



Phaseolin: A 47.5 kDa protein of red kidney bean (*Phaseolus vulgaris* L.) plays a pivotal role in hypersensitivity induction



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ARTICLE INFO

Article history:

Received 20 September 2013

Received in revised form 11 January 2014

Accepted 13 January 2014

Available online 24 January 2014

Keywords:

Allergens

Food allergy

Legumes

Peptide mass fingerprinting

Transcription factors

ABSTRACT

Red kidney bean (*Phaseolus vulgaris* L.), a protein rich legume, is consumed globally due to its delicacy. This study was aimed to purify, characterize and assess allergenicity of one of its clinically relevant allergens, later identified as phaseolin. This study was carried out using clinical, in vivo and ex vivo approaches. Phaseolin, an abundant protein of red kidney bean, was purified by column chromatography and reverse-phase-HPLC techniques and characterized by peptide mass fingerprinting. The IgE immunoblotting using red kidney bean allergic patients sera showed phaseolin as a major IgE binding protein of red kidney bean. Phaseolin treated mice demonstrated enhanced levels of specific IgE and IgG1, mouse mast cell protease-1, mRNA expressions of *IL-4*, *IL-5*, *IL-13* and *GATA-3* in the lungs, spleen and intestine along with anaphylactic symptoms indicative of allergic responses. Further, flow cytometry analysis and immunohistochemical studies indicated increased levels of *IL-4*, *IL-5*, *IL-13* and *GATA-3*, respectively as compared to controls. The level of Foxp3 was found suppressed in the intestine of phaseolin treated mice when compared to the control. Further, phaseolin treated mice showed positive results in type 1 skin test. Bone marrow derived mast cells (BMMCs) and rat basophilic leukemia (RBL-2H3) cells showed enhanced release of allergic mediators like β -hexosaminidase, histamine, cysteinyl leukotrienes and prostaglandin D₂. Taken together, phaseolin was found to possess characteristics of a potential allergen that may lead to hypersensitivity responses in the susceptible individuals and this may be one of the major proteins responsible for allergenicity of red kidney bean.

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1. Introduction

The prevalence of food allergy is an increasing health concern and encompassing developed as well as developing parts of the world [1]. Food induced allergic reactions may cause complications like breathing difficulties, bronchial asthma (BA), allergic rhinitis (AR), swelling of the lips and throat, abdominal cramps, vomiting and atopic dermatitis (AD) and sometimes death in susceptible individuals. It has been estimated that approximately 125 deaths per year occur in the United States due to food induced anaphylaxis [2]. The overall frequency of food induced anaphylaxis is 49.8 per 100,000 people per year where peanuts and tree nuts have been found to be the major players (33.2%) of such reactions [3]. Most of the legume allergic manifestations are mediated via immunoglobulin E (IgE), but sometimes non IgE mediated allergic reactions are also evident [4]. Food allergic reactions are directed by type 2 helper T (Th2)-cells and cytokines like *IL-4* and *IL-13* [5]. The

transcription factors *GATA-3* and *T-bet* have been found to be responsible to shift toward Th2 and Th1 reactions, respectively [5]. Oral tolerance is a naturally acquired potential to tolerated adverse immune effect of food allergens. In food allergic individuals, either lack of oral tolerance or impaired oral tolerance has been frequently reported [6]. The transcription factor Foxp3 is found to be involved in the regulation of oral tolerance [7]. Upon exposure of food allergens basophils and mast cells release allergic mediators, such as histamine, cytokines and leukotrienes in atopic individuals suffering from food or other allergies [8].

Legumes constitute a major proportion among allergenic foods due to the presence of high protein content. The prevalence, identification and characterization of allergens from peanut, soybean, chickpea, red gram, lentil, beans, green gram, black gram and other legumes have been well studied [9]. Allergens from several legumes including peanut (Ara h1–Ara h7), soybean (Gly mBD), green gram (Vig r2–Vig5), red gram (Caj c1–Caj c6) and green bean (Pha v3) have been identified, purified and characterized [9,10]. Studies indicated that either Ara h 2 or Ara h 6 allergens of peanut independently can induce most of the allergenic activity in a crude protein extract [11]. Therefore, allergenicity assessment of the purified food allergens are very important aspect to understand the allergenicity potential of certain foods. Several techniques

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have been used to purify food allergens including column chromatography and reverse-phase HPLC [12]. The characterization of the purified food allergens can be performed by various mass spectrometric techniques including liquid chromatography tandem mass spectrometry (LC–MS/MS) and peptide mass fingerprinting (PMF) [13].

Red kidney bean (*Phaseolus vulgaris* L.) is a common bean, commercially produced and consumed globally, including several countries of Asia, Africa, Europe, Oceania, South and North America [14]. Recently, the allergenicity assessment along with identification of IgE binding protein components of red kidney bean was unraveled in BALB/c mice and red kidney bean sensitive bronchial asthma and allergic rhinitis patients [15,16]. Rouge et al. has reported that the phaseolin and phytohemagglutinin (PHA) may be the culprit proteins responsible for allergic manifestation in a clinical study [17]. Kasera et al. have reported that PHA, phaseolin, alpha-amylase inhibitor precursor and group 3 late embryogenesis abundant proteins of kidney bean may cause allergic reactions [18]. The role of PHA in red kidney bean induced allergic manifestations has also been reported in a murine model [19]. Recently, phytohemagglutinin, a 31 kDa allergen of kidney bean has been purified and reported to manifest cross-reactivity to peanut and black gram [20]. However, the purification and characterization of other clinically relevant allergens and basic immunological events behind the purified red kidney bean's allergens have not been explored, so far.

Therefore, in this study, purification and characterization of phaseolin, a major protein of red kidney bean was carried out using proteomic and bio-informatic approaches coupled with evaluation of allergenic potential of this purified protein using clinical, in vivo and ex vivo approaches.

2. Materials and methods

2.1. Ethical permissions

Human studies were carried-out with the informed patient's consent and the study protocol was approved by the human ethics committee of the King George Medical University, Lucknow, India (Ref: IEC No. 2938/R. Cell-11). Animal study was carried out after approval by an Institutional Animal Ethics Committee of CSIR-IITR, Lucknow, India (IITR/IAEC/27/11-05/2011).

2.2. Preparation of red kidney bean crude protein extract

Crude protein extract of red kidney bean crude protein extract was prepared as per the method described earlier [21].

2.3. BALB/c mice

Healthy 6–8 week old female BALB/c mice (18 ± 3 g) were obtained from an animal breeding colony of CSIR-IITR, Lucknow, India. Mice were maintained under standard laboratory conditions in a pathogen free environment and on red kidney bean and peanut free diet.

2.4. Allergic patients, skin prick test (SPT), immunoglobulins and IgE immunoblot

A total of 350 patients suffering from some of the symptoms of urticaria, dermatitis, itch, angioedema, nasobronchial asthma, allergic rhinitis, shortness of breath, nausea and vomiting were studied at the outpatient department of Pulmonary Medicine, King George Medical University, Lucknow, India since Jan, 2009–June, 2012. Allergen extracts of different foods (Alcit India Private Limited, New Delhi) including red kidney bean allergen extract were used for skin prick test (SPT). Blood was collected from red kidney bean SPT positive (RKB SPT + Ve) patients and normal healthy volunteers ($n = 10$). Sera were separated from whole blood and stored at -80°C until the further use. The total IgE and specific IgE assays as well as IgE immunoblot were carried out

according to the earlier described methods [15]. Out of 20 red kidney bean allergy patients, patients 1 to 17 and control ($n = 10$) were included from our earlier study [15]. For details of patient numbers 18, 19 and 20 see Supporting Table 1.

2.5. Purification of the red kidney bean's IgE binding protein

The purification of red kidney bean protein was carried out using a combination of fractionation, anion exchange chromatography and reverse phase HPLC techniques. In brief, red kidney bean crude protein extract supplemented with 5 mM PMSF or phenylmethylsulfonyl fluoride (Sigma, St. Louis, United States), was used to concentrate in Amicon centrifugal tubes (Millipore, USA) in a step wise manner (Amicon Ultra-15, Mr. 100,000 cut-off followed by Amicon Ultra-15, Mr. 50,000 cut-off and further, Amicon Ultra-15, Mr. 30,000 cut-off). Further, the desired fractions were subjected to HiTrap anion exchanger 5.0 ml column (GE Healthcare, Little Chalfont, United Kingdom) using Econo gradient pump and ultraviolet (UV) monitor (Bio-Rad, Hercules, California, U.S.) with a speed of 5 ml/min and gradient solution of Tris and 50 mM NaCl solution (pH 8.02). Fractions obtained, along with a protein ladder ranged 170 kDa to 10 kDa (Fermentas, Vilnius, Lithuania) were resolved on a 12% separating gel and stained with G-250 dye. The fractions were further tested for IgE-binding potential on immunoblot using pooled sera of red kidney bean allergy patients. Fractions containing a distinct band of approximately 50 kDa were subjected further to a reverse phase-HPLC system (Waters Associates, Vienna, Austria) in a semi preparative column (Waters Associates, Vienna, Austria) and monitored at 280 nm. The column was equilibrated with solvent A {0.1% Trifluoroacetic acid (TFA) in H_2O } at a flow rate of 1 ml/min, and a non-linear gradient elution was used with solvent B (0.1% TFA in acetonitrile) in A, with 5% B at time (T) = 5 min; 70% B at T = 27 min, 80% B at T = 60 min and 100% B at T = 69 min.

