriene (CysL) levels. (C) Histamine, (D) Prostaglandin D₂ (PGD₂) levels in control, PHA-P, RKB-A and RKB challenged RBL-2H3 cells. Data represented in mean $\pm$ SE; n=3; (*p<0.05; **p<0.01; *p<0.001).**

The IgE dot blot with pooled sera of mice sensitized with PHA-L and PHA-E demonstrated IgE binding capacity at 2, 4 and 6 μ g while no such activity was observed in the case of control (Fig. 8D). The IgE binding capacity indicated the possibility of IgE mediated allergic reaction. It has been reported that most of the food allergic reactions are mediated via IgE. The IgE binding activity is a very common property of allergenic

proteins as reported in several food proteins including wheat allergens [52]. The IgE dot blot has been utilized in the identification of critical amino acids in an immunodominant IgE epitope of Pen c 13, a major allergen from *Penicillium citrinum* [53]. The outcomes of specific IgE/IgG1, cross-reactivity and IgE dot blot validated the possibility of the IgE mediated reactions by PHA-L and PHA-E.

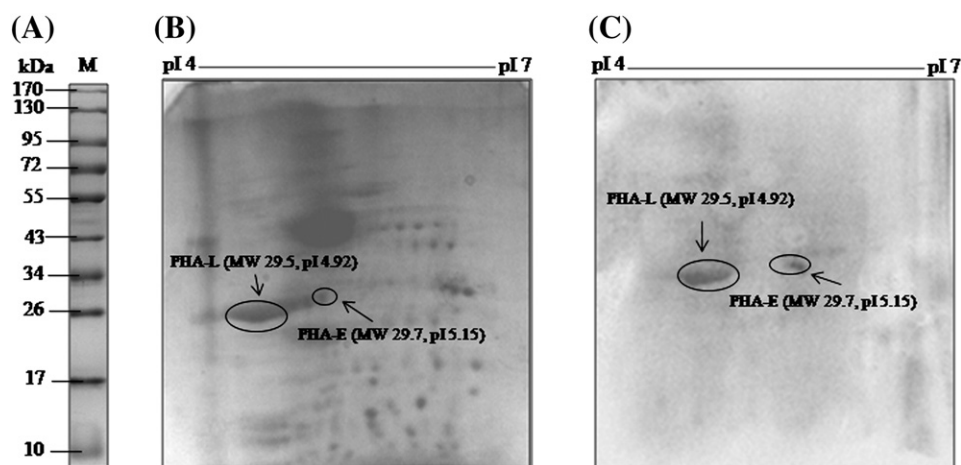


Fig. 7 – Identification and characterization of IgE binding proteins using PHA-P sensitized mice sera. (A) A protein ladder demonstrating a range of molecular weights. (B) 2-dimensional SDS-PAGE demonstrating RKB-CPE profile. (C) 2-dimensional IgE immunoblot demonstrating IgE binding proteins.

Table 1 – LC-MS/MS analysis of two IgE binding proteins.

S. N.	Tryptic peptide fragments	Sequence similarity with proteins and accession	Probability based MOWSE score	Expected mol. wt. (kDa)	pI
1.	K.TSFIVSDTVDLK.S K.GNVETNDVLSWSFASK.L K.LSDGTTSEGLNLNLVLNK.I	(Phaseolus vulgaris) Leucoagglutinating phytohemagglutinin; (PHA-L) Accession P05087	317	29.538	4.92
2.	K.TTTWDFVK.G R.HIGIDVNSIK.S R.LTNVNDNGEPTLSSLGR.A	(Phaseolus vulgaris) Erythroagglutinating phytohemagglutinin; (PHA-E) Accession P05088	158	29.728	5.15

Where: kDa=kilodalton; pI=Isoelectric point.

