



Allergic manifestation by black gram (*Vigna mungo*) proteins in allergic patients, BALB/c mice and RBL-2H3 cells

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ABSTRACT

The prevalence of black gram (*Vigna mungo*) induced allergic reactions are reported from several parts of the world including Asia and Australia. But, a thorough exploration of the allergic reactions induced by black gram proteins is still lacking. Therefore, efforts have been made to explore black gram allergy using in vivo and in vitro approaches. In this study, Simulated Gastric Fluid (SGF) assay and IgE immunoblotting were carried out to identify clinically relevant allergens of black gram. BALB/c mice and RBL-2H3 cells were used for elucidation of allergenic reactions of black gram proteins. Further, this study was extended to screen black gram sensitive patients among nasobronchial allergic patients on the basis of clinical history, skin prick test (SPT), specific IgE levels and IgE immunoblotting. Enhanced levels of specific IgE, IgG1/IgG2a ($p < 0.05$), histamine ($p < 0.05$), clinical symptoms, pathological indications in the lungs, intestine and spleen were evident in black gram sensitized BALB/c mice. Moreover, the expression of Th2 cytokine transcripts and GATA-3/T-bet ratio was found enhanced in the treated group. In vitro studies on RBL-2H3 cells, showed increased release of β -hexosaminidase ($p < 0.05$), histamine ($p < 0.05$), cysteinyl leukotriene ($p < 0.05$) and prostaglandin D2 ($p < 0.05$). Further, 8.5% of screened patients were found allergic to black gram and concomitant sensitization with other allergens has shown the possibility of further enhancement in allergenic problem. Conclusively, the present study suggested that black gram consumption may be responsible for inducing immediate type of allergic sensitization in susceptible subjects.

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1. Introduction

Nowadays, food allergy is a worldwide health issue induced by certain food in susceptible individual. Most of the allergic reactions have been found to mediate via IgE immunoglobulins and occur within minutes to hours of food consumption [1]. Legume allergy prevalence reports have taken the lime light nowadays from several parts of the world. Legume has been found to contribute a major proportion of 'big eight' allergic foods. The allergenicity assessment along with identification, characterization and purification of allergens from several legumes including peanut, soybean, lentil, chickpea, green gram, red kidney bean and red gram has been well studied [2]. Black gram (*Vigna mungo*,

family Leguminosae), a native of India, is commonly known as 'urad', 'urid bean', 'black matpe bean' and 'black lentil'. It is widely cultivated in India, Pakistan, Sri Lanka, Thailand, Indonesia, Malaysia and to a lesser extent in Australia and other Asian and South Pacific countries. Black gram is a protein-rich staple food containing about 24% protein [3]. Black gram is used in different ways as a whole seed or after splitting into two as 'dal' and in culinary preparations. It is also recommended for diabetic patients. Furthermore, neutral detergent fiber isolated from black gram possesses significant hypolipidemic, hypoglycemic and protective effect against colon cancer [4]. But, high consumption of any proteinaceous food including leguminous crops may increase the probability of sensitization against the potentially allergenic proteins in susceptible individuals [2,5].

Earlier study on black gram has mainly focused on the IgE-mediated sensitization in patients with asthma and allergic rhinitis [6]. Recently, 28 kDa IgE binding protein from black gram has been identified and characterized [7]. Several reports have suggested that black gram has allergenic peptides but mechanistic aspects behind its hypersensitivity remain unknown, therefore the present study aimed to explore black gram allergy using several pivotal allergic parameters.

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In this study, human subjects were diagnosed for black gram allergy and related allergic manifestations have been presented. The SGF (Simulated Gastric Fluid) assay and IgE immunoblotting were carried out to identify clinically relevant allergens of black gram. BALB/c mice were used as a model to assess the allergenic potential of black gram. Further, RBL-2H3 cells were used for the estimation of mast cell mediators such as β -hexosaminidase, histamine, cysteinyl leukotriene and prostaglandin D₂. Therefore, this study will be helpful to provide an overview of immunological parameters modulated during the elicitation of black gram hypersensitivity reactions. Moreover, identification and characterization of allergens may be useful in the development of non-allergenic transgenic variety as well as for therapeutic purpose.

2. Materials and methods

2.1. Screening of black gram allergy in human subjects

Two hundred bronchial asthma and allergic rhinitis patients at the outpatient department (OPD) of the Pulmonary Medicine, King George Medical University (KGMU), Lucknow have been included in this study. Black gram allergic patients were screened on the basis of their clinical history which was further confirmed by skin prick test (SPT), and specific IgE levels. The age of the patients included in the study ranged from 16 to 75 years. SPT and sera collection were carried-out with patient's consent and the study protocol was approved by the Human Ethics Committee of the KGMU, Lucknow (IEC No. 2938/R. Cell-11).

SPT was performed with allergens of pollens, fungi, insects, dust, danders and food extracts (Alcit India Private Limited, New Delhi). Glycerinated buffer saline and histamine acid phosphate were used as negative and positive controls respectively. The results were read 20 min after skin prick. Blood was collected from black gram SPT positive patients ($n = 17$). Patients showing no weal against black gram were designated as black gram SPT negative patients ($n = 183$) and they were not included in this study. Serum was separated from the whole blood and stored at -80°C . Specific IgE level was determined as described earlier [8] and results were expressed in terms of absorbance at 492 nm.

2.2. Preparation of black gram crude protein extracts (BG-CPE)

Black gram seeds were purchased from a local certified seed vendor. Seeds were put in a waterproof container with a thin layer of colored silica gel desiccant as indicator of dryness. BG-CPE was prepared as described earlier [9,10]. Briefly, powdered seeds were defatted with *n*-hexane. Defatted flour was macerated in the phosphate buffer (20 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture was agitated overnight at 4°C , centrifuged for 30 min at the $10,000 \times g$ and supernatant was recovered, filtered through a $0.45 \mu\text{m}$ syringe filter and stored as aliquots at -80°C until used.

2.3. Thermal stability assay

To resist the change in biological activity under high temperature conditions indicates that protein may have the ability to provoke allergic responses and this is checked by incubating protein at a range of temperature. Briefly, BG-CPE was incubated at a temperature range from 25°C to 95°C up to 30 min. Samples were mixed with stopping solution that is $2\times$ Tris-Tricine SDS sample buffer (containing 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 100 mM Tris, pH 8.8), boiled for 5 min and loaded on 12% SDS-PAGE to analyze the effect of thermal treatment on the stability of black gram protein [11].

2.4. Identification of clinically relevant allergens of black gram using Pepsin Digestibility Assay and IgE immunoblotting

In vitro SGF assay was performed as described earlier [12]. The amount of pepsin used in the SGF assays was about 13 times more than that of test proteins (by weight) to ensure sufficient degradation. Boiled samples ($20 \mu\text{L}/\text{well}$) were subjected to 12% SDS-PAGE at 22 mA for 4 h 30 min. Standard molecular weight markers (M) were included to estimate the mol wt of the proteins. Coomassie Brilliant Blue (G-250) stain was used for staining the gel. Gel image was captured and densitometry analysis was performed for validation of SGF results using Syngene Bio Imaging System (Syngene, Cambridge, UK).

To detect IgE binding proteins of black gram, IgE immunoblotting was performed [13]. BG-CPE was resolved on 12% SDS-PAGE and electrophoretically transferred on PVDF membrane using a semidry blotting unit (Amersham Biosciences, St Francisco, USA). Blots were blocked with 3% BSA in PBS-T buffer (pH 7.4) and kept overnight at 4°C . Pooled black gram sensitized mice and SPT positive human sera were used as primary antibodies while goat anti-mice IgE peroxidase conjugate and goat anti-human IgE peroxidase conjugate (Sigma Chemical Company, dilution 1:1000 in PBST + 1% BSA) were used as secondary antibodies respectively.

2.5. BALB/c mice and sensitization protocol

Healthy 6–8 weeks old female BALB/c mice ($22 \pm 3 \text{ g}$) were obtained from the CSIR-IITR, Lucknow, India animal breeding colony. Mice were maintained under standard laboratory conditions in specific pathogen free environment and on black gram and peanut free diet. Animal study was performed after approval of Animal Ethics Committee of CSIR-IITR, Lucknow (ITRC/IAEC/28/2011).