2.6. Characterization of the purified protein by peptide mass fingerprinting

Peptide mass fingerprinting (PMF) was carried out to characterize the purified protein. In brief, following trypsin digestion, the peptides obtained were mixed with α -cyano-4-hydroxycinnamic acid (HCCA) matrix in 1:1 ratio and 2 μl of the resulting mixture was spotted onto the matrix-assisted laser desorption/ionization (MALDI) plate. After air drying the sample, it was analyzed on the MALDI TOF/TOF ULTRAFLEX III instrument (MALDI-TOF/TOF MS Bruker Daltonics ULTRAFLEX III) using the flex control software. Further analysis was done with the flex analysis software for obtaining the PMF. Search parameters used for PMF is given below:

Enzyme: Trypsin; fixed modifications: Carbamidomethyl (C); variable modifications: Oxidation (M); mass values: Monoisotopic; protein mass: Unrestricted; peptide mass tolerance: ± 0.5 Da; peptide charge state: 1+; max missed cleavages: 1; number of queries: 71; selected for scoring: 57.

Protein score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 73 are significant ($p < 0.05$). Database: NCBI nr. The masses obtained in the PMF were submitted to Mascot search (<http://www.matrixscience.com/>) in a NCBI nr 20121103 (21332039 sequences; 7307542895 residues) protein database for identification of the protein.

2.7. Allergenicity prediction of the purified protein using bioinformatics

Search tools for prediction of allergenicity of protein like AllergenOnline (www.allergenonline.org/), Allpred (<http://www.imtech.res.in/raghava/allpred/>) and SDAP—structural database of allergenic proteins (<https://fermi.utmb.edu/SDAP/>) were used for in silico prediction of phaseolin allergenicity according to the methods described earlier [19,22,23].

2.8. In vitro digestibility assay of the purified protein

The SGF was prepared according to the guideline of the United States Pharmacopeia in which 3.2 mg pepsin (approximately 3460 U activity/mg) was dissolved in 1 mL of 34 mM NaCl and 0.7% HCl, pH 1.2 and further, in vitro digestibility assay of phaseolin in the SGF were carried out as described earlier [24].

2.9. IgE immunoblotting and specific IgE level using red kidney bean allergic human subjects' sera

To detect IgE binding potential of phaseolin, estimation of specific IgE and IgE-immunoblotting were carried out using sera of red kidney bean allergic patients according to the methods described earlier [22].

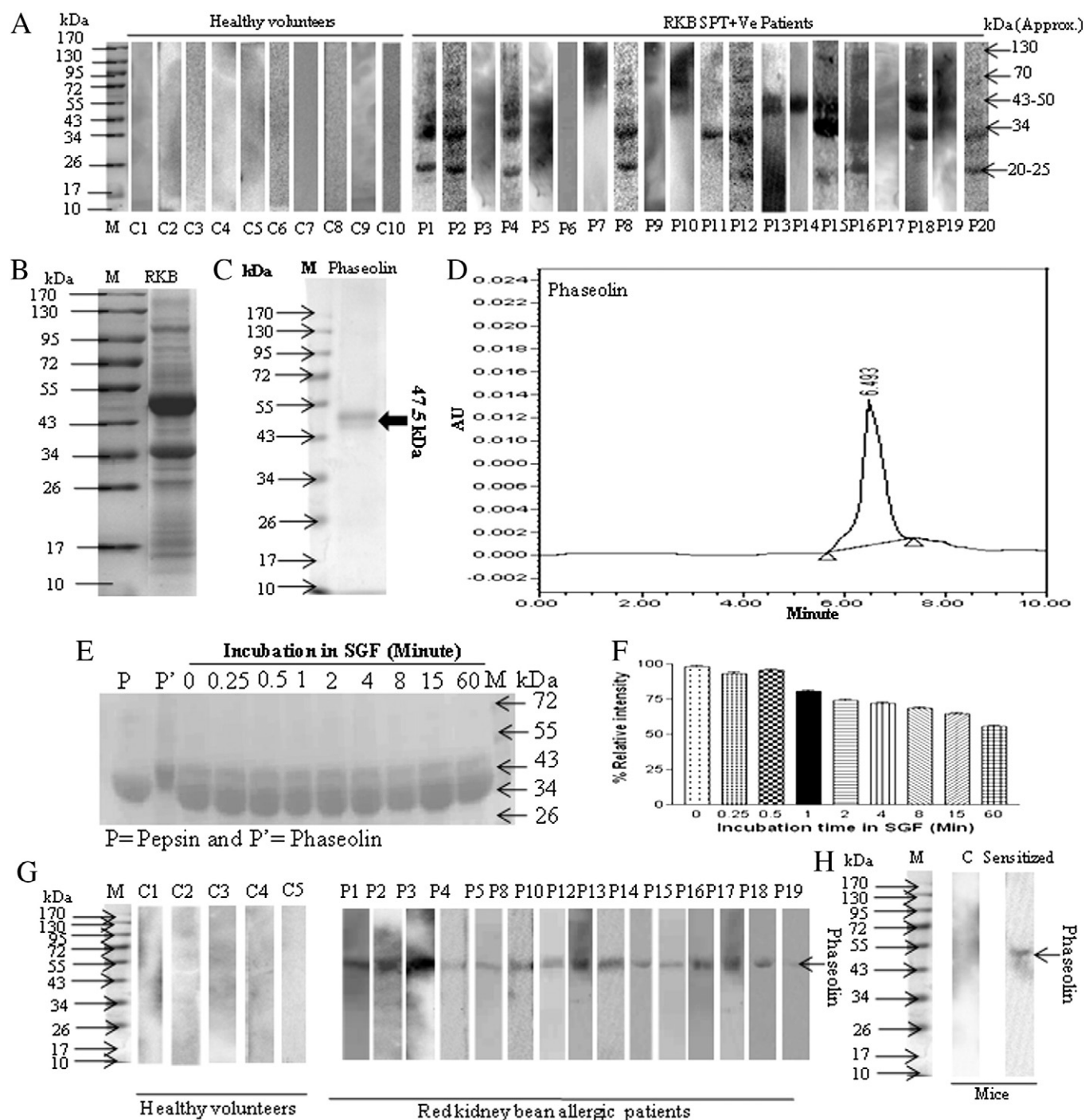


Fig. 1. Purification, identification and characterization of IgE binding proteins. (A) IgE immunoblot using individual control and red kidney bean allergic patient serum to observe IgE binding proteins of red kidney bean crude protein extract. (B) SDS-PAGE showing red kidney bean crude protein extract profile. (C) SDS-PAGE profile of purified protein. (D) Reverse phase high pressure liquid chromatography (RP-HPLC) chromatogram of purified protein. (E-F) SGF assay and densitometry analysis of phaseolin. (G) IgE immunoblot using control and red kidney bean allergic human subjects. (H) IgE immunoblot using control and phaseolin sensitized mice sera. Where M = mol. weight; kDa = kilo Dalton; where, P = patients; C = normal healthy volunteers with their respective number e.g., P1–P20 or C1–C10.

2.10. Allergenic assessment of phaseolin in BALB/c mice

Mice were randomly divided into three groups ($n = 15/\text{group}$) and sensitized orally with cholera toxin (Sigma-Aldrich, St. Louis, Mo) according to the earlier described method [25]. Briefly, mice were treated with 100 μl PBS + 10 μg of cholera toxin (control group), 1 mg of phaseolin + 10 μg of cholera toxin (phaseolin group) and 1 mg of ovalbumin (OVA) + 10 μg cholera toxin (OVA group), respectively once per week up to 4 weeks. Two weeks after the last sensitization, mice ($n = 5/\text{group}$) were challenged orally with 10 mg of phaseolin and OVA, respectively. Before, the start of treatment, purified phaseolin and OVA were tested for any endotoxin contamination using limulus amebocyte lysate (LAL) assay (Lonza India Private Ltd., Mumbai, India) and found free from endotoxin contaminations.

2.11. IgE immunoblotting using phaseolin treated mice sera

To detect IgE binding potential of phaseolin, IgE-immunoblotting was carried out using sera of phaseolin treated mice according to the methods described earlier [26,27].

2.12. Specific IgE and IgG1 levels

Specific IgE and IgG1 levels in the sera of control and phaseolin treated mice were estimated according to the earlier described methods [16].

2.13. Monocyte chemotactic protein-1 and mouse mast cell protease-1 levels

The levels of monocyte chemotactic protein-1 and mouse mast cell protease-1 were quantified in the sera of phaseolin treated and control mice using commercially available ELISA kits (BD Bioscience, USA and eBioscience, Inc. CA, USA, respectively) according to the manufacturer's instructions.

2.14. Anaphylaxis scores, rectal temperature and histopathology

The systemic anaphylaxis symptoms were measured in phaseolin treated mice as per the earlier described scoring system [28]. In brief, score 0 = no visual symptoms; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; score 3 = wheezing, labored respiration, and cyanosis around the mouth; 4 = symptoms as in no-3 with loss of consciousness, tremors, and/or convulsion; 5 = death. Rectal temperatures of phaseolin treated and control mice were measured before and after the challenge using a digital rectal thermometer (Bioseb, France). For histopathological studies, the lungs, intestine and spleen of control

and phaseolin treated mice were fixed in 10% formalin in phosphate buffer saline (PBS), embedded in paraffin, and cut into 3–5- μm thick sections.

2.15. Type 1 skin test against phaseolin

To evaluate the in vivo relevance of phaseolin induced specific antibodies, type I skin tests were carried out according to the earlier method [29]. Compound 48/80 (Sigma-Aldrich, St. Louis, Mo), a known inducer of mast cell degranulation was given as a positive control and the result was evaluated after 20 min.