3.11. Clinical symptoms in PHA-L and PHA-E treated mice

In this study, PHA-L and PHA-E groups showed prominent clinical symptoms indicating the allergic potential of PHAs. Mice in the PHA-L treated and challenged group exhibited symptoms of scratching around the throat and mouth (score 2, 20%); labored respiration (score 3, 20%); and near fatal reactions such as loss of consciousness or little activity despite gentle prodding (score 4, 60%). The anaphylactic manifestations induced by PHA-E demonstrated score 2 in 20% mice, score 3 in 40% mice and score 4 in 40% mice (Fig. 9A). The control mice did not show any symptom of anaphylaxis. The rectal or core body temperature was found to be decreased by 2 to 5 °C below the normal range of the control in PHA-L and 2 to 3 °C below the normal range of the control in PHA-E challenged mice (Fig. 9B). It has been well documented that the anaphylaxis symptoms accompanied with decreased body temperatures reflect the severity of systemic anaphylaxis in mice [17,37]. The prominent anaphylactic symptoms are again validating the possibility towards allergic manifestations induced by PHA-L and PHA-E.

3.12. Histopathology of intestine in PHA-L and PHA-E treated mice

We further, extended our study to understand mucosal allergic manifestations in PHA-E and PHA-L sensitized and challenged mice. The PHA-L and PHA-E group mice showed exfoliation in the epithelium along with prominent infiltration of leukocytes in the intestine (Fig. 9C). The presence of anaphylaxis symptoms along with abrupt changes in the intestine indicated the severity of PHA-L and PHA-E induced allergic reactions.

3.13. β -hexosaminidase release from RBL-2H3 cells after PHA-L and PHA-E exposure

We extended this study in RBL-2H3 cells to elucidate β -hexosaminidase release following PHA-L and PHA-E exposure. The release of β -hexosaminidase has been considered pivotal to study the *in vitro* degranulation of mast cells/basophils. Up to 77% β -hexosaminidase release was observed in PHA-L treated RBL-2H3 cells while up to 69% β -hexosaminidase release was evident in RBL-2H3 cells exposed

Table 2 – Bioinformatic analysis of PHA-L and PHA-E according to AllergenOnline.

Details	PHA-L	PHA-E
Accession no.	P05087	P05088
Allergenicity (8 mer)	No sequences found with an exact 8mer match	No sequences found with an exact 8mer match
Allergenicity (80 mer)	gi 253289 gb AAB22817.1 peanut agglutinin precursor (38.20%)	gi 253289 gb AAB22817.1 peanut agglutinin precursor [Arachis hypogaea] (68.0% similar)
Allergenicity (Full length)	gi 253289 gb AAB22817.1 peanut agglutinin precursor [Arachis hypogaea] (69.1% similar) gi 168314 gb AAA63278.1 pollen allergen [Lolium perenne] (52.6% similar) gi 75274600 sp Q9SC98 Q9SC98_LOLPR Pollen allergen (52.6% similar) gi 33149333 gb AAP96759.1 group 1 allergen Dac g 1.01 precursor [Dactylis glomerata] (52.6% similar) gi 168316 gb AAA63279.1 pollen allergen [Lolium perenne] (52.6% similar) gi 126385 sp P14946.2 MPAL1_LOLPR RecName: Full=Pollen allergen Lol p 1; A (52.6% similar) gi 1171005 sp P43216.1 MPAH1_HOLLA RecName: Full=Major pollen allergen Hol(50.0% similar)	gi 253289 gb AAB22817.1 peanut agglutinin precursor [Arachis hypogaea] (68.0% similar)

Similarity to other proteins is given with gene bank and accession numbers.

Table 3 – Bioinformatic analysis of PHA-L and PHA-E according to AlgPred.

Details	PHA-L	PHA-E
Accession no.	P05087	P05088
Mapping of IgE epitopes and PID	No experimentally proven IgE epitope	No experimentally proven IgE epitope
MEME/MAST motif	Non allergen	Non allergen
SVM module based on amino acid composition	Allergen	Allergen
SVM module based on dipeptide composition	Non allergen	Non allergen
Blast search on allergen representative peptides (ARPs)	Allergen	Non allergen
Hybrid Approach (SVMc+IgE epitope+ARPs BLAST+MAST)	Allergen	Non allergen and allergen

Where: PID=Percentage Identity; MEME=Maximum Entropy for Motif Elicitation; MAST=Motif Alignment and Search Tool; SVM=Support Vector Machine-based; ARPs=Allergen Representative Peptides; BLAST=Basic Local Alignment Search Tool.

with PHA-E in comparison to control. Further, the release of β -hexosaminidase in the presence of piceatannol (an IgE inhibitor) was evaluated [54]. In the presence of piceatannol, up to 63% and 54% β -hexosaminidase releases were evident in PHA-L and PHA-E group, respectively when compared to control (Fig. 10A). Our result suggested a similar outcome as reported in the case of potato lectin where activation and degranulation of both mast cells and basophils has been reported [55]. This study revealed that both IgE and non IgE mediated allergic reactions are induced by PHA-L and PHA-E and the degree of induction was more in case of PHA-L.

