

Allergenicity assessment of *Buchanania lanzan* protein extract in Balb/c mice

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ARTICLE INFO

Keywords:

Buchanania lanzan

Tree nuts

Allergy

Cytokines

Histamine

PGD₂

ABSTRACT

Tree nuts are among “Big Eight” and have been reported globally for causing allergy. *Buchanania lanzan* (Bl) is one of the major tree nuts consumed by Indian population. However, very little is known about *B. lanzan*'s induced allergic manifestation. Therefore, evaluation of its allergenic potential was undertaken. Bl-crude protein extract sensitized BALB/c mice sera were used to identify the allergic proteins by its IgE binding capability. The major IgE binding proteins found with molecular weight of 11, 20, 23, 25, 48, 54, and 65 kDa. Specific IgE, specific IgG1, MCPT-1, PGD₂ and histamine were assessed in mice sera. Enormous amount of mast cell infiltration was noted in different organs. The levels of Th1/Th2 transcription factors GATA-3, SOCS3 and STAT-6 were found upregulated, whereas T-bet was downregulated. Furthermore, elevated Th1/Th2 cytokine responses were observed in mice sera. All together, these reactions developed systemic anaphylaxis upon Bl-CPE challenge in sensitized BALB/c mice. In order to confirm the evidences obtained from the studies carried out in BALB/c, the investigation was extended to human subjects as well. Control subjects and allergic patients were subjected to skin prick test (SPT). Later sera collected from those positive to SPT along with controls were used for IgE immunoblotting. The study evaluated the allergic manifestation associated with Bl, and identified its proteins attributing Bl-mediated allergy. This work may help in managing tree nuts mediated allergies especially due to *Buchanania lanzan* sensitization.

1. Introduction

Food allergy is a widespread health concern all over the world with an increasing prevalence. The increased prevalence can be attributed to many factors like genetic, environmental and processing of food [1]. In India, the prevalence of food allergy has not been systematically studied and not much awareness about food hypersensitive reactions in Indian sub-continent is there. Food allergy is simply a reproducible hypersensitive reaction of the immune system following ingestion of food or its components, and this abnormal response can either be IgE or non-IgE mediated in nature [2]. Various food components have been linked with fatal IgE mediated immune response, but major focus is given to mainly four food items that are cow's milk, hen's egg, peanut/tree nuts, and fish/shellfish [3]. Out of these peanut and tree nut together correspond to 70–90% of recorded anaphylactic reactions and tree nut alone summed up to 18–40% with almost every tree nut capable of

inducing fatal allergic response [4]. Tree nuts that account for maximum reported allergic responses are walnut with 34%, cashew with 20%, almond with 15%, and pistachio with 7% [5]. Major tree nut derived food allergens from cashew nut (Ana o 1, Ana o 2, Ana o 3) and pistachios (Pis v1, Pis v 2) are well identified and characterized [5, 6]. Cashew nut and pistachios, both belong to Anacardiaceae family and exhibit cross reactivity to each other. *Buchanania lanzan* (Bl) is another tree nut from this family that is enriched with the 22% protein, 59% lipid, 12% carbohydrate and 4% fibres [7]. Bl is native to India, Australia, Thailand, Burma, Nepal and Pacific islands [8–10]. Bl is also called Chironji, or Charoli in India, Priyala in ayurveda (branch of Medicine practised in India) and Almodette in English [11]. It is used as a key ingredient in many Indian traditional cuisines, sweet dishes, for thickening of various savoury sauces, to flavour batters, to sprinkle fruit salads, soups, meaty kormas and in stewed rice. The Bl seed is also used as a substitute for almonds and the oil extracted from it is used in the

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<https://doi.org/10.1016/j.intimp.2018.07.039>

Received 9 May 2018; Received in revised form 30 July 2018; Accepted 30 July 2018

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place of both almond/olive oil [12]. It's high protein content (~22%) and widespread use in foods has raised concerns that it may cause IgE-mediated allergic reactions in sensitive individuals. Also, Bl has been reported to accelerate cell-mediated immunity and humoral immunity in BALB/c mice along with increased macrophage migration index (MMI), hemagglutination antibody (HA) titres and plaque-forming cell count [13]. Thus, it is perfect case to assess the allergenic potential of Bl.

Food allergy varies according to the food habits of different region/country. Indian population being major consumer of Bl (as flavoring agent) increases the probability of prevalence of allergy to this dry fruit and therefore this may be a cause of concern. Barely, any mechanistic studies have been carried out on allergens from Bl. Therefore, evaluation of allergenic potential of Bl followed by exploration of involved mechanistic pathways, are required in order to develop appropriate strategies to ameliorate its allergenicity. As mostly protein components of foods cause sensitization and result in allergy as well as severe anaphylaxis therefore, the aim of the current study was to explore the allergenicity potential of Bl and find out the culprit proteins that are responsible for its allergenicity. Female BALB/c mice were selected due to their immune responsiveness and were administered with Bl crude protein extract to mimic different parameters of allergenicity on humans as well. Present investigation is an effort towards assessment of allergenic potential of tree nuts, which would be an important contribution in the direction to develop specific immunotherapy as therapeutic approach to the problem.

2. Material and methods

2.1. *Buchanania lanzan* (Bl), cashew, pistachio and peanut crude protein extraction

Seeds of all the nuts were obtained from supermarket (Big Bazaar) (Bl- Batch no. RAI 1958; cashew-OF/117/05; pistachio-B/0418; peanut-B102016), and the same lot of seeds was used in the entire study. The crude protein extract was prepared as described in the previous method [14]. Precisely seeds were milled in a food processor and the powder so produced was defatted using of n-hexane (Merck, Worli, Mumbai, India), after which it was softened by PBS (phosphate buffer saline, 20 mM Na₂HPO₄, 2 mM KH₂PO₄, 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture formed was flustered overnight at 4 °C followed by centrifugation at 10,000 × g for 30 min. After that the resulting supernatant was collected and filtered via a 0.45 µm filter and stored (in aliquots) in a –80 °C freezer till the time of use.