2.16. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

A semi-quantitative RT-PCR analysis of Th2 cytokines (*IL-4*, *IL-5* and *IL-13*) in the lung, spleen and intestine of mice and transcription factors *GATA-3*, *T-bet* and *Fox p3* in the intestine were carried out using gene-specific primers in control, phaseolin and OVA treated groups. The primer sequences were as follows: *IL-4*: sense 5'-TCGGCATTTGAAC GAGGTC-3', antisense 5'-AAAAGCCCGAAAGAGTCT C-3'; *IL-5*: sense 5'-TCACCGA GCTCTGTGACAA-3', antisense 5'-CCACACTTCTCTTTT GGCG-3'; *IL-13*: sense 5'-GACCCAGAGGATAT TGCATG-3', antisense 5'-CCAGCAAAGTCTGATGT GAG-3'; β -Actin: sense 5'-GATTACTGCTCTGG CT CCTAG C-3', antisense 5'-GACTCAT CGTACTCTGCTTGC-3'; *GATA-3*: sense 5'-TCTCACTCTCGAGGC AGCATGA-3', antisense, 5'-GGTAC CATCTCGCCGCCACAG-3'; *T-bet*: sense 5'-TCCCATTCCTGTCCTT CA-3', antisense 5'-GCTGCCTTCTGCCTTTC-3'; *Foxp3*: sense 5'-GCTCCCGGCC TG GTCTGCTC-3', antisense 5'-AGGTGGCGGGGTGTTTCTGA-3'; *GAP DH*: sense 5'-TTCACCACCATG GAGAAGGC-3, antisense 5'-GG CATGGA CTGT GGTCTATGA-3. Total RNA from the lungs, spleen and intestine were isolated with RNAzol®RT (Molecular Research Center, Inc. Cincinnati, OH). The RT-PCR was carried out using a Verso One-Step RT-PCR kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. After completion of PCR cycles PCR products was analyzed on 2% agarose gel electrophoresis. The density of each band was estimated by the GeneTools software (Syngene). The β -actin and GAPDH were taken as endogenous controls and its respective densitometry values were used for normalizing different mRNA cytokines in the lungs, spleen and intestine, respectively. Normalized values were used for plotting the bar graphs.

2.17. GATA-3 and Foxp3 levels in the organs

The levels of GATA-3 in the lungs, spleen and intestine of control and phaseolin treated mice were detected by IHC according to the method described earlier [16]. In brief, goat antimouse GATA-3 antibody (BD

Table 1
Peptide mass fingerprinting (PMF) of the purified RKB protein.

S. N.	Tryptic peptide fragments	Sequence similarity with proteins and accession no.	Score	Expected mol. wt. (kDa)	Calculated mol. wt. (kDa)
1.	K.LMNK.Q K.EDLR.I R.LVEFR.S R.KGAFV.Y K.FEEINR.V K.NQYGHIR.V R.LQNLEDYR.L K.TDNVISIGRA K.RLQNLEDYR.L K.GNKETLEYESYR.A R.SGSAILVLVKPDDRR.E R.LQNLEDYRLVEFR.S R.EYFFLTSNDPIFSHQK.I K.IPAGTIFVLVNPDPKEDLR.I R.SKPEITLLPQQADAELLVVR.S	Phaseolin [<i>Phaseolus vulgaris</i>] Accession P02853	81	50	47.54

Peptide sequences obtained after tryptic digestion, accession number, score, expected and calculated and molecular weight in kDa (kilo Dalton).

Table 2

Details of IgE binding proteins and phaseolin specific IgE in red kidney bean allergic human subjects.

Patient ID	Binding with RKB-CPE (mol. wt in kDa)	Phaseolin specific IgE (OD 450 nm)
Control-1	None	0.099
Control-2	None	0.034
Control-3	None	0.064
Control-4	None	0.042
Control-5	None	0.076
Control-6	None	0.039
Control-7	None	0.067
Control-8	None	0.043
Control-9	None	0.075
Control-10	None	0.088
Patient-1	130, 70, 43–50, 34, 20–25	0.943*
Patient-2	130, 70, 43–50, 34, 20–25	1.457*
Patient-3	43–50, 34	1.534*
Patient-4	130, 70, 43–50, 34, 20–25	1.619*
Patient-5	43–50, 34, 20–25	1.224*
Patient-6	34	0.133*
Patient-7	130, 70	0.424*
Patient-8	130, 70, 43–50, 34, 20–25	1.720*
Patient-9	70	0.813*
Patient-10	70, 43–50	1.428*
Patient-11	34	0.708*
Patient-12	130, 70, 43–50, 34, 20–25	1.269*
Patient-13	43–50	1.496*
Patient-14	43–50	1.253*
Patient-15	43–50, 34	1.457*
Patient-16	130, 70, 43–50, 34, 20–25	0.973*
Patient-17	43–50, 34	1.619*
Patient-18	43–50, 34	1.224*
Patient-19	70, 43–50, 34	0.875*
Patient-20	34, 20–25	0.375*

The IgE binding proteins and phaseolin specific IgE. The specific IgE presented in mean $\pm$ SEM (p value < 0.05 has been indicated by *).

Biosciences, San Jose, CA) was used as primary antibody (dilution 1:200) while goat antimouse HRP conjugated antibody (Santa Cruz biotechnology, Inc. Santa Cruz, CA) was used as secondary antibody (dilution 1:500). Western blot analysis was also carried out to elucidate Foxp3 levels in the intestine of control and phaseolin treated mice according to method described earlier [16].

2.18. Splenocytes culture and cytokines

Splenocyte culture using the spleens of control and phaseolin treated mice as per the method described earlier [22]. In brief, spleens were isolated from the control and phaseolin treated mice and single cell suspension was prepared. Further, 1×10^6 cells/well was seeded in a 24 well plate and challenged after 12 h with phaseolin (50 μ g) and PBS, respectively. The supernatants were collected after 72 h and cytokines levels were estimated. The levels of IL-4, IL-5, and IL-13 were estimated by

Table 3

Bioinformatics analysis of phaseolin according to AllergenOnline.

S. N.	Allergen	Sequence similarity
1.	gi 256427 gb AAB23463.1 CG4 beta-conglycinin [<i>Glycine max</i>]	80.1%
2.	gi 18536 emb CAA35691.1 unnamed protein product [<i>Glycine max</i>]	78.2%
3.	gi 149208401 gb ABR21771.1 conglutin beta [<i>Lupinus angustifolius</i>]	74.2%
4.	gi 42414627 emb CAF25232.1 Vicilin [<i>Pisum sativum</i>]	75.2%
5.	gi 29539109 emb CAD87730.1 allergen Len c 1.0101 [<i>Lens culinaris</i>]	74.4%
6.	gi 149208403 gb ABR21772.1 conglutin beta [<i>Lupinus angustifolius</i>]	72.5%
7.	gi 29539111 emb CAD87731.1 allergen Len c 1.0102 [<i>Lens culinaris</i>]	75.2%
8.	gi 169950562 gb ACB05815.1 conglutin beta [<i>Lupinus angustifolius</i>]	73.3%
9.	gi 42414629 emb CAF25233.1 vicilin [<i>Pisum sativum</i>]	75.2%
10.	gi 1168390 sp P43237.1 ALL11_ARAHY RecName: Full = Allergen Ara h 1, clone P	71.6%

Similarity with other proteins is given with gene bank and accession numbers. Search options, E-Value Cutoff = 0.01, max. Alignments: 20, (FASTA version 35.04 Jan. 15, 2009).

Table 4

Bioinformatics analysis of phaseolin according to Algpred tools.

S. N.	Details	Phaseolin
1.	Mapping of IgE epitopes and PID	Allergen
2.	MEME/MAST motif	Non allergen
3.	SVM module based on amino acid composition	Allergen
4.	SVM module based on dipeptide composition	Allergen
5.	Blast search on allergen representative peptides (ARPs)	Allergen
6.	Hybrid approach (SVMc + IgE epitope + ARPs BLAST + MAST)	Allergen

Where: PID = Percentage Identity; MEME = Maximum Em for Motif Elicitation; MAST = Motif Alignment and Search Tool; SVM = Support Vector Machine-based; ARPs = allergen representative peptides; BLAST = Basic Local Alignment Search Tool.

cytokine bead array (CBA) kits (BD Biosciences, San Jose, California) using flow cytometry (BD FACS Canto II, USA).

2.19. BMMC culture and β -hexosaminidase release assay

BMMC culture was performed as per the previously described method [30]. Briefly, mice from control and phaseolin treated groups were sacrificed and femur and tibia were collected. Bone marrow cells were cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 50 ng/ml IL-3 (Santa Cruz biotechnology, Inc.) and 10 ng/ml stem cell factor or SCF (Sigma Life Science, USA). After 4 weeks, BMDCs were stained with toluidine blue (Sigma-Aldrich, St. Louis, MO) to confirm its morphology. The β -hexosaminidase release in BMDCs following the exposure of 10, 20, 30, 40 and 50 μ g phaseolin was studied according to the previously described method [19].

Table 5

Full FASTA alignment sequence homology of phaseolin using SDAP allergens database.