Mice were sensitized according to the earlier described protocol [14]. In brief, mice were randomly divided into three groups ($n = 10/\text{group}$). Groups of mice were injected intraperitoneally with $100 \mu\text{L}$ phosphate buffered saline (PBS), $100 \mu\text{g}$ black gram protein in $100 \mu\text{L}$ PBS and $100 \mu\text{g}$ peanut protein in $100 \mu\text{L}$ PBS respectively once in a week for 7 weeks. Blood samples were collected from retro-orbital sinus to measure black gram specific IgE, IgG1 and IgG2a antibodies on 15, 43 and 59 days. On day 60, groups of mice ($n = 10/\text{group}$) were challenged intraperitoneally with 10 mg CPE. Tissue and blood samples were collected as per schedule as depicted in Fig. 1. Peanut seeds were chosen as positive control as allergenic potential of peanut protein is well documented [15–17].

2.6. Specific IgE, IgG1 and IgG2a level estimation

Specific IgE, IgG1 and IgG2a levels were estimated with the earlier described methods [8,18]. Briefly, microtiter plates (Maxisorp; NuncTM Immunomodule, Roskilde, Denmark) were coated with $1 \mu\text{g}$ black gram protein in $100 \mu\text{L}$ per well in carbonate buffer (pH 9.6). Non-specific sites were blocked with 3% BSA, washed and incubated with the sera of the black gram treated mice (1:10 dilution). The sera from PBS treated mice were treated as control. After washing, the plates were incubated for 2 h with anti-mice IgE-HRP (1:1000 v/v; Southern Biotech, Birmingham, USA) and the absorbance was read at 492 nm (Biotek, Power Wave XS2). Measurement of specific IgG1 and IgG2a in sera samples was also performed by ELISA. In brief, $1 \mu\text{g}$ black gram protein in $100 \mu\text{L}$ per well in carbonate buffer was coated onto 96-well microplates and kept overnight at 4°C and then blocked with $200 \mu\text{L}$ of 3% BSA. Diluted serum samples (1:1000 dilutions for specific IgG1 and specific IgG2a) were added to each well and incubated. The plates were incubated for 2 h at 37°C and then washed 3 times with washing buffer (PBS-T). To each well, $100 \mu\text{L}$ of HRP conjugated goat antimouse IgG1 and IgG2a antibodies (1:1000; Southern Biotech, Birmingham, USA) was added and incubated for 1 h at 37°C . The plates were washed with washing buffer. $50 \mu\text{L}$ substrate solution (5 mg ortho-

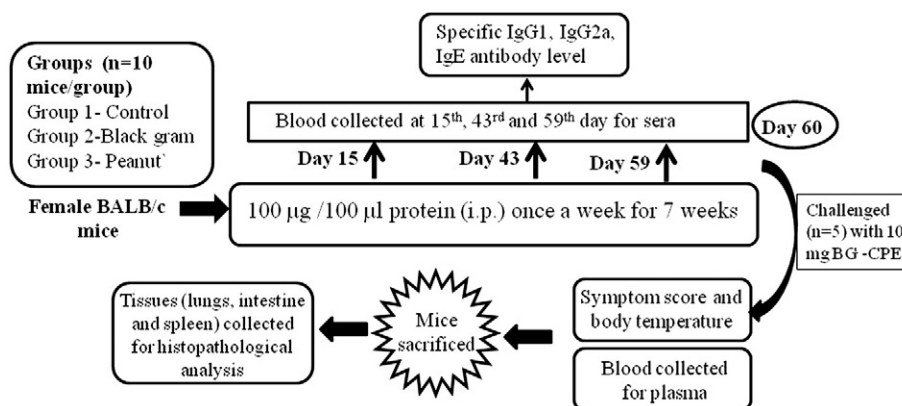


Fig. 1. Diagrammatic representation of animal treatment protocol.

phenylenediamine in 10 mL substrate buffer and 10 µL H₂O₂) was added to each well and the plate was incubated for 30 min at 37 °C in the dark. The reaction was stopped by the addition of 50 µL of stopping solution (5 N H₂SO₄) and the absorbance was taken at 492 nm in ELISA plate reader (Biotek, Power Wave XS2).

2.7. Systemic anaphylaxis score, rectal temperature and histamine level

Anaphylactic reactions were scored using the scoring system described earlier by Li et al. [19]:

0 – no symptoms; 1 – scratching and rubbing around the snout and head; 2 – puffiness around the eyes and snout, pillar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; 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 temperature was monitored 20 min after challenge using digital rectal thermometer (Bioseb, France). Plasma histamine level was determined 30 min after challenge using ELISA (SPI-BIO, Montigny le Bretonneux, France) following the manufacturers' instructions.

2.8. Histopathological analysis of tissues

The mice were challenged and later sacrificed by cervical dislocation. The spleen, lungs and intestine were taken for histopathology. Tissues were fixed in 10% formalin in PBS, embedded in paraffin, and cut into 3–5-µm thick sections.

2.9. Semi quantitative polymerase chain reaction (PCR)

A semi-quantitative reverse transcriptase polymerase chain reaction analysis of Th2 cytokines (IL-4, IL-5 and IL-13) and Th2/Th1 transcription factors (GATA-3, and T-bet) in the intestine of all treated groups was carried out by gene specific primers according to the method described earlier [20]. The primer sequences were as follows: IL-4: sense 5'-TCGGCATTTTGAAC GAGGTC-3', antisense 5'-AAAAGCCGAAAGAGT CT C-3'; IL-5: sense 5'-TCACCGA GCTCTGTTGACAA-3', antisense 5'-CCACACTTCTTTTT GGCG-3'; IL-13: sense 5'-GACCCAGAGGATAT TGCATG-3', antisense 5'-CCAGCAAAGTCTGATGT GAG-3'; GATA-3: sense 5'-TCTCACTCTCGAGGC AGCATGA-3', antisense, 5'-GGTAC CATC TCGCCGCCACAG-3'; T-bet: sense 5'-TCCCATTCCTGTCTT CA-3', antisense 5'-GCTGCCTTCTGCCTTTC-3'; and GAPDH: sense 5-TTCAACACCA TG GAGAAGGC-3, antisense 5-GG CATGGA. Total RNA from intestine was isolated with RNeasy® (MRC, OH, USA) according to the manufacturers' instructions (Molecular Research Center, Inc. Cincinnati, OH). The RT-PCR was performed by commercially available RT-PCR kit (QIAGEN, Netherlands). The program of thermal cycler was as follows;

reverse transcription at 50 °C for 30 min and subsequent 40 cycles of 94 °C denaturation for 30 s, 55 °C annealing for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Further, 8 µL of PCR product was run on 2% agarose gel containing ethidium bromide. The GAPDH gene was chosen as an endogenous control. Densitometry of each band was done by the Gene tools software (Syngene, Cambridge, UK).

2.10. Western blots for GATA-3, SOCS3, STAT-6 and T-bet transcription factors

The levels of Th1/Th2 transcription factors GATA-3, SOCS3, STAT-6 and T-bet in the intestinal proteins of black gram sensitized mice were detected by immunoblotting according to the method described earlier [21]. Briefly, intestinal proteins of control and black gram treated groups were extracted in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, pH 7.4). Intestinal proteins were resolved on 12% SDS-PAGE and electrophoretically transferred to a PVDF (Immobilon P 0.45 µm, cat no – IPVH00010, Millipore, Bangalore) using a semi-dry blotting unit (Amersham Biosciences, St Francisco, USA). Blocking was done with 3% BSA and kept for 2 h at 37 °C. Washing was done by PBS-T (20 mM Na₂HPO₄, 2 mM KH₂PO₄, 5.4 mM KCl, 0.5 M NaCl, pH 7.0 and 0.5% of Tween 20) for 4–5 times. The goat anti-mouse GATA-3, SOCS3, STAT-6, T-bet antibodies (dilution 1:200 in PBS-T + 3% BSA) were used as primary antibodies, respectively. The goat anti-mouse IgG peroxidase conjugate was used as a secondary antibody. The β-actin (Santa Cruz biotechnology, CA, USA) was used as an endogenous control. Images were captured using Syngene gel documentation system equipped with a CCD camera (Syngene, Cambridge, UK). Densitometry of each band was performed by the Gene tools software (Syngene, Cambridge, UK).