2.2. Ethical approval

Skin prick test (SPT) and blood collection were carried out with patient's consent and the study protocol was approved by the human ethics committee of King George's Medical University, Lucknow, India (Ref. no.-2931/Ethics/R.Cell-18). Animal study was accomplished after the approval of Institutional Animal Ethical Committee of CSIR-IITR, Lucknow, India (IITR/IAEC/56/17).

2.3. Animal treatment protocol

Female BALB/c mice of 6–8 weeks old, weighing 22 ± 3 g were received from the animal breeding colony of Indian Institute of Toxicology Research, Lucknow. These mice were put in standard laboratory conditions in animal care facility of CSIR-IITR, M.G. Road campus, Lucknow and treated according to the method mentioned earlier [15, 16]. Briefly, mice were sensitized with either PBS, or Bl-CPE (*Buchanania lanzan*-crude protein extract) or Pn-CPE (Peanut-crude protein extract), where Pn-CPE was taken as positive control for the study. BALB/c mice (n = 15/group) were divided into three above mentioned subgroups and were administered with 100 µg protein

intraperitoneally once in a week. Blood samples were collected via retro-orbital sinus to measure serum IgE and IgG1 antibodies on day 59. On day 60, the experimental mice were challenged i.p. with 1 ml of 5 mg/ml of Bl-CPE intraperitoneally. Tissue and blood samples were collected for further experiments.

2.4. Screening of *Buchanania lanzan* sensitive patients

Skin prick test (SPT) were executed with the allergen kit (All cure Pharma Pvt. Ltd. New Delhi, India) on allergic patients (n = 100) and normal, healthy individual (n = 10) at the Department of Pulmonary and Critical Care Medicine, King George's Medical University (KGMU), Lucknow, India. Briefly, glycerol histamine acid phosphate and glycerinated buffer saline (1:100) were used as positive and negative, respectively. Comparison was done with reference to the weal diameter and scoring was done as: smaller than histamine weal then +1, equal +2, larger +3 and for bigger with pseudopodia then +4. Blood was collected from Bl SPT positive patients and normal healthy volunteers. Patients showing no weals against Bl were assigned as Bl SPT negative patients and they were omitted from the study. Serum was separated from whole blood and stored at –80 °C for further analysis [17].

2.5. IgE immunoblots analysis

To identify IgE specific protein in Bl crude protein extract, IgE immunoblotting was accomplished by the method described earlier [18, 19].

2.6. Measurement of anaphylaxis score and rectal temperature

The systemic anaphylaxis index was measured in Bl treated mice as defined formerly on the scale of 0–5, where 0 stands for no symptoms, 1 for scratching and rubbing around the nose and head, 2 for puffiness around the eyes and mouth, diarrhoea, and reduced activity or standing still with an increased respiratory rate, 3 for wheezing, labored respiration, and cyanosis around the mouth, in score 4 symptoms are same as in No. 3 with loss of consciousness, tremor, and/or convulsion and 5 for mortality (death) [20]. Rectal thermometer (Bioseb, France) was used to record rectal temperature in Bl protein administered mice and their respective controls 20 min prior and subsequent to challenge.

2.7. Detection of cross-reactive proteins with cashew, pistachio and peanut using Bl-sensitized mice sera

Proteins extracts of cashew, pistachio and peanut were separated by SDS-PAGE and electroblotted to polyvinylidene difluoride (PVDF) membrane. A serum pool containing IgE specific to the major allergens of Bl was used to incubate the immunoblots. Detection of IgE was carried out by the method discussed earlier [18, 19].

2.8. *Buchanania lanzan* protein specific IgE and IgG1 measurement in mice sera

Assessment of specific IgE and IgG1 levels in control and Bl protein treated mice sera was conducted using previously illustrated method [15].

2.9. Estimation of MCPT-1 and TSLP

Blood was collected from the all the experimental groups after 30 mins of challenge and serum was collected to be used for the assay. The serum levels of mouse mast cell protease-1 (MCPT-1) and thymic stromal lymphopoietin (TSLP) were assessed in Bl protein treated and control mice using ELISA (for MCPT-1: Gen Asia Biotech Co. Ltd. Shanghai China and for TSLP: Wuhan Fine Biotech Co., Ltd., Wuhan, China) according to the manufacturer's instructions. The results so

obtained were expressed in terms of absorbance.

2.10. Assessment of PGD₂, histamine and cysteinyl leukotriene

Prostaglandin D₂ (PGD₂), histamine and cysteinyl leukotriene (CysL) levels in the sera of BI treated mice with their respective controls were analysed by ELISA kit (for histamine: Gen Asia Biotech Co. Ltd. Shanghai, China, and for CysL and PGD₂: Cayman Chemical, USA) in accordance with the manufacturer's instructions.

2.11. Histopathological studies of different organs

Intestine, lungs and spleen were collected for the histopathological studies. The samples were fixed in 10% formalin in PBS and then stained with hematoxylin and eosin. The sections after staining were subjected to the microscopic analysis.