Alignment	Allergen	Sequence	% Sequence identity
1.	Vig r 2.0101	Q198W3	63.90
2.	Vig r 2.0201	B1NPN8	62.95
3.	Gly m conglycinin	AAB23463	57.72
4.	Gly m 5.0201	Q9FZP9	54.16
5.	Gly m 5.0101	O22120	53.92
6.	Gly m conglycinin,	CAA35691	53.92
7.	Len c 1.0101	CAD87730	46.32
8.	Len c 1.0102,	CAD87731	46.08
9.	Lup an 1.0101,	ACB05815	44.89
10.	Ara h 1,	P43237	43.23
11.	Ara h 1	P43238	43.23
12.	Gly m conglycinin,	AAA33947	24.23
13.	Ses i 3,	AAK15089	33.25
14.	Jug n 2,	AAM54366	35.63
15.	Jug r 2,	AAF18269	35.39
16.	Gly m conglycinin,	AAB01374	54.63
17.	Ana o 1.0101,	AAM73730	30.88
18.	Ana o 1.0102,	AAM73729	30.64
19.	Pis v 3.0101,	EF116865	30.88
20.	Cor a 11	AAL86739	30.88
21.	Gly m Bd28K,	BAB21619	24.94
22.	Ara h 3	O82580	15.44
23.	Ara h 3	AAC63045	15.44
24.	Ara h 4,	AAD47382	12.11
25.	Gly m glycinin G2	CAA68460	11.88
26.	Gly m 6.0201,	P04405	11.88
27.	Gly m 6.0301,	P11828	18.53
28.	Sin a 2.0101,	Q21LW0	11.88
29.	Cor a 9,	AAL73404	13.78
30.	Gly m 6.0101,	P04776	13.06
31.	Gly m glycinin G1,	AAA33966	13.06

Where, SDAP: structural database of allergenic proteins. The FASTA alignments between the query sequence and all SDAP allergens have an E score higher than 0.010000.

2.20. Mediators release from RBL-2H3 cells

The release of β -hexosaminidase, histamine, prostaglandin D₂ and cysteinyl leukotriene following the exposure of phaseolin was studied in vitro using RBL-2H3 cells (ATCC, Manassas, VA). RBL-2H3 cells were primed with the sera (dilution 1:10) of phaseolin sensitized and control mice, respectively according to the earlier described method [29]. The cells were challenged with different concentrations (10, 20, 30, 40 and 50 μ g) of phaseolin for 90 min. The release of β -hexosaminidase was estimated according to the earlier described protocol [31]. The levels of histamine, prostaglandin D₂ and cysteinyl leukotriene were also estimated following 50 μ g of phaseolin in the passively sensitized RBL-2H3 cells using commercially available ELISA kits (Cayman, Ann Arbor, Michigan; SPI-bio, Massy Cedex, France and eBioscience, San Diego, CA, respectively) according to the manufacturer's instructions.

2.21. Statistical analysis

Significant differences between mean values were assessed by means of ANOVA, followed by Bonferroni's multiple comparison test. The p values < 0.05 were considered statistically significant.

3. Results

3.1. IgE immunoblotting of red kidney bean crude protein extract showed five IgE binding proteins

Initially, we have focused our study to find out the clinical relevance of red kidney bean allergy in human subjects. For the fulfillment of this study red kidney bean allergic human subjects were targeted using clinical history, SPT, total IgE, specific IgE, and IgE immunoblot as deciding

criteria. Twenty (5.7%) out of 350 allergic patients had qualified the criteria (clinical history, SPT, total IgE, specific IgE, and IgE immunoblot) of being susceptible to red kidney bean. These patients were suffering from AR, BA and dermatitis. As per the standard questionnaire, none of the patient has any history of anti-histamine or IgE therapy. Considering the immediate onset 28.6% of the red kidney bean allergic patients (n = 6) had claimed symptoms of abdominal pain and cramps, indigestion and diarrhea on ingestion of red kidney bean dishes. Twenty allergic patients showed positive SPT results (+1 to +3) against red kidney bean allergen extract. Significantly increased levels of total IgE and specific IgE in RKB SPT + Ve patients were observed when compared to that of the normal healthy volunteers (for details of patients 18–20, see Supporting Table 1 and see Reference [15] for the details of control 1 to 10 as well as patients 1–17). The IgE binding red kidney bean proteins with approximate mol. wt. 130 kDa, 70 kDa, 43–50 kDa, 34 kDa and 20–25 kDa were recognized by individual serum of RKB SPT + Ve patients. The sera of healthy volunteers did not show any reactivity to red kidney bean proteins (Fig. 1A). The protein with mol. wt. 43–50 kDa was evident in the 15 (75%) red kidney bean allergy patients and this happened to be a major protein of red kidney bean having IgE binding capacity.

3.2. Purified protein was characterized as phaseolin

In the studies of Section 3.1, we have found that red kidney bean proteins induce allergic symptoms in human subjects, we next intended to find out one of the major IgE binding proteins. Considering this objective we used purification and characterization along with IgE immunoblotting. The SDS-PAGE of red kidney bean crude protein extract showed the presence of a wide range (170–10 kDa) of proteins (Fig. 1B). The protein of approximately mol. wt. 50 kDa protein appeared in the unbound fraction as a single band

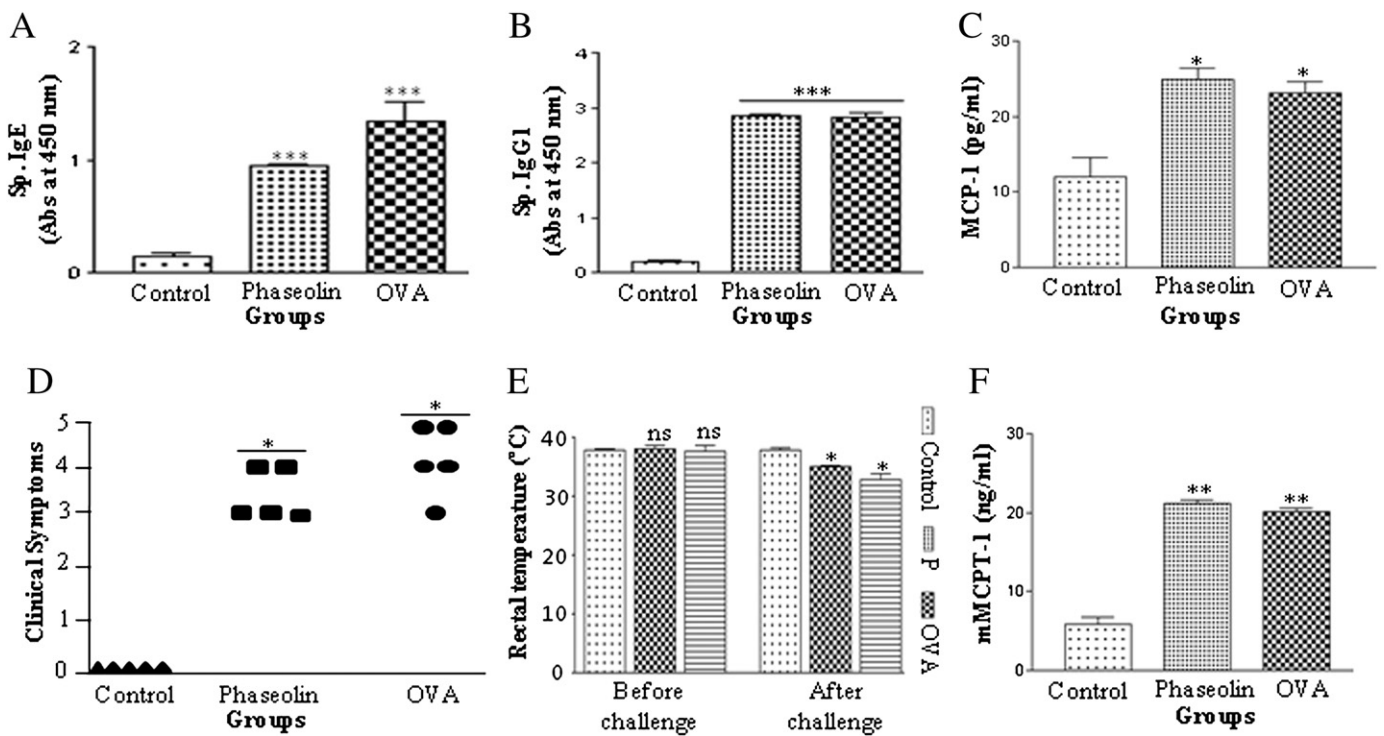


Fig. 2. Allergenicity assessment of phaseolin in BALB/c mice. The allergenicity potential of phaseolin was assessed in the BALB/c mice following oral exposure along with cholera toxin as an adjuvant. The pooled sera of control, phaseolin treated and OVA treated groups were used to estimate immunoglobulins and mediator levels. Two weeks after the last sensitization, anaphylactic symptoms including rectal temperature were measured (n = 5 mice/group) following oral challenge with 10 mg of phaseolin or OVA, respectively. (A) Level of phaseolin specific IgE (sIgE) and (B) specific IgG1 or sIgG1 and (C) monocyte chemotactic protein-1. (D) Anaphylactic or clinical symptoms (where; score 0 = no visual symptoms; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; score 3 = wheezing, labored respiration, and cyanosis around the mouth; 4 = symptoms as in no-3 with loss of consciousness, tremors, and/or convulsion; 5 = death). (E) Rectal temperature or core body temperature. (F) Mouse mast cell protease-1 (where; *p < 0.05; **p < 0.01 ***p < 0.001). ns = nonsignificant.