2.11. Mediators' release assay in RBL-2H3 cell line

RBL-2H3 cells (ATCC, USA) were used to find out the level of β-hexosaminidase, histamine, prostaglandin D2 and cysteinyl leukotrienes. β-Hexosaminidase release assay was performed according to the previously described protocol with slight modifications [22]. In brief, pooled sera of black gram sensitized mice were added in RBL-2H3 cells. The IgE-sensitized RBL cells attached to microtiter wells were washed twice in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose, 20 mM HEPES, and 1 mg/mL BSA at pH 7.4) and stimulation was initiated by the addition of 250 µL of serially diluted antigens (25, 50, 75, 100 and 125 µg) in Tyrode's buffer. The samples were then incubated for 1 h at 37 °C, and

degranulation was stopped by placing the cells on ice. To determine the amount of β -hexosaminidase activity released by the cells, 25 μ L of supernatant and 100 μ L of substrate buffer {1.2 mM β -hexosaminidase-substrate (4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide), in 0.05 M sodium acetate buffer (pH 4.4)}, were mixed in a separate 96-well plate and incubated for 30 min at 37 °C. The reaction was quenched by addition of 175 μ L of quenching buffer (0.1 M glycine-carbonate buffer, pH 10.0). Controls without antigen were used to measure spontaneous release. Total β -hexosaminidase release was obtained by lysing the cells with 0.1% Triton-X 100. β -Hexosaminidase assay was performed according to previously described methods using 5 different concentrations of BG-CPE (25 μ g, 50 μ g, 75 μ g, 100 μ g and 125 μ g). β -Hexosaminidase activity in the supernatant was quantified by measuring the absorbance intensity of the hydrolyzed substrate in ELISA microplate reader (Biotek, Power wave XS2) at 360 nm and 450 nm wavelength. Level of histamine, prostaglandin D2 and cysteinyl leukotrienes in the RBL-2H3 was estimated according to the manufacturers' instructions (Cayman Chemicals, East Ellsworth Road, USA).

2.12. Statistical analysis

The statistical significance of the data obtained was determined using a software package from InStat versions 3.0 and 5.0 (Graph pad, San Diego, CA, USA; <http://www.graphpad.com>) using the Bonferroni analysis of variance (ANOVA) test. Values for all measurements are expressed as mean $\pm$ SEM. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Marked positive skin reaction to black gram was observed in 8.5% of screened allergic patients

Two hundred patients were screened to know the scenario of black gram induced allergenicity. Out of 200 patients, seventeen (8.5%) showed a marked positive skin reaction to black gram. SPT, specific IgE and allergic symptoms along with related information regarding allergy in screened patients are presented in Table 1. In black gram sensitive patients, specific IgE (OD) was found to range between 0.424 and 1.51. In addition, black gram allergic patients had concomitant sensitization to other allergens as well (Table 2). Black gram sensitive individuals were also found to be allergic to pollens, fungi, insects, dust and dander (Table 3). Out of 17 patients, 15 showed positive SPT with pollens while 14 showed sensitization with insects. All black gram

Table 2

Prevalence of concomitant sensitization of black gram sensitive patients (n = 17) to other allergens.

Groups	Allergens	Patients
1	Pollens	15
2	Fungi	5
3	Insects	14
4	Dust	8
5	Dander	5
6	Non-leguminous food items	11
7	Animal products	4
8	Other legumes	17

allergic patients also had sensitivity to other leguminous crops as evident by marked positive skin reactions elicited by different legumes (Table 4).

3.2. No significant change in black gram protein profile on thermal treatment

To identify the allergenic proteins in the BG-CPE, thermal stability assay was performed in the range of temperature 25 °C to 95 °C and no significant change was found between the protein profiles of heat treated protein at different temperatures (25, 37, 55, 75 and 95 °C) and untreated protein (Fig. 2a).

3.3. Pepsin Digestibility Assay revealed two stable protein components in SGF

Two proteins with approximate mol wt of 50 kDa and 28 kDa were found to be pepsin resistant as shown in pepsin digestibility profile of the BG-CPE in Fig. 2b. Proteins of 50 kDa and 28 kDa were found stable in SGF up to 30 min and 2 min respectively. Densitometry analysis revealed that the percentages of 50 kDa protein that remained undigested were 98.5, 98, 93, 75, 61, and 28, up to 30 min in SGF. Density of 28 kDa protein was found to decrease down to 88.5, 87 and 86.5% at 0.25, 1, and 2 min respectively and degraded within 8 min (Fig. 2c).

3.4. IgE immunoblotting of BG-CPE showed three IgE-binding proteins

Immunoblotting of BG-CPE with sera of black gram-sensitized mice showed IgE binding at 50 kDa, 28 kDa and 26 kDa proteins i.e., allergenic proteins in BG-CPE (Fig. 3a). Further, western blot analysis with sensitive patient's sera had indicated 50 kDa, 28 kDa and 26 kDa bands as major IgE binding proteins (Fig. 3b). However, no IgE binding

Table 1

Details of black gram-sensitive respiratory allergy patients.

S.N.	Age (years)/sex	Residence	Diet	Family history of atopy	SPT (weal)	Specific IgE (OD)	Allergic symptoms
Patient-1	43/M	Urban	Non-vegetarian	Genetic	+2	1.007733	AR, D
Patient-2	19/F	Rural	Vegetarian	Genetic	+2	1.0574	BA, D
Patient-3	41/M	Urban	Vegetarian	Genetic	+1	0.424733	AR
Patient-4	23/M	Rural	Vegetarian	Genetic	+3	0.668067	AR, BA, D
Patient-5	20/M	Urban	Vegetarian	Genetic	+2	1.0454	AR, BA
Patient-6	19/F	Urban	Non-vegetarian	Genetic	+2	0.849067	AR, BA
Patient-7	30/M	Rural	Non-vegetarian	–	+2	0.989	AR, BA
Patient-8	30/M	Urban	Vegetarian	–	+2	0.927067	AR, BA
Patient-9	13/M	Urban	Non-vegetarian	Genetic	+1	0.724	AR
Patient-10	35/F	Rural	Vegetarian	–	+2	0.4844	AR, D
Patient-11	50/M	Rural	Non-vegetarian	Genetic	+3	1.164067	AR, BA
Patient-12	21/F	Urban	Non-vegetarian	–	+2	1.488	AR
Patient-13	20/F	Urban	Non-vegetarian	–	+3	1.418067	AR, BA, D
Patient-14	35/F	Rural	Non-vegetarian	–	+1	1.023733	AR, BA
Patient-15	26/M	Rural	Non-vegetarian	Genetic	+3	1.512067	AR, BA
Patient-16	21/F	Urban	Vegetarian	Genetic	+3	1.034067	AR, BA, D
Patient-17	22/M	Rural	Non-vegetarian	Genetic	+1	0.8224	AR, BA

M, male; F, female; IgE, immunoglobulin E; SPT, skin prick test; AR, allergic rhinitis; BA, bronchial asthma; D, dermatitis; SPT (skin prick test) measured according to weal size (in mm). Specific IgE was presented in terms of absorbance at 492 nm.

Table 3

Prevalence of concomitant sensitization of black gram sensitive patients (n = 17) to other non-food allergens.