2.12. Detection of mast cells in different organs

The detection of mast cells in lung, spleen and intestine was carried out by toluidine blue staining method in all experimental groups. Briefly, the tissue sections from all groups (3–5 µm thickness) were rehydrated in xylene (twice for 5 mins each) followed by 100, 90, 70 and 50% ethanol in a serial manner. After rehydration, sections were rinsed with PBS once and stained with toluidine blue dye (Sigma Aldrich, USA) for 30 min. Then the sections were kept under running water till the excess stain was washed off the section. Further dehydration process was carried out using 50, 70, 90 and 100% ethanol in sequential manner and xylene, respectively. After completion of dehydration step, slides were mounted with DPX (BDH, India) and images were captured by microscope (Nikon Eclipse TE2000-S microscope) [16].

2.13. Measurement of Th1/Th2 cytokines levels in mice serum

The measurement of serum cytokines was done by flow cytometry (BD FACS CANTO II) using Th1/Th2 cytokines bead array kit (BD Bioscience, San Jose, CA). Serum was collected prior to animal sacrifice at the end of scheduled treatment and subjected to estimation of serum cytokines like IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and IL-17. The sample for cytokine assessment was prepared in accordance with manufacturer's instructions and examined on the same day. CB FCAP assessed the levels of cytokines present in the samples on the ground of the standard curve acquired for each cytokine [21].

2.14. Splenocyte culture

Splenocyte culture was performed according to the method described previously [22]. Briefly, spleens were isolated from the different groups and single cell suspension was prepared. The splenocytes were further analysed for specific surface markers present on T and B cells.

2.15. Immunophenotyping studies

The levels of FITC tagged CD4+, APC tagged CD8+ T-cells and AF700 tagged CD19+ (BD Biosciences, San Jose, CA) B-cells populations in splenocytes were analysed using specific antibodies by flow cytometry according to the protocol described earlier [22].

2.16. The mRNA levels of cytokines and transcription factors in intestine

A semi-quantitative RT-PCR estimation of Th1/Th2 transcription factors and Th2 cytokines (IL-4, IL-13, and IL-5) in the intestine of BI treated mice with their respective controls was carried out by gene specific primers of transcription factors and cytokines in accordance with formerly defined protocol [23]. The primer sequences used were

IL-4: sense 5'-TCGGCATTGTAACGAGGTC-3', antisense 5'-AAAAGCC CGAAAGAGTCTC-3'; IL-5 sense 5'-TCACCGAGCTCTGTTGACAA-3', antisense 5'-CCACACTTCTCTTTTGGCG-3'; IL-13: sense 5'-GACCCAG AGATATTGCATG-3', antisense 5'-CCAGCAAAGTCTGATGTGAG-3'; GATA-3 sense 5'-TCTCACTCTCGAGGCAGCTGA-3', antisense 5'-GGTA CCATCTCGCGCCACAG-3'; T-bet: sense 5'-TCCCATTCTGTCTCT TCA-3', antisense 5'-GCTGCCTTCTGCCTTTC-3'; GAPDH: sense 5'-TTC ACCACCATGGAGAAGGC-3, antisense 5'-GGCATGGACTGTGGTCA TGA-3'.

2.17. Western blot analysis of transcription factors

The protein level expression of several Th1/Th2 transcription factors like, GATA-3, T-bet, SOCS3, and STAT-6 in intestinal protein of BI treated mice with their respective controls were estimated by immunoblotting in accordance with formerly described protocol [16].

2.18. Statistical analysis

The data were expressed significantly as mean value ± SD. The whole data was evaluated by Instant version 5.0 (GraphPad, San Diego, CA, USA; <http://www.graphpad.com>). Differences between values were compared by one-way ANOVA with the Bonferroni as a post-hoc test. A value of p < 0.05 was considered statistically significant and it has been mentioned for each experiment.

3. Results

3.1. IgE immunoblotting of Buchanania lanzan crude protein extract

IgE binding ability of BI was ascertained by IgE immunoblot using sera of sensitized mice and BI-SPT positive human patients. Seven major IgE binding proteins with M.W. of 11, 20, 23, 25, 48, 58, and 65 kDa were observed in IgE immunoblot. No IgE binding protein band was found in control sera of mice and healthy human subjects (Fig. 1A–C).

3.2. To examine systemic anaphylaxis score and body temperature

BI crude protein extract (5 mg/ml) was administered into five mice from each group intraperitoneally [16]. Clinical symptoms started to appear after 15–20 min of the challenge, among the five mice from BI crude protein sensitized (Group 2) mice developed puffiness around the eyes and mouth, diarrhoea, and reduced activity or remained stand still with an increased respiratory rate that is the symptom number-2. Another two mice belonging to the same group showed wheezing, labored respiration, and cyanosis around the mouth that is a symptom number-3 along with the death of one mouse which is symptom number-5. No signs of anaphylaxis were observed in the mice belonging to the control group after being challenged with the same amount of BI crude protein extract (Fig. 1D, E). The body temperature dropped below the normal range by 3–4 °C after being challenged with BI crude protein extract, whereas no such drop in temperature was noted in the control group of mice. With the progression of anaphylaxis after challenge, group of sensitized animals challenged with BI-CPE recorded sharp drop in core body temperature.