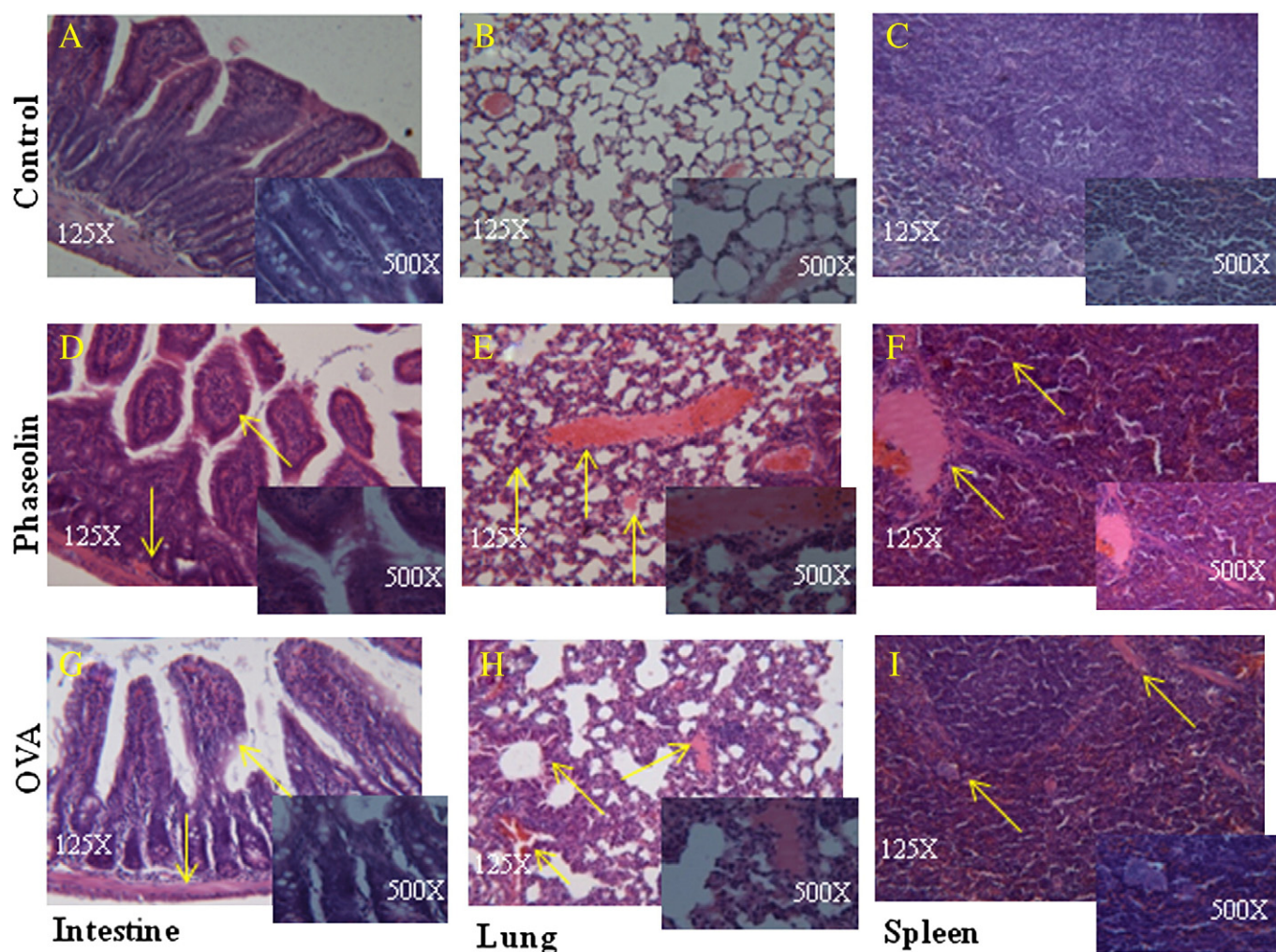


Fig. 3. Histopathological studies. Histopathology of the intestine, lungs and spleen from control, phaseolin, and OVA treated mice was carried out to find out any histopathological change. For histopathological studies, the lungs, intestine and spleen of different groups were fixed in 10% formalin in phosphate buffer saline (PBS), embedded in paraffin, and cut into 3–5- μ m thick sections. (A–C) No prominent histopathological changes were observed in the control group. (D–I) The intestine of phaseolin and OVA treated mice showed mild goblet cell hyperplasia, exfoliation in the intestinal wall, while the spleen showed lymphoid hyperplasia, megakaryocytes and edema (see arrows). The lungs of phaseolin and OVA treated mice showed congestion, thickening in the alveolar septa and edema (see arrows).

on 12% SDS-PAGE (Fig. 1C) and a single peak at a retention time 6.493 min on RP-HPLC (Fig. 1D). The PMF studies suggested the purified protein to be phaseolin with molecular weight 47.53 (Table 1).

3.3. Phaseolin showed pepsin resistant property and IgE binding activities

Following purification and characterization of one of the IgE binding proteins of red kidney bean i.e., phaseolin, we sought to explore its

Table 6
Scoring table for the histopathological changes in the organs.

S. N.	Organs	Symptoms	Control	Phaseolin	OVA
1.	Intestine	Mild goblet cell hyperplasia	+	++	+++ / ++++
		Exfoliation in the intestinal wall	0	++	+
2.	Spleen	Lymphoid hyperplasia	+	++	+
		Megakaryocytes	+	++	+
		Edema	0	+	0
		Congestion	+	++	+
3.	Lungs	Thickening in the alveolar septa	+	++	+++ / ++++
		Edema	0	+	+

Histopathology of the intestine, spleen and lung of control, phaseolin and OVA treated mice. Where: S.N. = serial number; OVA = ovalbumin; + = mild symptoms; ++ = moderate symptoms; +++ = severe symptoms; 0 = no symptoms. Results were estimated after the observations of three samples of a single group.

allergenic properties like resistance to proteolytic digestion, IgE binding potential, and specific IgE levels. Phaseolin showed resistance to pepsin up to 60 min in the SGF. The percentage density of remnant phaseolin following pepsin digestion were 99.0, 91.6, 96.5, 81.5, 75.0, 73.0, 69.5, 65.7 and 56.5% of the incubation time 0, 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min, respectively (Fig. 1E and F). The purified allergen was recognized by 75% (15/20) of the individual sera from patients with red kidney bean allergy. Individual healthy volunteer's serum did not show any binding with phaseolin (Fig. 1G). Red kidney bean allergic patients showed increased specific IgE against phaseolin when compared to that of healthy volunteers (Table 2). Sera of phaseolin sensitized mice also showed the IgE binding with phaseolin, while control mice sera did not show any such binding (Fig. 1H). This study showed that similar to human subjects, the sensitized mice also showed IgE against phaseolin. This study intended us to continue the allergenic assessment of phaseolin in mouse model.

3.4. Bioinformatics analysis predicted phaseolin as a potential allergen

We further, analyzed the allergenic potential of phaseolin using in silico approaches. So far, phaseolin has not been included as an allergen in any of the allergenic databases like AllergenOnline, structural database of allergenic proteins (SDAP), international union of immunological societies (IUIS) and Allergome. In our study we have selected three

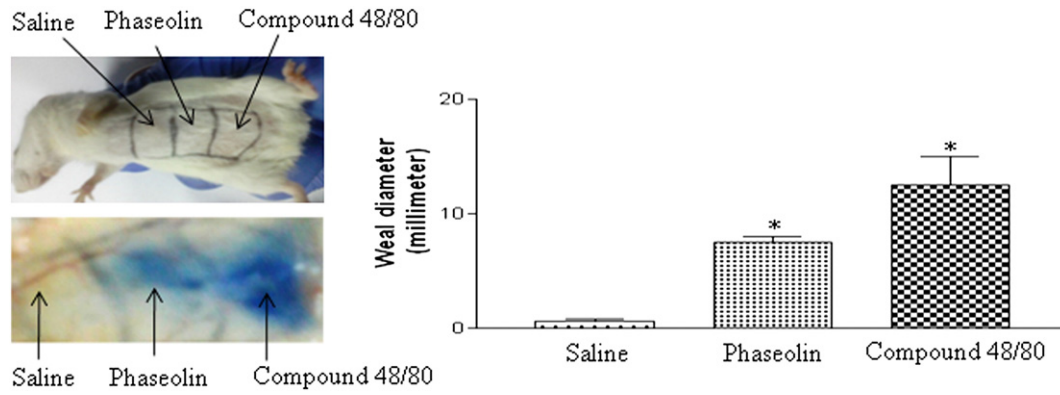


Fig. 4. Type 1 skin test. The type 1 skin test was carried in the phaseolin treated mice. The mice were treated with normal saline, phaseolin and compound 48/80 compound on the abdominal surface (as marked in the mouse shown in the figure). Following 20 min after exposure the skin samples were removed, diameter was measured and images were taken. Normal saline was taken as control while compound 48/80 as a positive control. The diameter was measured in millimeter ($n = 5$) using a weal measuring scale (where, * $p < 0.05$).

different bioinformatic tools i.e., AllergenOnline, AlgPred, and SDAP for the prediction of allergenic properties of phaseolin. As per the analysis

of AllergenOnline phaseolin showed similarity with the allergenic proteins. The 8mer ($N = 414$; number of sequences with at least one 8mer