Patients	Pollens	Fungi	Insects	Dust	Dander
Patient-1	–	<i>Rhizopus nigricans</i> , <i>Trichoderma species</i>	Cockroach (male), cockroach (female), cricket, grass hopper	Paper dust	Buffalo dander, Cow dander
Patient-2	<i>Adhatoda vasica</i> , <i>Ailanthus encelsa</i>		Cockroach (male), cockroach (female), cricket	–	–
Patient-3	<i>Gynandropsis gynandra</i>	–	Cockroach (male), cockroach (female), grass hopper	Grain dust	–
Patient-4	–	–	Cockroach (male), cockroach (female)	–	–
Patient-5	<i>Ailanthus encelsa</i> , <i>Amaranthus spinosus</i> , <i>Brassica compestris</i> , <i>Chenopodium murale</i> , <i>Cyperus rotundus</i> , <i>Ricinus communis</i> , <i>Rumex dentatus</i> , <i>Sorghum vulgare</i> , <i>Zea mays</i> , <i>Carsia siamea</i> , <i>Pennisetum thyphoids</i>	<i>Alternaria teneis</i> , <i>Aspergillus flavus</i> , <i>Aspergillus fumigatus</i> , <i>Aspergillus niger</i> , <i>Neurospora sitophila</i> , <i>Penicillium spp.</i> , <i>Trichoderma species</i>	Cockroach (male), cockroach (female)	Cotton mill dust, house dust	Cow dander
Patient-6	<i>Prosopis juliflora</i> , <i>Ricinus communis</i> , <i>Holoptelea inlegrefcelia</i>	<i>Aspergillus flavus</i> , <i>Cladosporium herbarum</i>	–	–	–
Patient-7	<i>Adhatoda vasica</i> , <i>Dodanaea viscosa</i>	–	Cockroach (male), cockroach (female), grass hopper	–	–
Patient-8	<i>Argemone maxicana</i> , <i>Azadirachta compestris</i> , <i>Brassica compestris</i> , <i>Gynandropsis gynandra</i> , <i>Parthenium hysterophorus</i> , <i>Prosopis juliflora</i> , <i>Putranjiva roxburghii</i> , <i>Ricinus communis</i> , <i>Rumex dentatus</i> , <i>Pennisetum thyphoids</i> , <i>Holoptelea inlegrefcelia</i>	–	Cockroach (male), cockroach (female), cricket, grass hopper	Cotton mill dust, house dust, grain dust	Buffalo dander
Patient-9	<i>Putranjiva roxburghii</i> , <i>Ricinus communis</i> , <i>Pennisetum thyphoids</i>	–	Cockroach (male), cockroach (female), cricket, grass hopper	–	–
Patient-10	<i>Ailanthus encelsa</i> , <i>Amaranthus spinosus</i> , <i>Brassica compestris</i> , <i>Cannabis sativa</i> , <i>Prosopis juliflora</i>	–	Cockroach (male), cockroach (female), grass hopper	Grain dust	–
Patient-11	<i>Adhatoda vasica</i> , <i>Amaranthus spinosus</i> , <i>Azadirachta compestris</i> , <i>Eucalyptus teritocornis</i> , <i>Gynandropsis gynandra</i> , <i>Prosopis juliflora</i>	<i>Alternaria teneis</i> , <i>Aspergillus flavus</i> , <i>Aspergillus niger</i> , <i>Rhizopus nigricans</i> , <i>Trichoderma species</i>	Cockroach (male), cockroach (female), cricket	Cotton mill dust, house dust, paper dust, grain dust	–
Patient-12	<i>Adhatoda vasica</i> , <i>Ailanthus encelsa</i> , <i>Argemone maxicana</i> , <i>Azadirachta compestris</i> , <i>Brassica compestris</i> , <i>Chenopodium murale</i> , <i>Cyperus rotundus</i> , <i>Dodanaea viscosa</i> , <i>Gynandropsis gynandra</i>	–	–	–	–
Patient-13	<i>Amaranthus spinosus</i> , <i>Cannabis sativa</i> , <i>Chenopodium murale</i> , <i>Gynandropsis gynandra</i> , <i>Melia azedarch</i> , <i>Morus alba</i> , <i>Parthenium hysterophorus</i> , <i>Prosopis juliflora</i> , <i>Putranjiva roxburghii</i>	<i>Alternaria teneis</i> , <i>Helminthosporium specles</i>	Cockroach (male), cockroach (female), cricket	Cotton mill dust, house dust	–
Patient-14	<i>Amaranthus spinosus</i> , <i>Argemone maxicana</i> , <i>Cannabis sativa</i> , <i>Prosopis juliflora</i> , <i>Putranjiva roxburghii</i>	–	–	–	Cow dander, dog dander
Patient-15	<i>Prosopis juliflora</i> , <i>Sorghum vulgare</i>	–	Cockroach (male), cockroach (female), cricket, grass hopper	–	–
Patient-16	<i>Chenopodium murale</i> , <i>Cyperus rotundus</i> , <i>Prosopis juliflora</i> , <i>Carsia siamea</i> , <i>Pennisetum thyphoids</i>	–	Cricket, grass hopper	Cotton mill dust, house dust	Buffalo dander, cow dander
Patient-17	<i>Adhatoda vasica</i> , <i>Ailanthus encelsa</i> , <i>Argemone maxicana</i> , <i>Brassica compestris</i> , <i>Prosopis juliflora</i> , <i>Ricinus communis</i> , <i>Pennisetum thyphoids</i>	–	Cockroach (male), cockroach (female), cricket, grass hopper	–	–

Table 4

Prevalence of concomitant sensitization of black gram sensitive patients (n = 17) to other food allergens.

S.N.	Non-leguminous food items	Animal products	Other legumes
Patient-1	Coffee beans, apple, mustard, mustard leaves	Fish	Rajma, Groundnut, Chickpea, Masoor
Patient-2	Rice, mustard, mustard leaves	–	Rajma, Lobia, Dal raughi, Bengal gram
Patient-3	Rice	–	Arhar
Patient-4	Mustard	–	Rajma, Lobia, Groundnut, Chickpea, Soybean flour, Dal raughi, Arhar
Patient-5	–	–	Rajma, Lobia, Groundnut, Chickpea, Arhar, Soybean flour, Pea
Patient-6	Potato	–	Arhar
Patient-7	Mustard leaves	–	Rajma, Ground nut, Dal raughi
Patient-8	Mustard	–	Chickpea, Bengal gram, Dal moth
Patient-9	–	–	Rajma, Ground nut, Bengal gram, Arhar, pea, Dal moth
Patient-10	Mushroom	–	Bean fresh, Chickpea
Patient-11	–	–	Rajma
Patient-12	Wheat, coffee beans, rice, mustard leaves, banana	–	Rajma, Lobia, Groundnut
Patient-13	Rice, mustard leaves	Egg white, chicken, mutton	Rajma, Arhar, Bengal gram, Masoor, Soybean flour, Dal moth
Patient-14	–	Milk, chicken	Rajma, Lobia, Ground nut, Chickpea, Dal raughi, Dal masoor, Bengal gram
Patient-15	–	Fish	Rajma, Moong, Pea, Dal moth
Patient-16	–	–	Rajma, Arhar
Patient-17	Mustard	–	Dal raughi, Lobia, Dal moth

proteins were detected when the strip was incubated with healthy human and PBS treated mice sera.

3.5. Enhanced level of immunoglobulins in black gram sensitized mice sera

Significantly higher production of specific IgE was observed in BG-CPE sensitized mice ($p < 0.05$) as compared to control group (Fig. 4a).

Measurement of other immunoglobulins such as specific IgG1 and specific IgG2a also showed a significant increase ($p < 0.05$) in black gram treated mouse sera as compared to PBS treated group (Fig. 4b, c). The increase in the level of specific IgG1 was greater as compared to that of specific IgG2a level. All mice immunized with BG-CPE had ratios of IgG1/IgG2a ~2–3 and for peanut, it was ~4–5, indicating Th2 mediated immune responses (Fig. 4d).