3.3. Immunoblotting of cross reactive IgE-binding proteins in crude extract of cashew, pistachio and peanut using BI-sensitized mice sera

To study the possible cross-reactivity between BI and two other nuts including cashew and pistachio as well as peanut, the protein patterns of these nuts were first analysed. The IgE immunoblots depicted 8 cross-reactive allergenic fractions in 10–55 kDa range (10, 13, 20, 23, 26, 28, 30 and 54 kDa) in cashew (Fig. 2A), 5 cross-reactive allergenic fractions in pistachio within 10–55 kDa range (10, 12, 24, 40 and 54 kDa) (Fig. 2B) and 6 cross-reactive allergenic fractions in peanut within

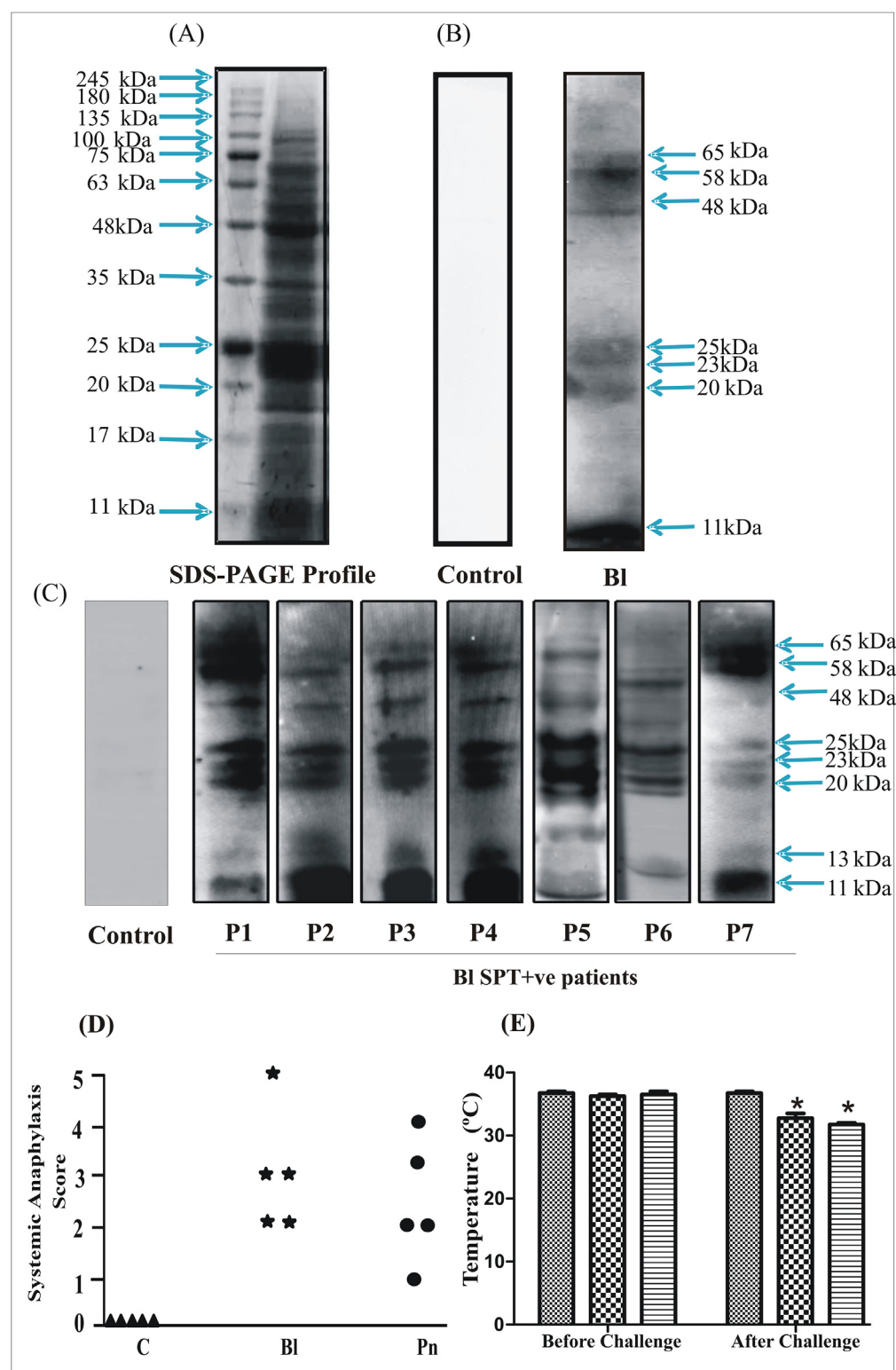


Fig. 1. (A) SDS-PAGE protein profile of crude protein extract of *Buchanania lanzan* (Bl). IgE immunoblot analysis of (B) *Buchanania lanzan* sensitized mice pooled sera with its respective control and (C) *Buchanania lanzan*-SPT positive human patients with its respective control, depicting seven major allergenic proteins present in the crude protein extract of *Buchanania lanzan* (Bl). (D) Anaphylactic symptoms (mice $n = 5/\text{groups}$) scored after 20–30 min following challenge. (E) Rectal temperature was measured on day 60 before and after 20 min of protein challenge. Data are presented in mean $\pm$ SD; (* $p < 0.0142$) of three independent experiments when compared to control with *Buchanania lanzan* (Bl) and Peanut (Pn) treated group.

range of 10–35 kDa range (15, 22, 25, 27, 33 and 35 kDa) (Fig. 2C).

3.4. Assessment of specific IgE and IgG1 in *Buchanania lanzan* sensitized mice sera

IgE and IgG1 are important parameters indicating allergy that should be taken into account while evaluating allergenic potential of any product. Specific IgE and IgG1 levels were significantly higher in

sera of Bl treated mice in comparison to controls (Fig. 3A, B).