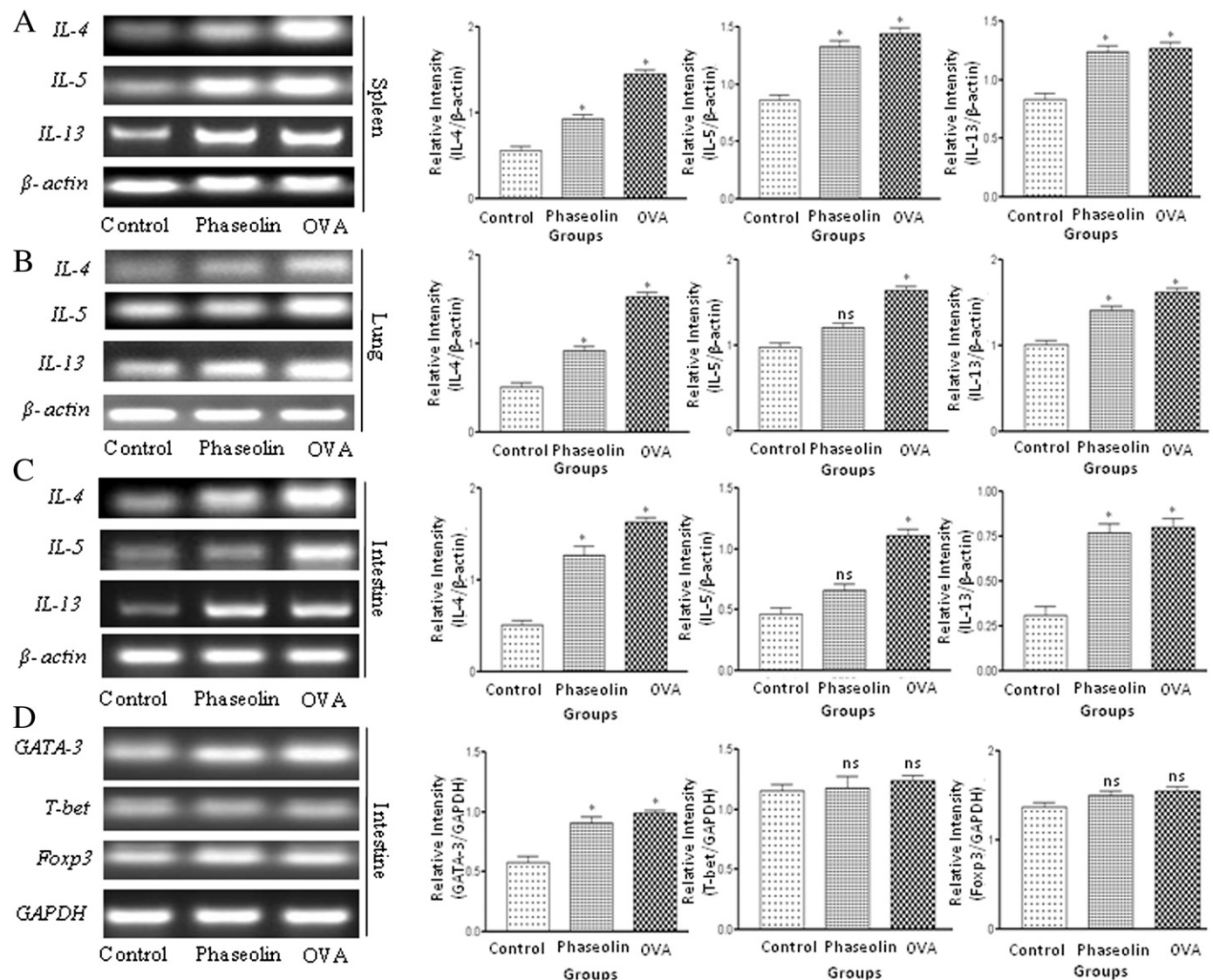


Fig. 5. The mRNA expressions of cytokines and transcription factors. A semi-quantitative RT-PCR analysis of Th2 cytokines (*IL-4*, *IL-5* and *IL-13*) in the lung, spleen and intestine of mice and transcription factors *GATA-3*, *T-bet* and *Fox p3* in the intestine were carried out using gene-specific primers in control, phaseolin and OVA treated groups. (A–C) *IL-4*, *IL-5* and *IL-13* in the spleen, lungs and intestine, respectively. (D) *GATA-3*, *T-bet* and *Foxp3* in the intestine, respectively (where, * $p < 0.05$). ns = nonsignificant.

match = 10) study revealed the similarity of phaseolin with the beta-conglycinin storage protein (*Glycine max*), Ara h 1 allergen (*Arachis hypogaea*) etc. Further, the 80mer (N = 342; number of sequences with hits 32) study revealed the similarity of phaseolin with CG4 β -conglycinin (*G. max*), allergen Len c 1.0102 allergen and Len c 1.0101 (*Lens culinaris*), Vicilin (*Pisum sativum*), conarachin (*A. hypogaea*), conglutin beta (*Lupinus angustifolius*), vicilin seed storage pr (*Juglans nigra*) and 7S globulin (*Sesamum indicum*). Furthermore, the full FASTA analysis using AllergenOnline also showed the similarity of phaseolin with other allergens as depicted in Table 3.

The Algpred results indicated phaseolin as an allergenic protein on the basis of mapping of IgE epitopes and PID, SVM module based on amino acid and dipeptide compositions, BLAST search (Hits found in ARPs database: PHFNSKAMVIVVVKGTGNLELVA) on allergen representative peptides (ARPs) and hybrid approach (SVMc + IgE epitope + ARPs BLAST + MAST). Further, phaseolin has sequences SYLQEFKHI at the position 180 which is very similar to IgE epitope SYLQEFSRNT of Ara h1 [32]. The details of Algpred results regarding the allergenicity prediction of phaseolin is given in Table 4.

The allergenicity of phaseolin was also studied using SDAP allergen database that indicated the possibility toward allergenic potential of phaseolin. The exact match for contiguous amino acids (n = 6), showed that phaseolin share similarity with known allergens like Vig r 2.0101, Lol p 11.0101, Phl p 11.0101, Gly m 5.0101, and Gly m conglycinin. FASTA alignments for 80 amino acids sliding window showed the similarity of phaseolin with Vig r 2.0101, Gly m conglycinin, Gly m 5.0201, Vig r 2.0201 etc. The full FASTA analysis for the prediction of allergenicity of phaseolin using SDAP tool is given in the Table 5.

3.5. Enhanced immunoglobulin levels, monocyte chemotactic protein-1, anaphylaxis scores and mouse mast cell protease-1 due to phaseolin exposure

After elucidation of allergenic potential of phaseolin in the clinical as well as in silico conditions, we have extended our study in the BALB/c mice. Using BALB/c mice, a high IgE responder mice strain, attempts have been made to explore the specific immunoglobulins, chemical mediators, anaphylactic symptoms affected by phaseolin exposure. Enhanced levels of specific IgE, specific IgG1, monocyte chemotactic protein-1, mouse mast cell protease-1, prominent anaphylaxis symptoms and reduced core body temperature were evident in the phaseolin treated mice when compared to control (Fig. 2A–F). The intestine of phaseolin treated mice showed mild goblet cell hyperplasia, exfoliation in the intestinal wall, while the spleen showed lymphoid hyperplasia, megakaryocytes and edema. The lungs showed congestion, thickening in the alveolar septa and edema. Similar histopathology features like

mild goblet cell hyperplasia, exfoliation in the intestine, lymphoid hyperplasia, megakaryocytes and edema in the spleen, and congestion, thickening in the alveolar septa and edema in the lungs were evident in the organs of OVA treated group. No prominent histopathological changes were observed in the control group (Fig. 3 and Table 6). A positive reaction to type I skin test was evident in the phaseolin challenged mice when compared to control (Fig. 4).

3.6. Upregulated Th2 cytokines and GATA-3 and suppressed Foxp3 levels

After elucidation of phaseolin induced immunoglobulins, mediators and anaphylaxis in BALB/c mice, we next intended to explore the molecular events behind these phenomena. Considering this objective, we targeted to explore the important cytokines as well as transcription factors in the phaseolin induced allergic manifestations. The mRNA levels of IL-4, IL-5 and IL-13 were found up-regulated in the lungs, spleen and intestine of phaseolin sensitized mice when compared to that of controls (Fig. 5A–C). The expression of GATA-3 was also found up-regulated in the intestine, while there were no significant changes in the expressions of Foxp3 and T-bet in comparison to controls (Fig. 5D). Phaseolin treated splenocytes secreted enhanced levels of IL-4, IL-5 and IL-13 when compared to respective control (Fig. 6A–C). The GATA-3 level was found significantly enhanced in the lungs, spleen and intestine of phaseolin treated mice when compared to that of control as depicted in the Fig. 7A. The level of Foxp3 was found suppressed in the intestine of phaseolin treated mice when compared to the control (Fig. 7B).

3.7. Increased mediators release from BMMCs and RBL-2H3 cells following phaseolin exposure

Following illustration of phaseolin induced allergic reactions in the clinical, in silico and in vivo conditions, we next sought to explore the allergic potential of phaseolin in ex vivo and in vitro conditions. For this purpose, BMMCs and RBL-2H3 cells were used to find out the mediator release following the exposure of phaseolin as ex vivo and in vitro tool, respectively. The BMMCs showed 43, 44, 47, 65 and 59% release of β -hexosaminidase following exposure of 10, 20, 30, 40 and 50 μ g phaseolin treatment over control (Fig. 8A). Further, phaseolin exposed RBL-2H3 cells showed 7, 60, 75, 80 and 87% increase in the release of β -hexosaminidase at the concentrations of 10, 20, 30, 40 and 50 μ g when compared to control (Fig. 8B). Furthermore, enhanced release of histamine, prostaglandin D2 and cysteinyl leukotriene were observed following exposure of phaseolin when compared to control (Fig. 8C–E).