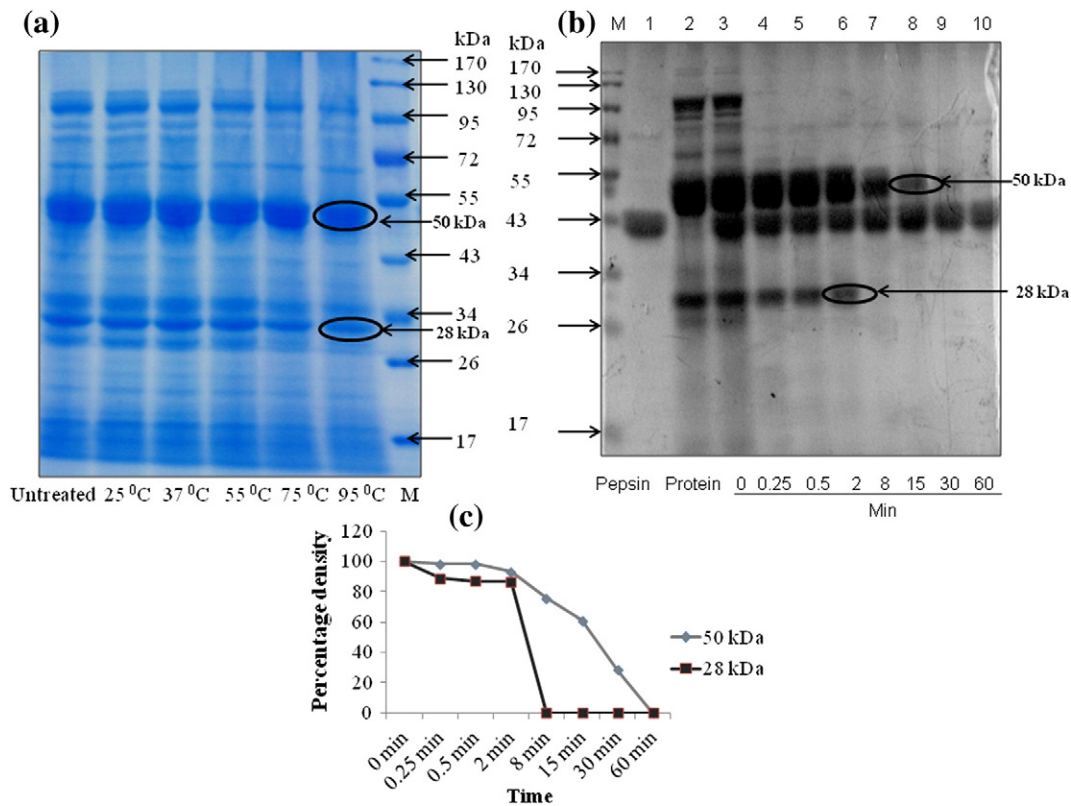


Fig. 2. Thermal Stability Assay and Pepsin Digestibility Assay of BG-CPE (a) BG-CPE was incubated at a temperature range from 25 °C to 95 °C up to 30 min and resolved on 12% SDS-PAGE to analyze the effect of thermal treatment on black gram protein profile (b). Pepsin digestibility profile of BG-CPE after incubation at 0, 0.25, 0.5, 2, 8, 15, 30 and 60 min in SGF (c). Densitometry plot of undigested proteins at abovementioned time points.

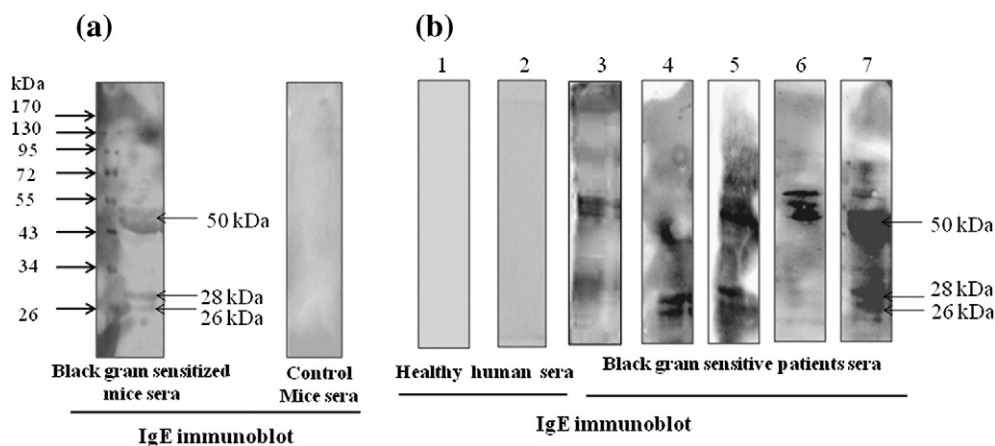


Fig. 3. IgE immunoblotting (a) detection of IgE binding proteins with black gram and PBS treated mice sera. (b) IgE immunoblot with black gram sensitive patients and healthy humans' sera. No IgE binding was found with control mice and healthy human sera.

3.6. Higher systemic anaphylaxis score and lower rectal temperature after BG-CPE challenge

BG-CPE sensitized mice showed higher anaphylactic score when intraperitoneally challenged with black gram protein on day 60. Out of five mice, one mouse showed a symptom score of 2, one mouse showed a symptom score of 3, one mouse showed a symptom score of 4 and the remaining two mice showed a symptom score of 5. A drop in rectal temperature in black gram sensitized group was found in the range of 4–8 °C. However, the control group did not show anaphylactic symptoms upon BG-CPE challenge (Fig. 4e, f).

3.7. Enhanced release of histamine due to black gram exposure

BG-CPE treated mice also showed a significant increase in plasma histamine level as compared to control mice ($p < 0.05$). The increase in plasma histamine was ~5 fold when compared to control group (Fig. 4g).

3.8. Prominent histopathological changes in different tissues in black gram sensitized mice

Histopathology showed abrupt changes in the lung, intestine and spleen in BG-CPE treated mice. The lungs from BG-CPE-sensitized mice showed hyperplasia and exfoliation of bronchiolar lining epithelium as well as thickening of alveolar septa. Control mice had normal histological structure of alveoli and bronchioles. Analysis of intestine of BG-CPE treated mice revealed destruction of mucosal lining and infiltration of mucosa with inflammatory cells while control mice had normal structure with no distortion in mucosa. The effect of BG-CPE treatment was also observed on spleen that had lymphoid hyperplasia. In contrast, normal histology of red pulp and white pulp was observed in control mice (Fig. 5).

3.9. Upregulated Th2 cytokines, GATA-3 and down regulated T-bet at m-RNA level

The mRNA levels of IL-4, IL-5 and IL-13 were found to be up-regulated in the intestine of black gram treated group when compared to the respective control. Moreover, the expression of GATA-3 was found to be up-regulated, while the expression of T-bet was down-regulated in the intestine of sensitized mice over the PBS treated mice. The expression levels of IL-4, IL-5, IL-13, and GATA-3 were found to be 2.38, 1.46, 1.52, and 1.27 fold respectively higher and T-bet was found to be

0.4 fold lower in black gram sensitized group when compared to control (Fig. 6).

3.10. Western blot analysis showed up-regulation of Th2 reaction favoring transcription factors

Upregulated expression of GATA-3, STAT-6, and SOCS 3 and lower expression of T-bet were observed in intestinal proteins of black gram treated mice when compared to the control group. The expression levels of GATA-3, SOCS 3 and STAT-6 were approximately 1.23, 1.26 and 1.21 fold respectively higher in black gram treated group when compared to control. The expression level of T-bet was 0.3 fold lower in black gram sensitized group as compared to control. β -Actin was taken as endogenous control (Fig. 7).

3.11. Histamine and other mediators released by RBL-2H3 cells

β -Hexosaminidase level was elevated ($p < 0.05$) (Fig. 8a) in stimulated-RBL-2H3 cells in a dose-dependent manner (25, 50, 75, 100 and 125 μ g) on exposure of black gram proteins. The levels of histamine ($p < 0.05$) (Fig. 8b), prostaglandin D2 ($p < 0.05$) (Fig. 8c) and cysteinyl leukotriene ($p < 0.05$) (Fig. 8d) were also found to be elevated in those cells when treated with black gram.

4. Discussion

Legume allergy has gained considerable attention in recent years as it is a serious growing problem worldwide. Several reports have been published regarding the allergenic potential of different legumes [2, 23]. Some preliminary studies regarding allergenicity of black gram reported that sensitization is IgE-mediated in patients of asthma and allergic rhinitis [6,7]. In spite of these reports, there is still a need to have a thorough exploration of the allergic reactions induced by black gram. Therefore, an attempt has been made to explore the immunological events to fulfill the gap in the understanding of black gram induced allergy. This study focused on black gram sensitive patients which will provide useful information regarding the causative factors of both food and non-food origin responsible for enhancing allergenicity. Further, BALB/c mice and RBL-2H3 cell lines were used for the elucidation of immunological events and to unravel the mechanistic aspect behind the black gram induced allergy.