3.14. β -hexosaminidase release by BMMCs after PHA-L and PHA-E exposure

The β -hexosaminidase release was also assessed in BMMCs following PHA-L and PHA-E exposure. Up to 78% β -hexosaminidase release was observed in PHA-L treated BMMC cells while, up to 68% β -hexosaminidase release was evident in BMMCs exposed with PHA-E in comparison to control. In the presence of piceatannol, up to 68% and 65% β -hexosaminidase releases were evident in PHA-L and PHA-E groups, respectively when compared to control (Fig. 10B). In

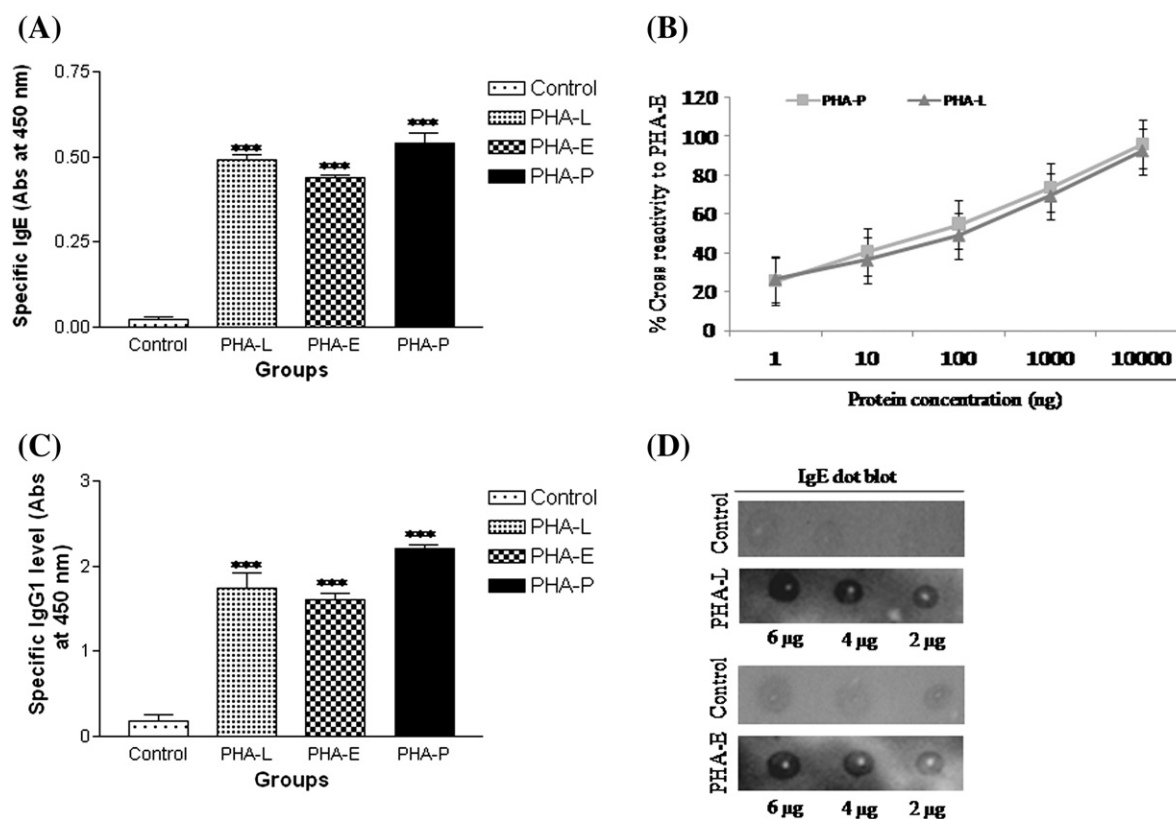


Fig. 8 – Serum immunoglobulins, cross-reactivity and IgE dot blot. (A) Level of specific IgE and (B) Cross-reactivity of PHA-E with PHA-L and PHA-P. (C) Specific IgG1 to PHA-L, PHA-E and PHA-P. (D) The IgE dot blot of the control, PHA-L and PHA-E. Data represented in mean $\pm$ SE; n=3; (*)p < 0.001.**