3.5. MCPT-1 and TSLP in sera of *Buchanania lanzan* sensitized mice

Mouse mast cell protease-1 (MCPT-1) known as β -chymase, mainly expressed in intestinal mucosal mast cells, increases the mucosal permeability in case of allergic hypersensitive reactions. Thymic stromal lymphopoietin (TSLP) expressed by epithelial cells also contribute in

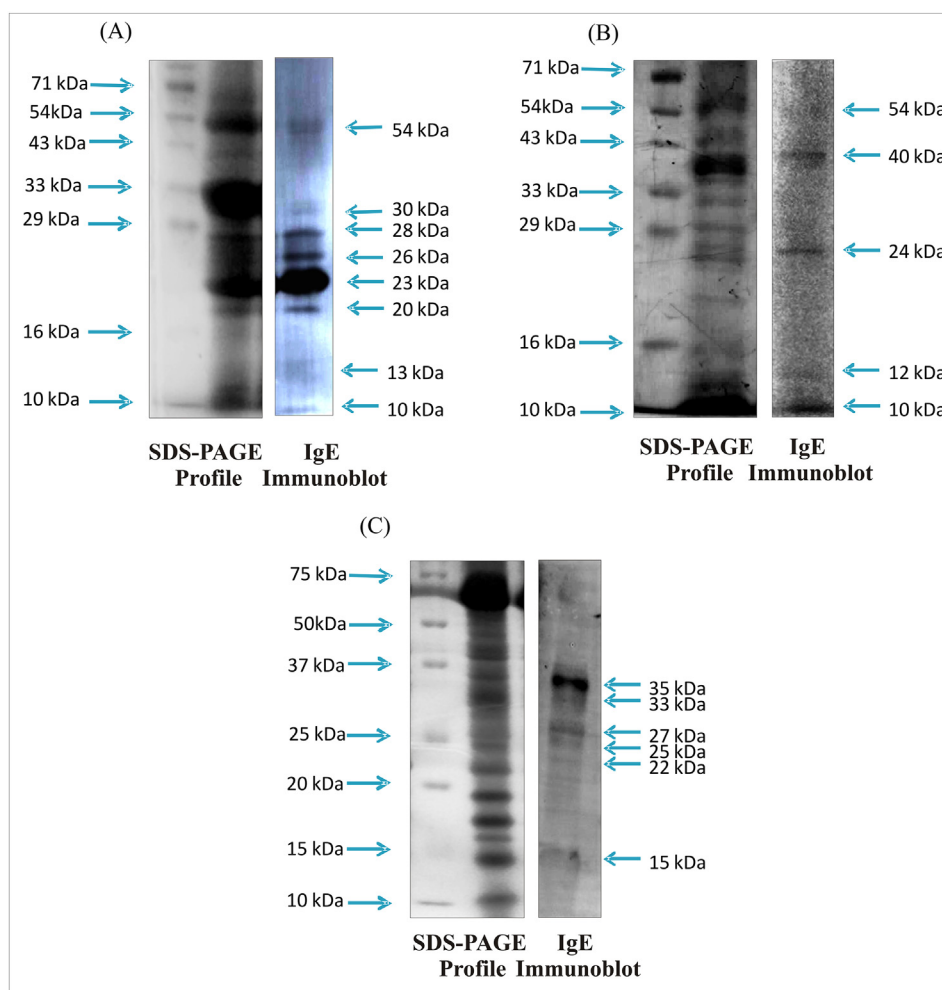


Fig. 2. Cross-reactivity blots with other nuts: (A) SDS -PAGE profile and IgE immunoblot with Cashew-crude protein extract (B) SDS-PAGE profile and IgE immunoblot with Pistachio-crude protein extract (C) SDS -PAGE profile and IgE immunoblot with Peanut-crude protein extract.

activation of mast cells in allergic individuals [14, 24]. MCPT-1 (~2 fold) and TSLP (~3.2 fold) were significantly upregulated in the serum of Bl and Peanut (Pn) treated groups in comparison to their respective controls (Fig. 3C, D).

3.6. Estimation of the histamine, cysteinyl leukotriene (CysL), and PGD₂ levels

Antigen mediated cross linking of IgE to mast cells/basophils result in degranulation and release of allergic mediators like histamine, PGD₂ and CysL in a sensitized individual [25]. Increased levels of histamine, PGD₂ and CysL were present in the sera of Bl treated mice when compared to their respective control (Fig. 3E,G).

3.7. Histopathological assessment of intestine, lungs, and spleen

Eminent histopathological changes in intestine, lungs and spleen were observed in Bl treated mice. The lungs of Bl treated mice showed the hyperplasia of bronchial lining epithelium and thickening of alveolar septa (Fig. 4B). Destructions of mucosal lining and inflammatory cell infiltration were obtained in the intestine of *Buchanania lanzan* protein administered mice (Fig. 4A). The spleen sections showed lymphoid hyperplasia and large-size megakaryocytes in Bl treated mice. Control mice retained normal structure of intestine, lungs and spleen (Fig. 4C).

3.8. Mast cell expansion in spleen, lungs and intestine

The number of mast cell was significantly higher in spleen, lungs and intestine of Bl and Pn treated group when compared to control (Fig. 5).

3.9. Western blots analysis of transcription factors

Transcription factors such as GATA-3, SOCS3 and STAT-6 are actively involved in systemic reactions in food allergy. Increased expression of transcription factors GATA-3 (~2.2 fold), SOCS3 (~1.8 fold) and STAT-6 (~2.4 fold) and decreased expression of T-bet (~2 fold in Bl and 4 fold in Pn) were presented in intestinal protein taken from Bl treated mice groups as compared to control groups (Fig. 6A, B).