It has been reported that most of food allergens are generally resistance to heat denaturation and have the potential to react with IgE [24]. It indicates that heat resistant proteins have more chances to provoke allergenic potential because these are not degraded during thermal processing e.g., boiling, cooking, autoclaving, and micro-wave

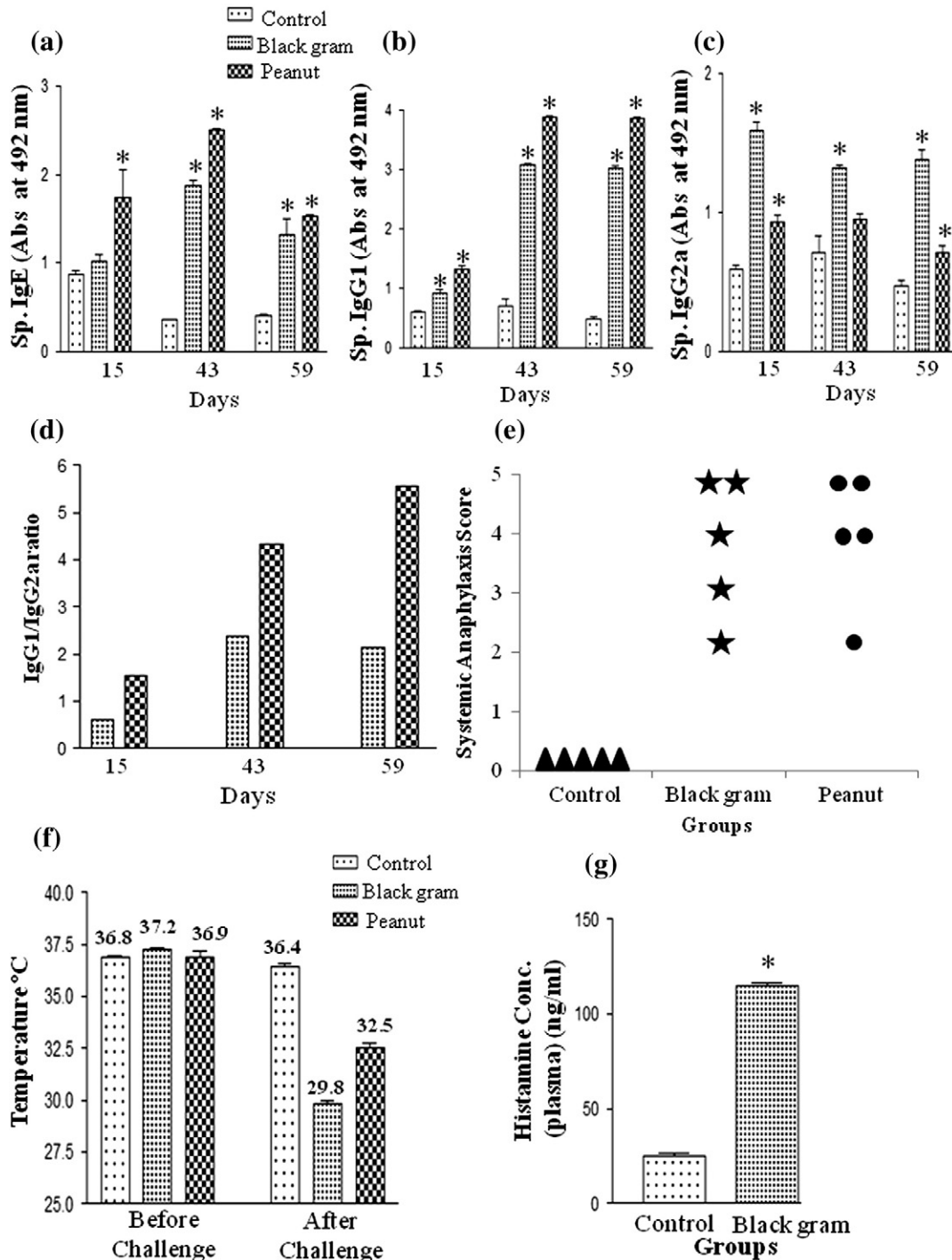


Fig. 4. Assessment of different allergic manifestations in BALB/c mice following black gram protein treatment (a, b, c). Serum levels of specific IgE, IgG1, and IgG2a responses at different time points (d). Ratio of IgG1/IgG2a at different time points (e). Anaphylactic symptoms scored after 20–30 min following challenge (f). Rectal temperature was measured before and 20 min after challenge on day 60 (g). Histamine levels were estimated in plasma collected after challenge. Data are presented as means $\pm$ SEM (* $p < 0.05$) versus control mice.

heating. According to our result, no significant change in protein profile between heat treated protein and untreated protein was observed, which indicated that majority of black gram proteins are thermally stable thereby enhancing the probability of allergenic sensitization even after cooking.

Pepsin Digestibility Assay is one of the standard experiments used for allergenicity studies. It is a well established fact that proteins showing stability in SGF for more than 2 min have more chances to elicit allergic responses [9,10,25]. Further, proteins with more than 10% stainable full-length protein band that remained up to or more than

30 min may be considered stable. Proteins reduced to less than 10% stainable band at 5 to 30 min can be considered of intermediate stability [11]. In BG-CPE, 50 kDa and 28 kDa proteins were found resistant to digestion against SGF, indicating that these proteins may sensitize the immune system after reaching mucosa, interact with immune system and may elicit adverse reactions. Through Pepsin Digestibility Assay, it can be inferred that SGF resistant proteins behave as an allergens as they have enhanced chances for absorption by the intestinal mucosa.

In the present study, based on questionnaire and SPT result, nasobronchial allergic patients have been screened to know the

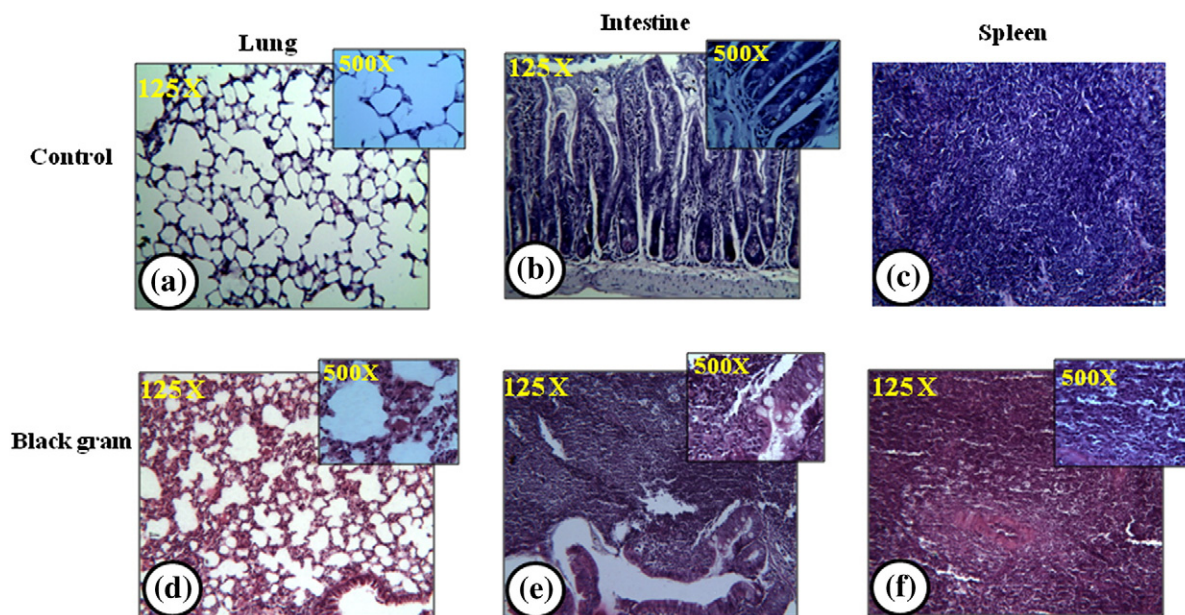


Fig. 5. Histopathological analysis of different organs following black gram proteins exposure in mice (a, b, c). Histopathological analysis of the lung, intestine and spleen of PBS treated mice (d, e, f) the lung, intestine and spleen of black gram treated mice.