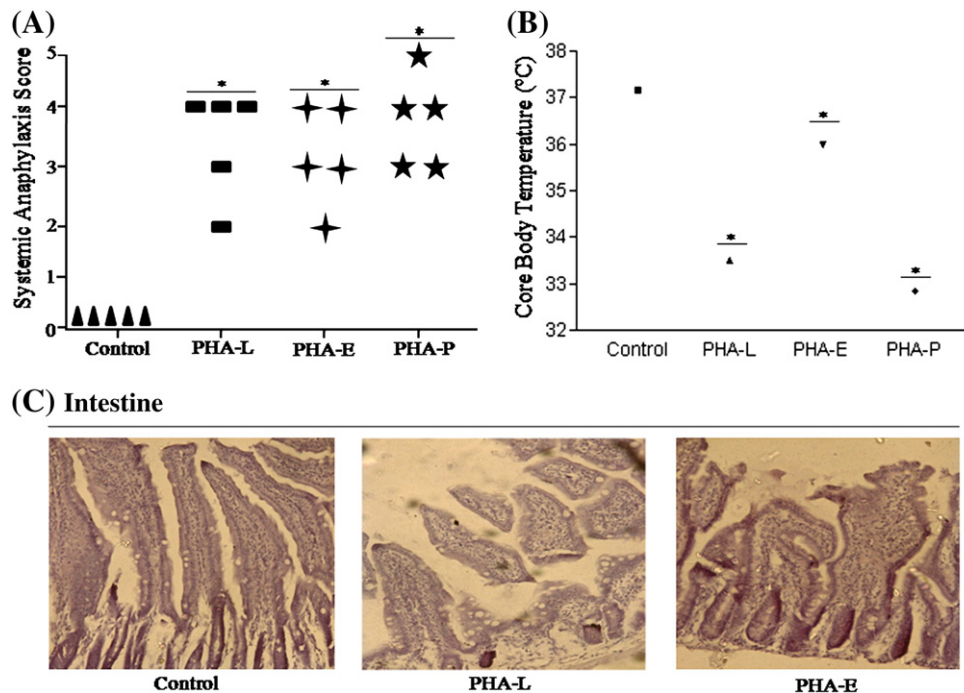


Fig. 9 – Clinical manifestations in PHA-L and PHA-E sensitized BALB/c mice. (A) Systemic anaphylactic score and (B) Core body temperatures of challenged mice after 40 min in control, PHA-L, PHA-E and PHA-P groups. (C) Histopathology of intestine in sensitized and challenged PHA-L and PHA-E treated mice. Data represented in mean $\pm$ SE; n=3; (*p<0.05).

accordance with previous reports, piceatannol is a naturally occurring stilbene that selectively inhibits SYK by competing for its substrate-binding site, effectively blocked the Ins-1, 4, 5-P3

production [54,56,57]. The outcome of this study further supported the mast cell degranulation properties of PHA-L and PHA-E.