3.10. Enhanced mRNA level of cytokines and transcription factors

GATA-3 and T-bet are two important regulators for Th1/Th2 balance in an individual. Elevated levels of GATA-3 expression were observed during Th2 differentiation, and T-bet is expressed specifically and predominantly in Th1 cells [26]. Cytokines play the critical role in induction of allergic reactions. IL-4, IL-5, and IL-13 are responsible for augmented allergic sensitivity [27]. The mRNA levels of cytokines IL-4 (~2.3 fold), IL-5 (~1.8 fold) and IL-13 (~1.5 fold) that constitute bunch of Th2 cytokine were significantly upregulated in Bl and peanut treated mice as compared to controls. The mRNA level of the

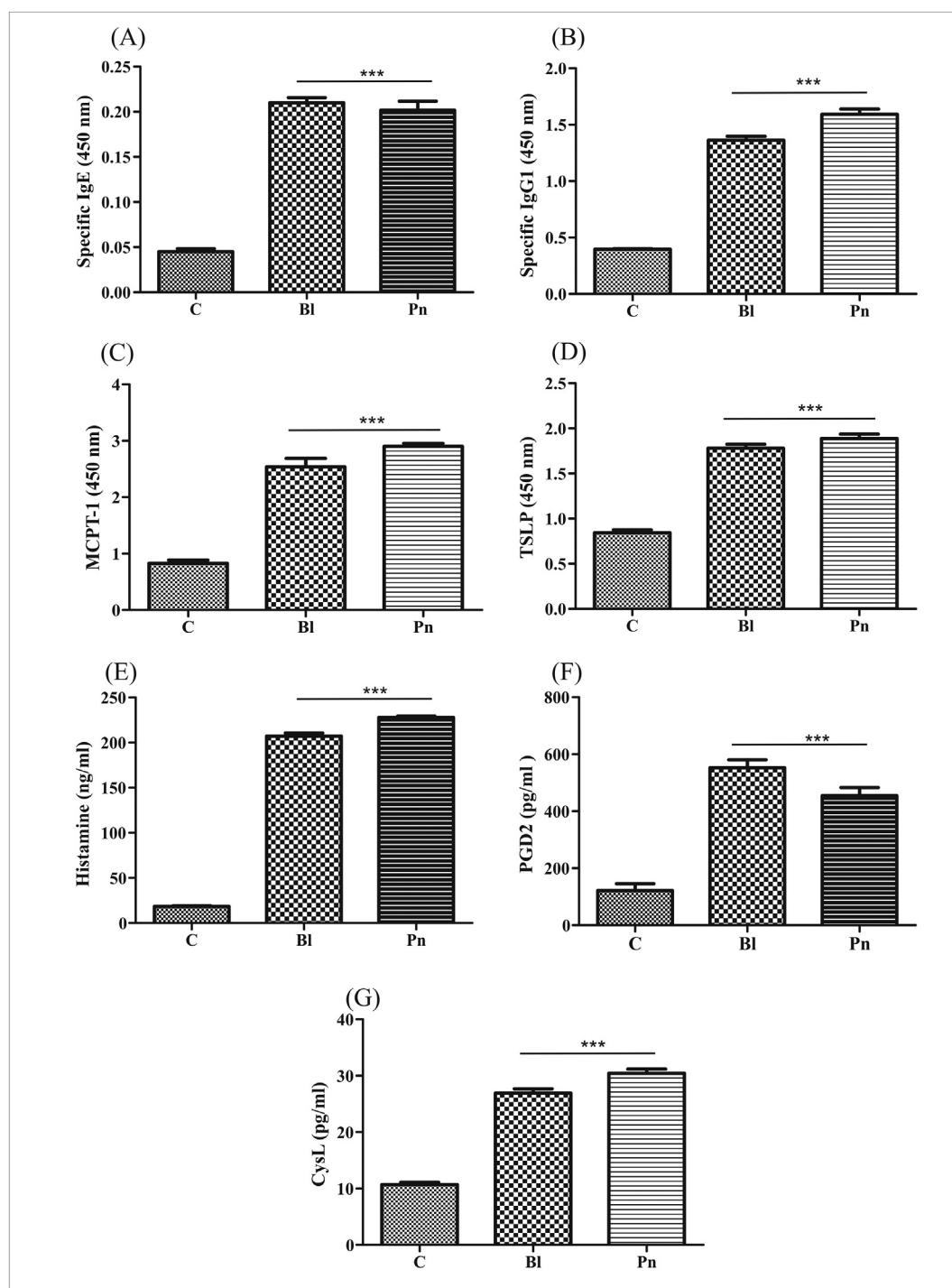


Fig. 3. Estimation of (A) Specific IgE, (B) Specific IgG1, (C) MCPT-1, (D) TSLP, (E) Histamine, (F) PGD₂ and (G) CysL in the pooled sera of all (n = 15/group) *Buchanania lanzan* (Bl) and Peanut (Pn) treated mice. Results are represented in mean $\pm$ SD; of three independent experiments with similar results (**p < 0.0001; ***p < 0.0001; ***p < 0.0001; ***p < 0.0001; ***p < 0.0001; ***p < 0.0001 respectively) when compared with control.

transcription factor for GATA-3 was also significantly increased (~2.5 fold) in treated groups along with decreased expression of T-bet (~2 fold) mRNA, indicating an inclination towards Th2 environment (Fig. 6C, D).

3.11. Upregulated level of CD4+ T-cells, CD8+ T-cells and B-cells

Immunophenotyping studies carried out indicate alterations in relative distributions of T and B cells. Both T and B cells play a contributing role in food allergy such as precipitating anaphylactic

reactions (T-cells) and producing IgE (B-cells). Treatment of Bl and positive control of peanut protein brought a significant upregulation in the populations of CD4+ T-cells (56% and 52% respectively), CD8+ T-cells (20.6% and 21.4% respectively) and B-cells (23.9% and 21.8% respectively) in comparison to control (Fig. 5A, C, Fig. 7).