sensitization pattern of black gram. Seventeen allergic patients (8.5%) showed positive SPT to black gram along with higher specific IgE levels. The patients of black gram allergy were suffering from allergic rhinitis, dermatitis and bronchial asthma. The allergic manifestations in black gram SPT positive patients were further enhanced by cross reactivity

with other known allergens. Black gram sensitive patients showing concomitant sensitization with other allergens especially higher with pollens, other common legumes and insects enhanced the probability of allergenic manifestations. Our results have been supported by the previous studies on the cross reactivity of black gram with chickpea, red

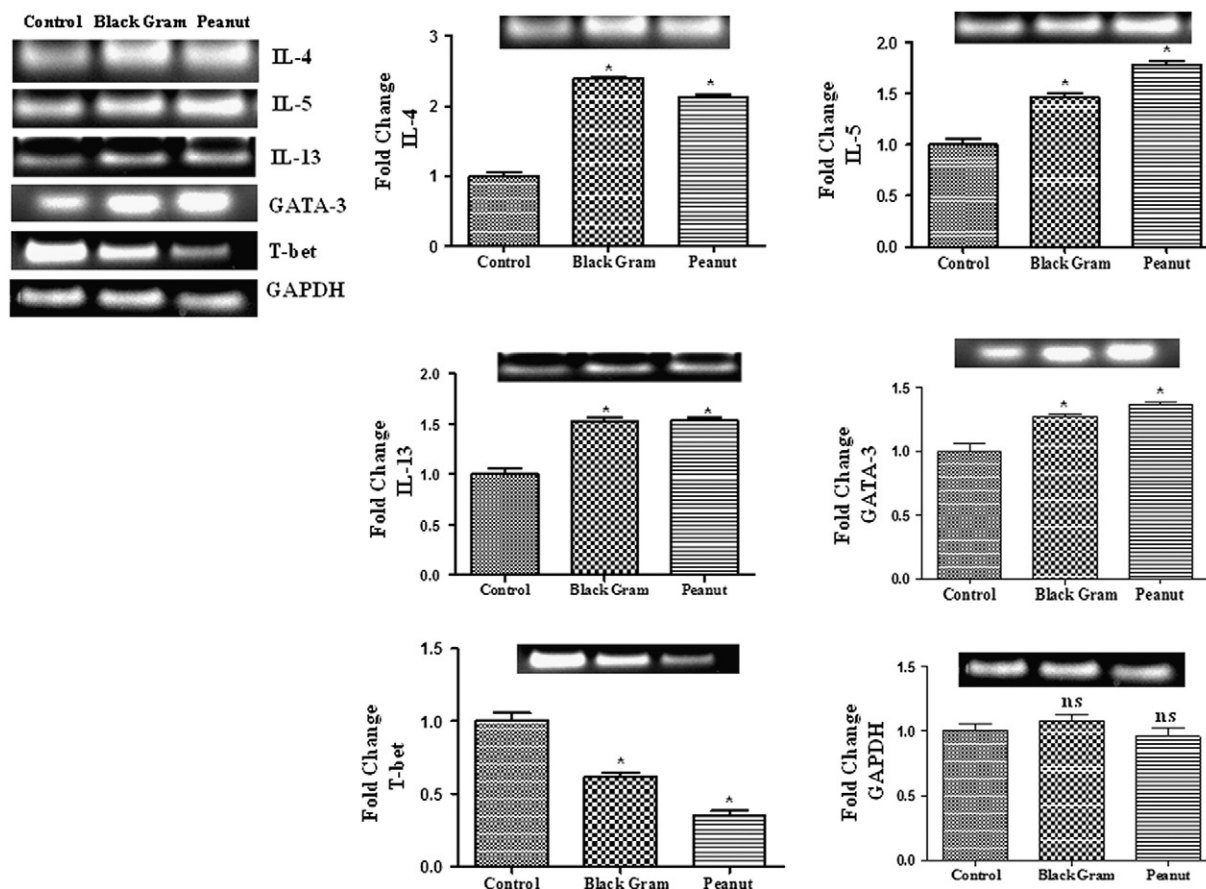


Fig. 6. The mRNA expressions of IL-4, IL-5, and IL-13 cytokines and GATA-3 and T-bet transcription factors in the intestine of control, black gram and peanut groups and their densitometry, where GAPDH was taken as internal control.

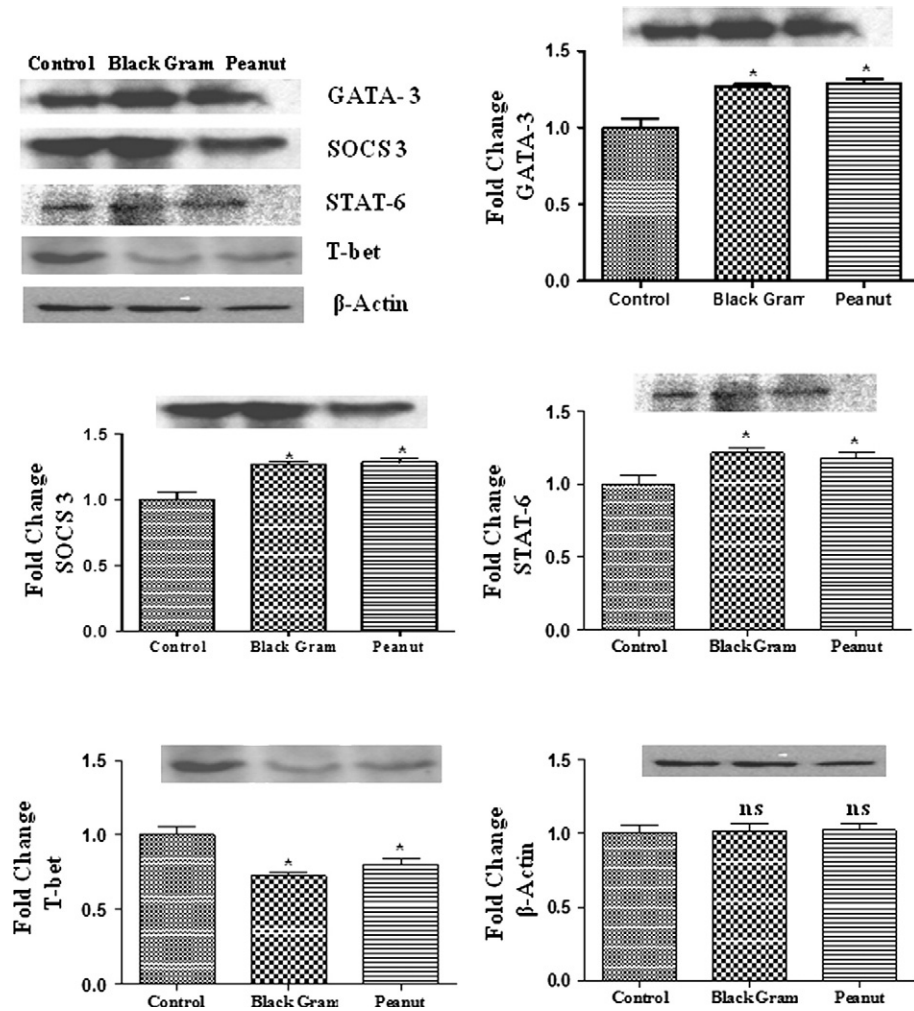


Fig. 7. Western blotting for GATA-3, SOCS 3, STAT-6 and T-bet transcription factors in the intestine of control, black gram and peanut groups and their densitometry, where β -actin was taken as internal control.

kidney bean, pea, lentil, etc. [6,13,18]. Therefore, black gram has the ability to induce allergic symptoms in sensitive individuals who may be allergic to other allergens as well.