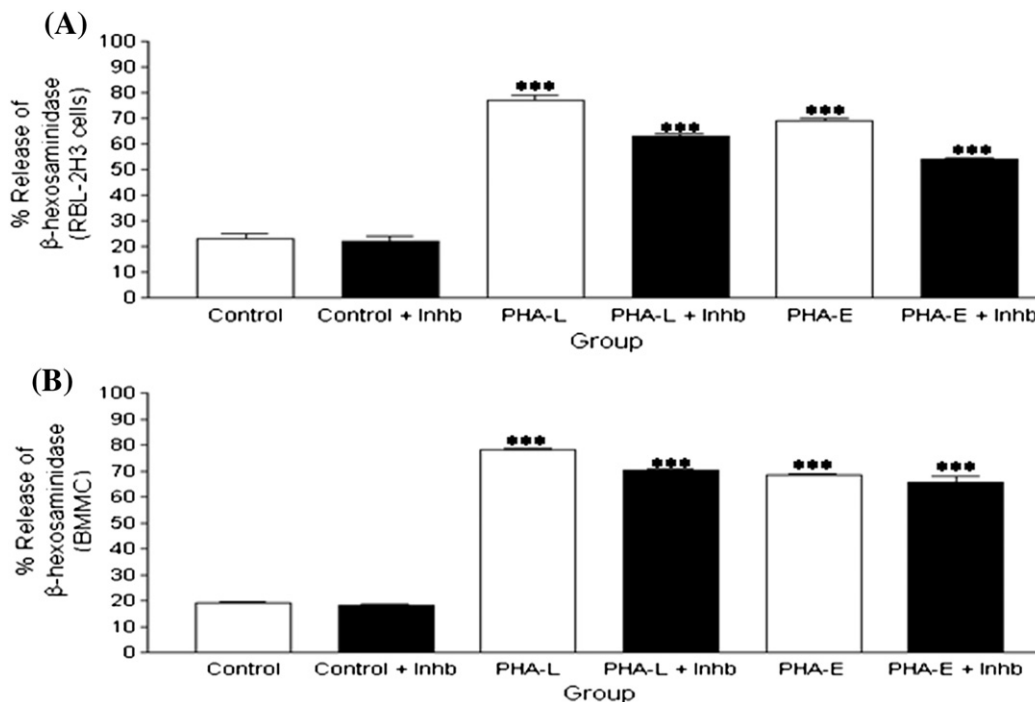


Fig. 10 – Release of β -hexosaminidase from RBL-2H3 cells and BMMCs. (A) Release of β -hexosaminidase following PHA-L and PHA-E exposure in the absence and presence of IgE inhibitor (Inhnb), piceatannol in RBL-2H3 cell. (B) Release of β -hexosaminidase following exposure of PHA-L and PHA-E in the absence and presence of IgE inhibitor (Inhnb), piceatannol in BMMC. Data represented in mean $\pm$ SE; n=3; (***)p<0.001).

3.15. GATA-3 and T-bet expressions in PHA-L and PHA-E treated BMMCs

GATA-3 and T-bet are key regulators in Th2 and Th1 reactions [2]. Therefore, expression of these two regulators was estimated to determine the fate of reactions induced after PHA-L and PHA-E exposure in BMMCs. The GATA-3 and T-bet levels showed significantly ($p < 0.05$) up regulated expressions in the BMMCs following PHA-L and PHA-E exposure as compared

to control (Fig. 11A and B). It has been reported that BMMCs are potent secretors of proinflammatory chemokines and cytokines, thus they can also demonstrate the clear picture about the up and down regulation of transcription factors [58]. Further, our results suggested a mixed fate of allergic reactions induced after exposure of PHA-L and PHA-E as supported by several other parameters measured in this study.

In summary, the presence of PHAs may have augmented the allergenic potential of red kidney beans, which was