3.12. Th1/Th2 cytokines levels of serum

Th1/Th2 cytokines (IL-2, IL-4, IL-6, IL-10, TFN- α , IFN- γ and IL-17) were analysed in the serum of Bl and peanut treated mice groups and

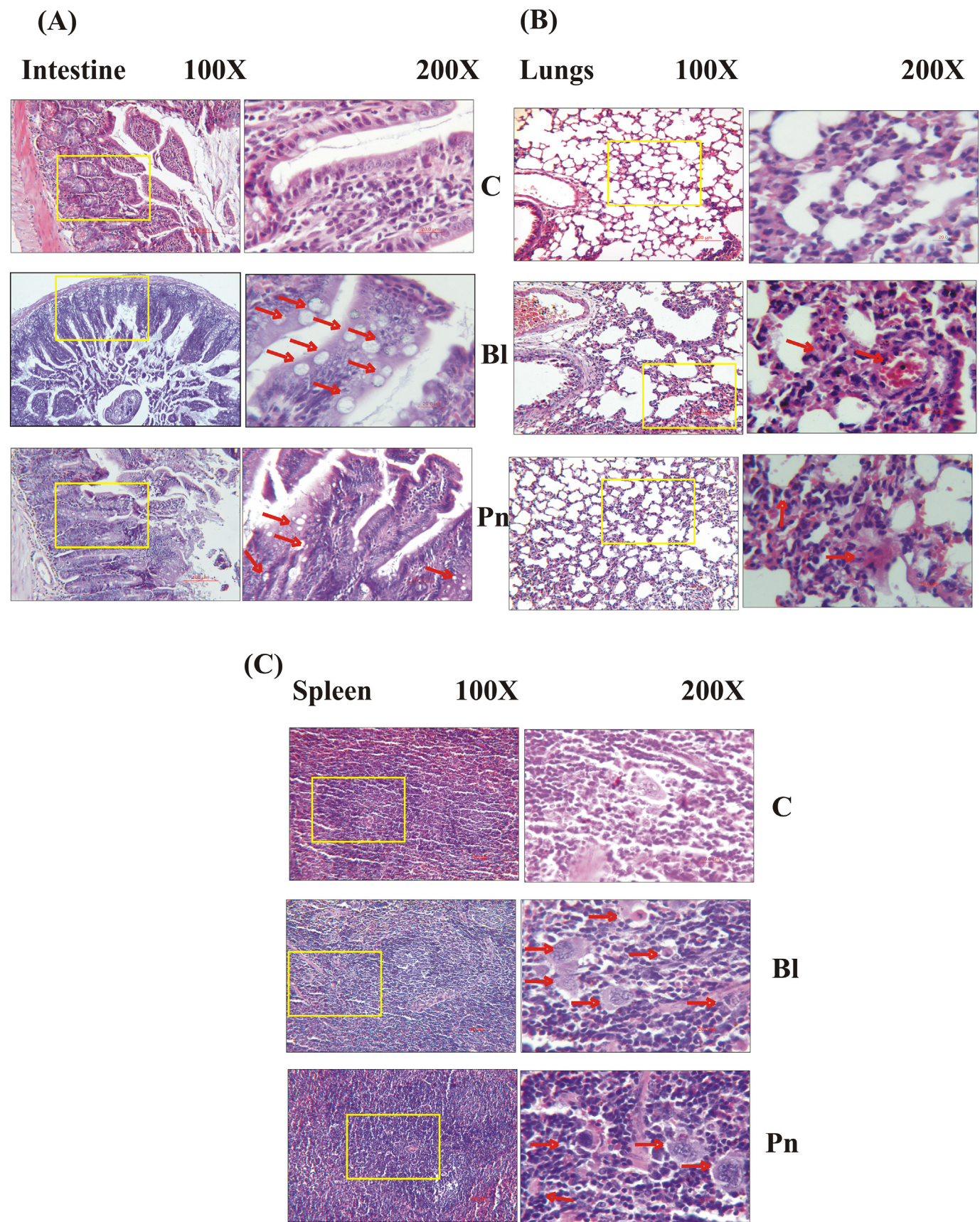


Fig. 4. Histopathological analysis of (A) intestine, (B) lungs and (C) spleen of control and treated mice groups (mice n = 5/group). There was no identifiable histopathological changes in control group, while intestine of *Buchanania lanzan* (Bl) and Peanut (Pn) treated group shows mild goblet cell hyperplasia, exfoliation of villi with infiltration of inflammatory cells, lungs showed thickening of alveolar septa and epithelium, and spleen depicted presence of megakaryocytes and lymphoid hyperplasia.