After confirming allergic potential of black gram in human subjects, the study was extended to mice and RBL-2H3 cell line. SDS-PAGE immunoblot with mice sera demonstrated that three proteins of 50, 28 and 26 kDa had an IgE-binding capacity. Further, this result was confirmed by immunoblotting with individual patient's sera where the same IgE binding proteins of 50, 28 and 26 kDa were found. This revealed that IgE-binding proteins are similar in both BG-CPE sensitized mice and sensitive patients showing similarities between mice and human immune systems. Recently, 28 kDa black gram protein has been characterized in which the N-terminus-12 residue sequence was 'GRREDDYDNLQL' that showed 87% homology with Rho-specific inhibitor of transcription termination and 77% homology with NBS-LRR type disease resistant protein from peanut [7]. Allergic response is characterized by class switching to IgE antibody and an increase in specific IgE level is indicative of involvement of specific allergen. In the present study, specific IgE level against black gram was found to be enhanced due to the presence of immunoreactive proteins in BG-CPE. Significant enhancement in IgG1 antibody level in the BG-CPE-treated group was an indication of favoring Th2-mediated reactions as supported by previous studies [26,27]. The subclass of IgG (ratios of IgG1 to IgG2a antibody) induced after antigen immunization will provide the clue for the Th2- versus Th1-type immune responses [28–30]. If the ratios of IgG1:IgG2a is >1 then it indicates primarily Th2-type antibody responses. All mice immunized with

BG-CPE had ratio of IgG1/IgG2a >1 indicating that Th2 mediated immune responses. These findings demonstrated that black gram protein has the ability to provoke allergenic manifestations in susceptible individual.

Allergen-mediated cross linking of mast cell-bound IgE leads to the release of histamine and other mediators that contribute to the symptoms of anaphylaxis [31]. Higher anaphylactic score (score 2–5) and drop in body temperature (4–8 °C) were observed in black gram treated mice as compared to control group. These both parameters are indicative of allergenic potential of black gram proteins to promote allergic reactions in sensitized individual.

Allergic response is demonstrated by inflammation and accumulation of inflammatory cells in affected organs [32]. Histopathological changes in the intestine, lungs and spleen were indicative of the involvement of black gram proteins in allergenic consequences. Prominent histological changes explain the modulation in the levels of cytokines and specific antibodies, and may be helpful in understanding black gram induced allergenic manifestations.

We have seen the allergenic effect of BG-CPE on intestinal immune system where GALT (gut associated lymphoid tissue), one of the major parts of intestine comprises of Peyer's patches (PP), mesenteric lymph nodes, M cells, etc. is there to present antigenic immune responses sufficiently. We have taken the small intestine as part for conducting experiments as it has PPs, which are highly specialized "gut-type" lymphoid follicle wall that contains naive B cells, follicular dendritic cells, and T cell rich areas [33]. Intestinal immune system

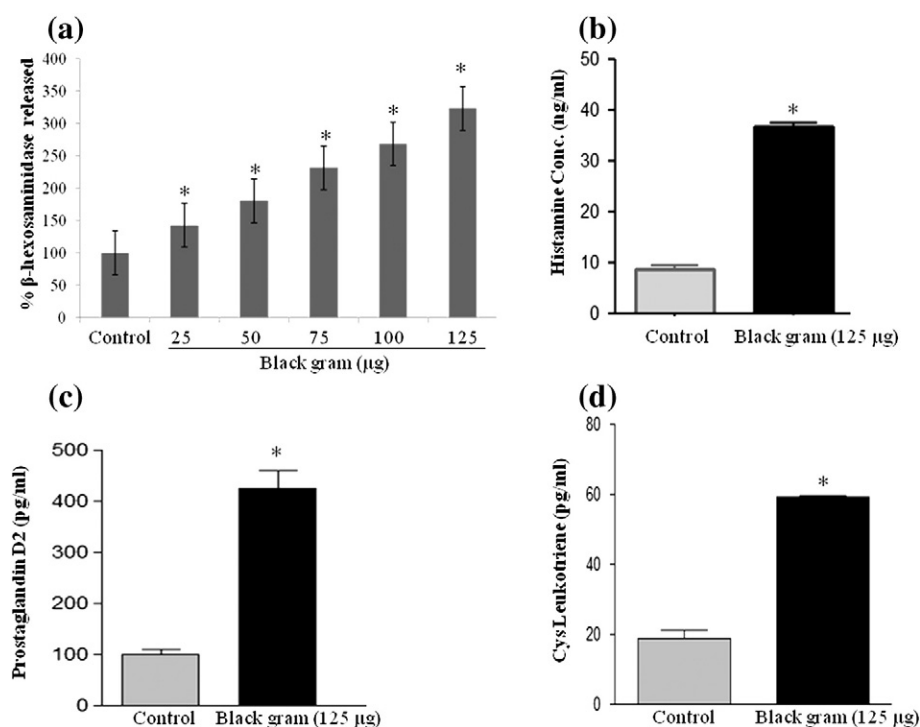


Fig. 8. Mediators released from RBL-2H3 cell lines primed with black gram treated mice sera (a) level of β -hexosaminidase release determined by different doses (25 μg –125 μg) of BG-CPE exposure (b) level of histamine (c) prostaglandin D2 and (d) cysteinyl leukotriene. Data are presented as means $\pm$ SEM (* $p < 0.05$) versus control mice.

fences off potentially harmful intestinal antigens from the systemic circulation and helps in the presentation of antigens to immune effector cells which are concentrated at sites of organized mucosal lymphoid follicles. Therefore, for this study, they seem critical for the decision between inflammation and tolerance, as the intestine of BG-CPE treated mice was found to be severely affected. Therefore, we used this organ for further experiments to see the expression of cytokines and transcription factors.

It is reported that the IL-4, IL-5 and IL-13 cytokines play a crucial role in the induction of allergic reactions [20,34]. The differentiated Th2 cells secrete cytokines IL-4 and IL-13 which induce class switching from IgG to IgE antibody which is responsible for allergic reactions [35]. It is known that the ratio of GATA-3/T-bet is an important aspect during the allergic manifestation. GATA-3 plays an important role in the shifting of Th2 reactions from Th1 by suppressing T-bet transcription factors [36]. GATA-3 promotes the secretion of IL-4, IL-5, and IL-13 cytokines from activated Th2 cells which favor the development of allergic manifestations [37]. Conclusively, the down regulation of T-bet expression along with increased expression of GATA-3, SOCS 3, STAT-6 and Th2 cytokines promote Th2-mediated pathway in food induced allergic reaction [1,38]. Consequently, our data also showed that the up-regulation of IL-4, IL-5, IL-13 and GATA-3 and down-regulation of T-bet at mRNA level were observed in black gram treated group as compared to control indicated that the black gram induced allergic manifestations mediated via Th2 response.

The allergenicity potential of black gram protein observed in mouse model was further confirmed by using RBL-2H3 cell line. RBL-2H3 cells have been taken as an important tool while assessing allergenicity under in vitro conditions, which supposed to mimic in vivo scenario up to the certain limit [39]. The β -hexosaminidase is used as a marker of basophil degranulation, which was found to be elevated after black gram treatment is indicating the degranulation in RBL-2H3 cells [40]. The higher concentration of mediators like histamine, prostaglandin D2 and cysteinyl leukotrienes confirmed black gram induced mediator release. The β -hexosaminidase, histamine, prostaglandin D2 and cysteinyl leukotrienes are the major allergic mediators involved in the

allergic reactions [41–43]. Similar findings noticed in RBL-2H3 cell line where enhanced level of histamine, prostaglandin D2 and cysteinyl leukotriene was found again supporting our earlier hypothesis.

5. Conclusion

The allergenic potential of black gram was assessed in BALB/c mice, RBL-2H3 cell line and the results were extended to human patients screening for black gram allergy. This work showed the black gram proteins as a causative factor for allergy in nasobronchial allergic patients. We have established a mouse model of black gram allergy assessment by demonstrating black gram induced enhancement in the total and specific IgE and IgG1 levels, histamine, Th2 cytokines and GATA-3/T-bet transcription factors' ratio. Higher anaphylaxis score, reduced body temperature and histopathological changes in tissues were also observed in black gram sensitized mice. Additionally, increased level of β -hexosaminidase, and other mediators has been found in black gram treated RBL-2H3 cells. Conclusively, these findings established the allergenic potential and mechanistic aspects behind black gram allergy. The study will be helpful for the development of diagnostic and therapeutic approaches to combat with black gram allergy. The data presented in this work will be helpful for developing genetically modified hypoallergenic black gram species.

Conflict of interest statement

The authors have declared no conflict of interest.

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