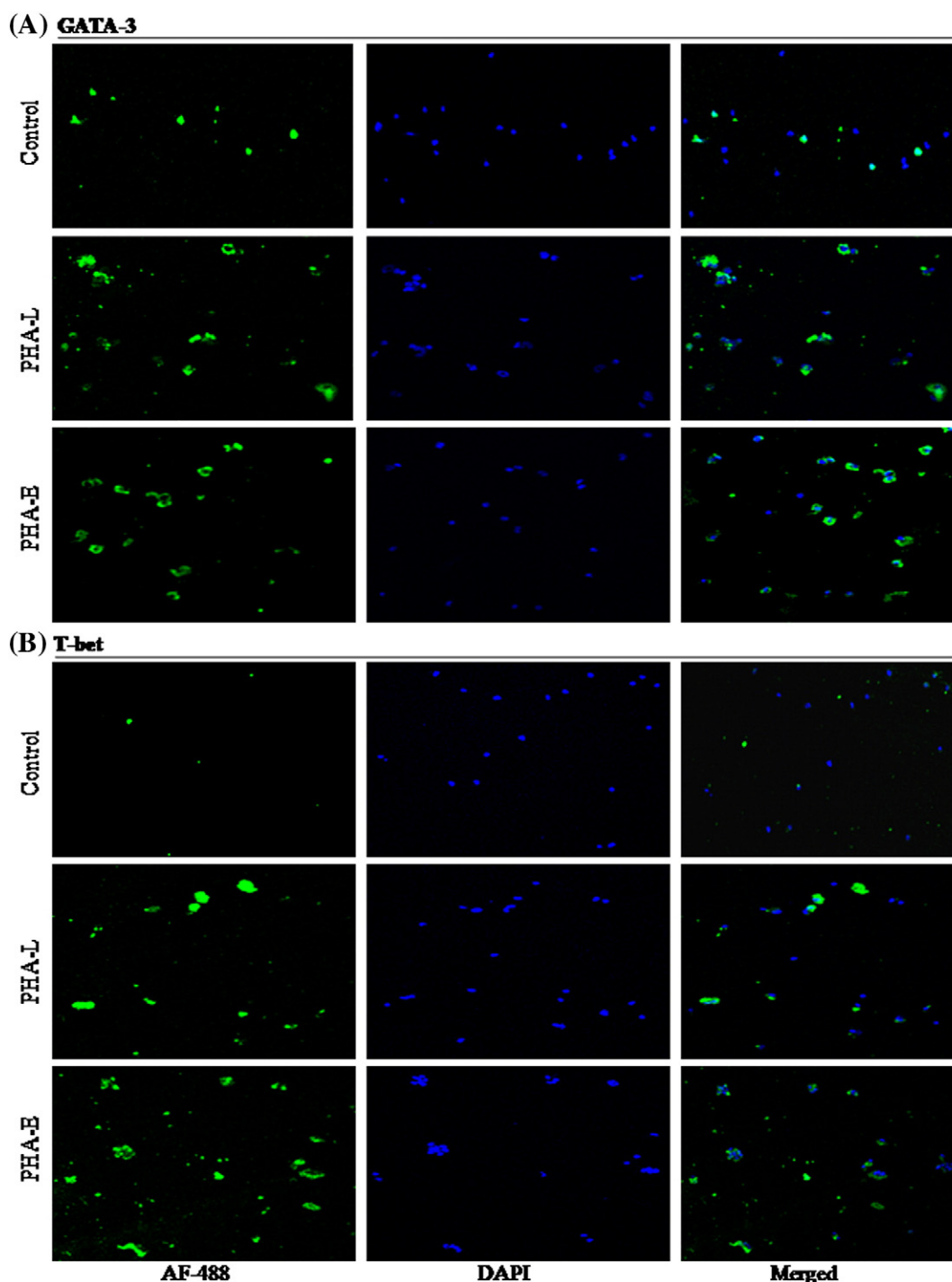


Fig. 11 – GATA-3/T-bet expressions in BMMCs. (A) Fluorescence illustration of GATA-3 and (B) T-bet expression in PHA-L and PHA-E treated BMMCs. Where, AF-488=Alexa fluor-488, and Merge means superimposed images with AF-488 and DAPI.

evident by enhanced levels of immunoglobulins like specific IgE/IgG1, prominent anaphylactic manifestations, release of β -hexosaminidase and other allergic mediators, and pathological symptoms of allergy in the intestine, lung and spleen in the BALB/c mice. The characterization of PHA-L and PHA-E validated the IgE binding potential of PHAs. Further, the increased level of specific IgE/IgG1 and β -hexosaminidase in the PHA-L and PHA-E treated groups confirmed the pivotal role of PHAs in the allergic manifestations induced by red kidney beans. But, in the presence of IgE inhibitor piceatannol, mild reduction in β -hexosaminidase indicated towards a non IgE mediated allergic response accompanying IgE mediated reactions. The up regulated expression of GATA-3 and T-bet in PHA-L as well as in PHA-E groups validated aforesaid possibility. Further, studies may be required to unravel molecular mechanism behind PHAs induced allergic reactions.

Acknowledgments

We are grateful to the Director of the Institute for his keen interest in this study. This work was financially supported by the Network Project InDepth (BSC 0111) of Council of Scientific and Industrial Research (CSIR), New Delhi. We would like to convey our heartiest gratitude to Dr. Pradeep K. Shrivastava for the editorial assistance. SK and AKV are thankful to CSIR, New Delhi for the award of their Senior Research Fellowships. AS and DK are thankful to CSIR and UGC, New Delhi for the award of their Junior Research Fellowships, respectively. Also, thanks are due to Pathway Builder 2.0. This is CSIR-IITR manuscript no #3085.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2013.02.003>.

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