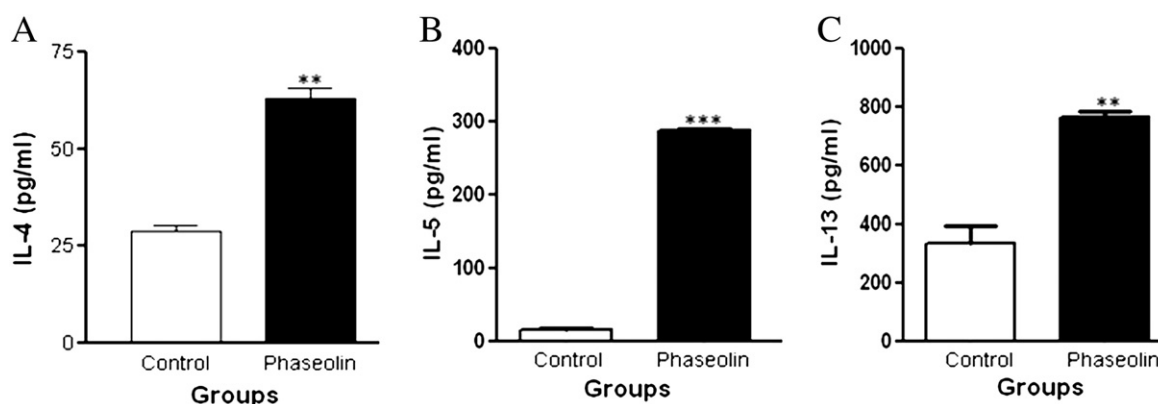


Fig. 6. The cytokine levels in the splenocytes culture supernatant. Spleens were isolated from the control and phaseolin treated mice and single cell suspension was prepared. The supernatants were collected after 72 h following the exposure of phaseolin and cytokines levels were estimated by flow cytometry. Levels of (A) IL-4, (B) IL-5 and (C) IL-13 in the control and phaseolin exposed groups (where, **p < 0.01***p < 0.001).

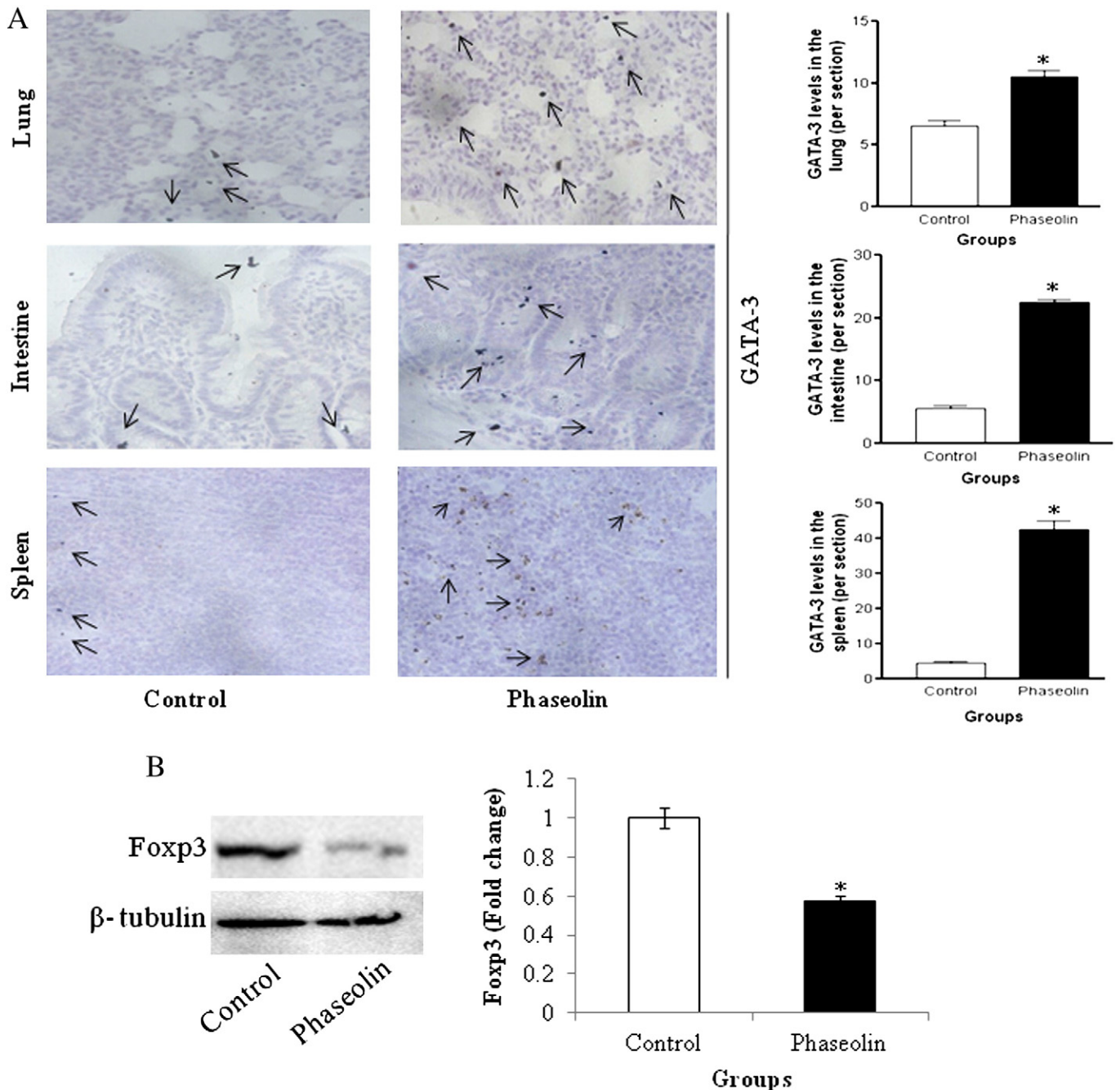


Fig. 7. GATA-3 and Foxp3 levels. The levels of GATA-3 in the lungs, spleen and intestine of control and phaseolin treated mice were detected by immunohistochemistry. Western blot analysis was also carried out to elucidate Foxp3 levels in the intestine of control and phaseolin treated mice. (A) Immunohistochemical analysis of GATA-3 levels in the lungs, spleen and intestine. (B) Western blot analysis of Foxp3 levels in the intestine of control and phaseolin treated mice (where, * $p < 0.05$).

4. Discussion

The red kidney bean is one of the world's most important grain legumes and considered as an important source of protein, some vitamins, minerals, fiber, and part of a balanced diet. But, prevalence of red kidney bean allergy may lead to a serious health concern like other allergic foods [14,16]. Prevalence of red kidney bean allergy in the patients suffering from bronchial asthma, allergic rhinitis and dermatitis was studied previously by our group [15]. The SPT and specific IgE levels have been recommended as an important diagnostic tool for food allergy [33]. Elevated levels of total IgE and specific IgE have been reported in case of several food allergies including cow's milk, hen's egg, wheat and soy allergy [34,35]. Additionally, an inclusion of IgE immunoblot for prediction of red kidney bean allergy strengthens

the diagnostic approach because IgE immunoblot has been reported as a diagnostic approach in the case of *Anisakis simplex* allergy [36]. In this present study, we have used sera of red kidney bean allergic patients to recognize IgE binding proteins. We have found a protein with approximate molecular weight 50 kDa as a major IgE binding protein of red kidney bean. Thus, we targeted this protein for the purification, characterization and allergenicity assessment.

Different chromatography and immuno-biochemical tools have been used for the isolation and characterization of allergenic proteins from food sources [37]. In the present study, we have purified phaseolin using a combination of approaches including fractionations, anion exchange chromatography, IgE-immunoblotting and reverse phase-HPLC similar to the purification of other allergens [38–40]. The allergenic proteins have been reported to possess resistance against proteolytic

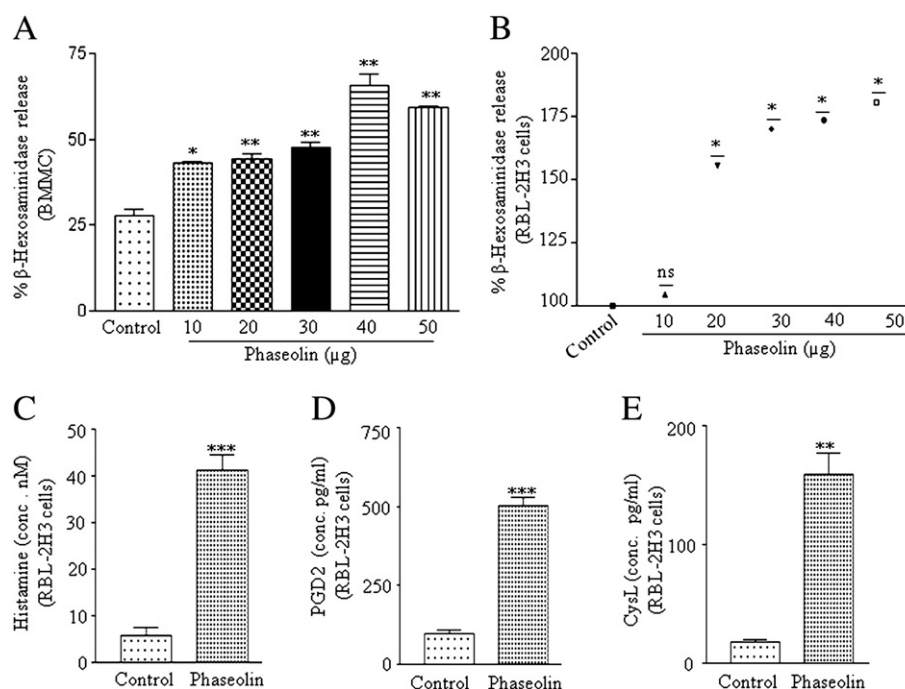


Fig. 8. Mediators release from bone marrow mast cells (BMMCs) and RBL-2H3 cells. Mice from control and phaseolin treated groups were sacrificed, and femur and tibia were collected. Bone marrow cells were cultured in Iscove's modified Dulbecco's medium supplemented with IL-3 and stem cell factor. The β -hexosaminidase release in BMMCs following the exposure of 10, 20, 30, 40 and 50 μ g phaseolin. The release of β -hexosaminidase, histamine, prostaglandin D_2 and cysteinyl leukotriene following the exposure of phaseolin was studied in vitro using RBL-2H3 cells. RBL-2H3 cells were primed with the sera of phaseolin sensitized and control mice, respectively. (A) β -Hexosaminidase release in BMMCs following phaseolin exposure, (B) β -hexosaminidase release in RBL-2H3 cells following phaseolin exposure, (C) histamine, (D) prostaglandin D_2 and (E) cysteinyl leukotriene levels in RBL-2H3 cells following phaseolin exposure (where, * p < 0.05, ** p < 0.01, *** p < 0.001).

enzyme pepsin [41]. Similar to phaseolin, several allergens from different foods including Pru ar 3 have showed resistance against gastrointestinal digestion [42]. The IgE immunoblot and enhanced level of phaseolin specific IgE using serum of individual red kidney bean allergic patients suggested the possibility of IgE mediated reactions in the susceptible individuals.

The use of proteomic and bioinformatic tools in the prediction of allergenic properties of proteins including B and T cell epitopes has been found to be of pivotal importance [43]. Phaseolin comes from cupin protein super family and its allergenicity has been reported in several foods including green gram [22]. The SDAP data analysis revealed the similarity of phaseolin with other food allergens from green gram, soybean, lentil, lupin, peanut etc. indicating the possibility of diverse cross-reactivity and risk in the susceptible patients. The amino acid sequence of phaseolin was similar to several food allergens including β -conglycinin of soybeans namely Gly m 5, Len c 1 allergen of lentil, Ara h1 of peanut and Ses i 3 of sesame. The allergenicity of all these proteins is well established [44–47].

Following allergenic assessment of phaseolin allergenicity in human subjects we moved to explore several immunological aspects behind the phaseolin induced allergenicity in the murine model. The allergenicity of phaseolin was evident by significantly enhanced levels of total IgE, specific IgE, monocyte chemotactic protein-1, mouse mast cell protease-1, prominent anaphylaxis symptoms and reduced core body temperature. Similar to our study the potential allergen of peanut Ara h2 and Ara h6 has been reported to exhibit very similar results in murine model [48]. Similar to red kidney bean allergic patients, IgE-immunoblotting with phaseolin sensitized mice pooled sera has also showed the IgE binding. The monocyte chemotactic protein-1 has been reported as an important mediator of monocyte and CD4⁺/CD8⁺ lymphocyte recruitment and a potent histamine-releasing agent for basophils and mast cells [49]. Effective production of mouse mast cell protease-1 together with specific IgE and IgG1 suggests a breakdown in oral tolerance to the allergen [50,51]. Histopathological changes observed in the phaseolin treated group indicated the allergic signs similar to other food allergens.

Symptoms have been reported in the mouse model of IgE-mediated cow's milk hypersensitivity that mimics the clinical features of immediate cow milk allergy in humans [52]. In our study, phaseolin treated mice showed positive type 1 skin test, which indicated the in vivo relevance of its allergenic potential. Type 1 skin test was also found positive in OVA treated mice corroborating our findings [28]. Enhanced mast cells are indicative of its important role in phaseolin induced allergic reactions (Supporting Fig. 1). Increased level of mast cells in the phaseolin treated mice validated the possibility of phaseolin induced allergenicity [8].

The expression of IL-4, IL-5 and IL-13 mRNA was found enhanced in the lungs, spleen and intestine tissue from mice sensitized with phaseolin indicated that phaseolin may induce Th2 type of reactions. Similar to our study the mRNA of IL-4, IL-5 and IL-13 has been reported to increase in the major food allergen shrimp tropomyosin [53]. Further, the enhanced expressions GATA-3 and no significant changes in the expression of Foxp3 and *T-bet* validated the phaseolin induced Th2 reactions. Immunohistochemical analysis revealed enhanced GATA-3 levels in the organs of phaseolin treated mice. This result further validated the role of GATA-3 in the phaseolin induced Th2 type allergic manifestation. At protein level the increased IL-4, IL-5 and IL-13 in the culture supernatants of the phaseolin exposed splenocytes further confirmed the role of these Th2 cytokines in phaseolin induced allergic manifestations [54,55]. We noticed reduced level of Foxp3 in the intestine of phaseolin treated mice that correlate the induction of allergy and impaired oral tolerance [56].

Phaseolin showed an elevated level of β -hexosaminidase release in BMMCs that further confirmed the allergenic potential of phaseolin in ex vivo condition. Recently, release of β -hexosaminidase in BMMCs following exposure of raw and autoclaved red kidney bean crude protein extract and PHA have been reported by our group [19]. RBL-2H3 has been reported to be an important tool to monitor IgE-mediated mast cell degranulation in vitro [57]. In this present study, enhanced levels of allergic mediators like β -hexosaminidase, histamine, prostaglandin D_2 and cysteinyl leukotriene following exposure of phaseolin indicated its mast cell degranulative property.

5. Conclusion

Taken together, the allergenicity potential of phaseolin can be evident by enhanced total and specific IgE, anaphylaxis, histopathological changes, gene expressions of *IL-4* and *IL-13* and *GATA-3* genes, Th2 cytokines in the splenocyte culture supernatants and β -hexosaminidase from BMMCs and RBL-2H3 cells. Overall, phaseolin may be responsible for potential allergenicity that may be the reason for allergenic responses in susceptible individuals.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2014.01.014>.

Conflict of interest statement

The authors have declared no conflict of interest.

Acknowledgments

We are grateful to the Director of the Institute for his keen interest in this study. This work was financially supported by the In Depth Project of Council of Scientific and Industrial Research (CSIR), New Delhi. Thanks are due to Prof. R. Prasad and Dr. Giridhar BH for their consistent support during SPT at CSM Medical University, Lucknow, India. Authors SK and AKV are thankful to CSIR, New Delhi for the award of their Senior Research Fellowships. AS and DK is thankful to CSIR and UGC, New Delhi for the award of their Junior Research Fellowships, respectively. Thanks are due to Dr. Pradeep K Shrivastava for his editorial assistance. We are thankful to Mrs. Sumita Dixit for her help in HPLC. This is CSIR-IITR manuscript No. 3064.

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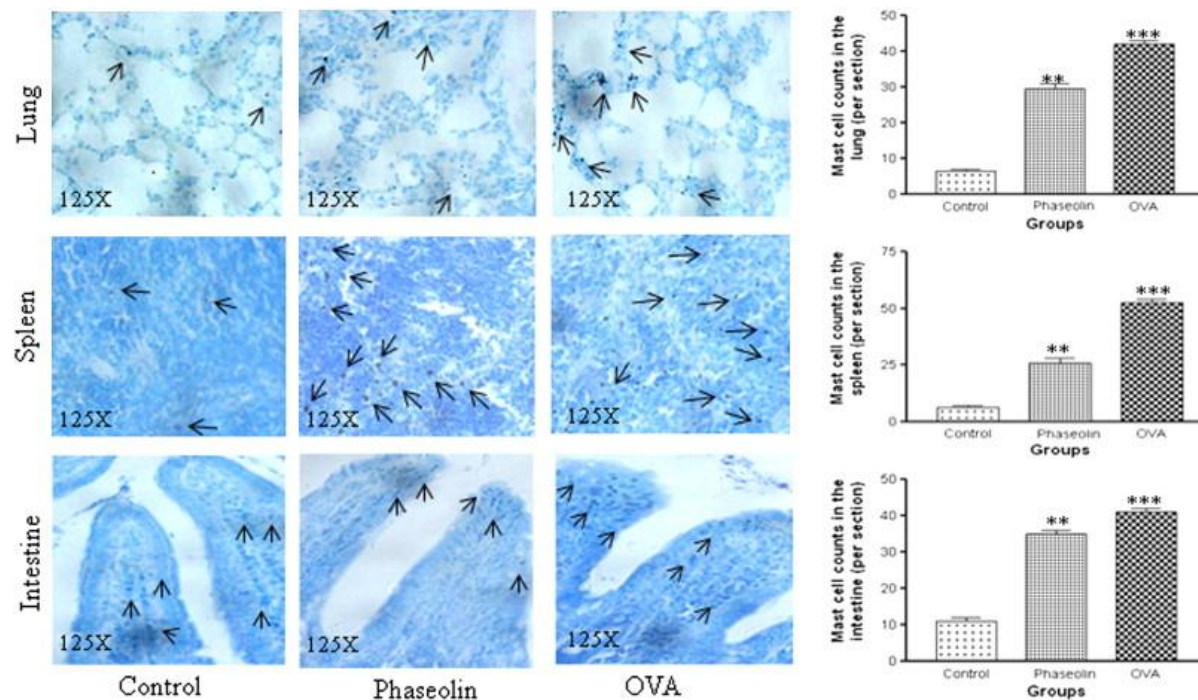
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Supporting Table 1: Details of healthy volunteers and red kidney bean allergic human subjects

Patient ID	Age/Sex	Allergic symptoms	SPT	Total IgE (kU/L)	Sp IgE kAU/L
Patient-18	28/M	BA, AD	+3	342*	5*
Patient-19	39/F	AR	+2	653*	3.5*
Patient-20	67/M	BA, AR, AD	+3	259*	3.8*

Supporting Table 1: Diagnosis of red kidney bean allergic patients. Where, Age in years, M = male, F = female, BA = bronchial asthma, AD = atopic dermatitis, AR = allergic rhinitis, SPT= Skin prick test, measured according to wheel size (in mm). Total and specific IgE presented in mean $\pm$ SEM (p value <0.05 has been indicated as *). For Control 1 to 10 and Patient 1 to 17 see reference [15].



Supporting Fig. 1. Illustration of mast cells in the lungs, spleen and intestine of phaseolin treated and control groups. Samples of the intestine, lungs and spleen from control, phaseolin, and OVA treated mice were collected and were fixed in 10% formalin in ...

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International Immunopharmacology, Volume 19, Issue 1, 2014, 178–190

<http://dx.doi.org/10.1016/j.intimp.2014.01.014>