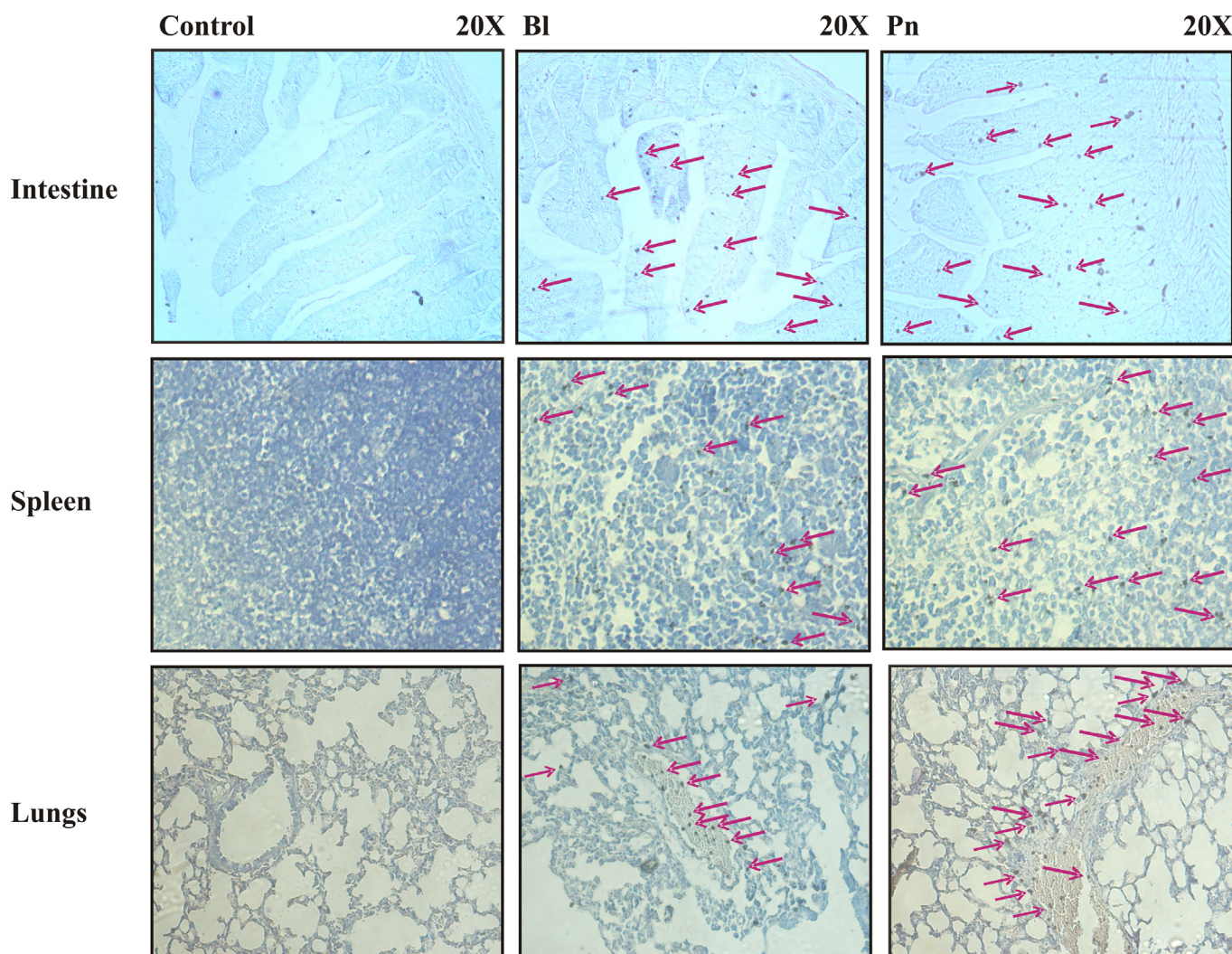


Fig. 5. Toluidine blue staining for mast cell detection in spleen, lung and intestine. A significant increase in mast cells is seen in the *Buchanania lanzan* (BI) and Peanut (Pn) treated groups which are indicated by arrows in the sections, whereas there are hardly any mast cell could be spotted in the control sections.

their respective control. The results showed the increased levels of all Th1/Th2 cytokines when compared to control. The level of IL-4 was almost equal to the level of peanut treated mice, depicting induction of similar intensity of allergic reaction. For the assessment of all these parameters sera from peanut sensitized mice were considered as positive control (Fig. 6A, G, Fig. 8).

4. Discussion

Tree nuts/seeds allergy is an emerging health concern and is escalating across the globe with time. Among the 8 major allergen types, tree nuts are one of them. According to several epidemiological studies, allergy to tree nuts has been reported from all over the world. Tree nuts allergy affects population of different countries, Germany- 8.5%, Israel- 4.1%, U.K.- 2.2%, Canada- 1.2% and U.S.- 0.5% [28]. Out of the affected population, 9% of the young children tend to outgrow the tree nut allergy [29]. BI (family-Anacardaceae) tree nut is used widely in various food preparations, sweet dishes and several other cuisines, including Indian cuisine. According to previous studies, major members of same family, i.e. cashew nut and pistachios are known to have allergic implications. Therefore, in this study an attempt has been made to identify the allergenic potential of BI and understand its mechanism.

The main attribute of a protein to be allergic is its IgE binding potential. In case of BI seven prominent IgE binding proteins could be

identified in sera of BI sensitized mice with M.W. of 11, 20, 23, 25, 48, 54 and 65 kDa, indicating that these proteins may possess capacity to evoke allergic reactions in humans also. To further confirm the allergenic potential of BI, experiments had been carried out taking female BALB/c mice. Female BALB/c mice are known to have augmented Th2-Type immune reaction and generation of antibody [30], making it more sensitive to Th2 driven allergic reactions as well as the absence of male testosterone hormone that tends to suppress such responses [25, 31]. Furthermore, female BALB/c mice reflect significantly upregulated specific IgE production in contrast to males [32]. The food allergens have a tendency to evoke increased production of specific IgE and IgG1. Significantly upregulated levels of specific IgE and IgG1 in the sera of mice treated with BI-CPE highlighted the allergy causing capacity of BI that requires further authentication.

The results of this study also suggest that BI shows cross-reactivity among the tree nuts (cashew, pistachio and peanut). The obtained results shows 5–8 cross reactive allergic fractions that may induce allergic manifestations in the individuals ingesting these nuts as well. The probable reason of cross reactivity could be related to botanical family associations: pistachio and cashew belong to the same botanical family, Anacardiaceae [33].

In the pre-exposed individuals, the allergenic protein present in food induces an effector phase leading cross-linking between the two adjacent IgEs present on the surface of sensitized mast cells or basophils

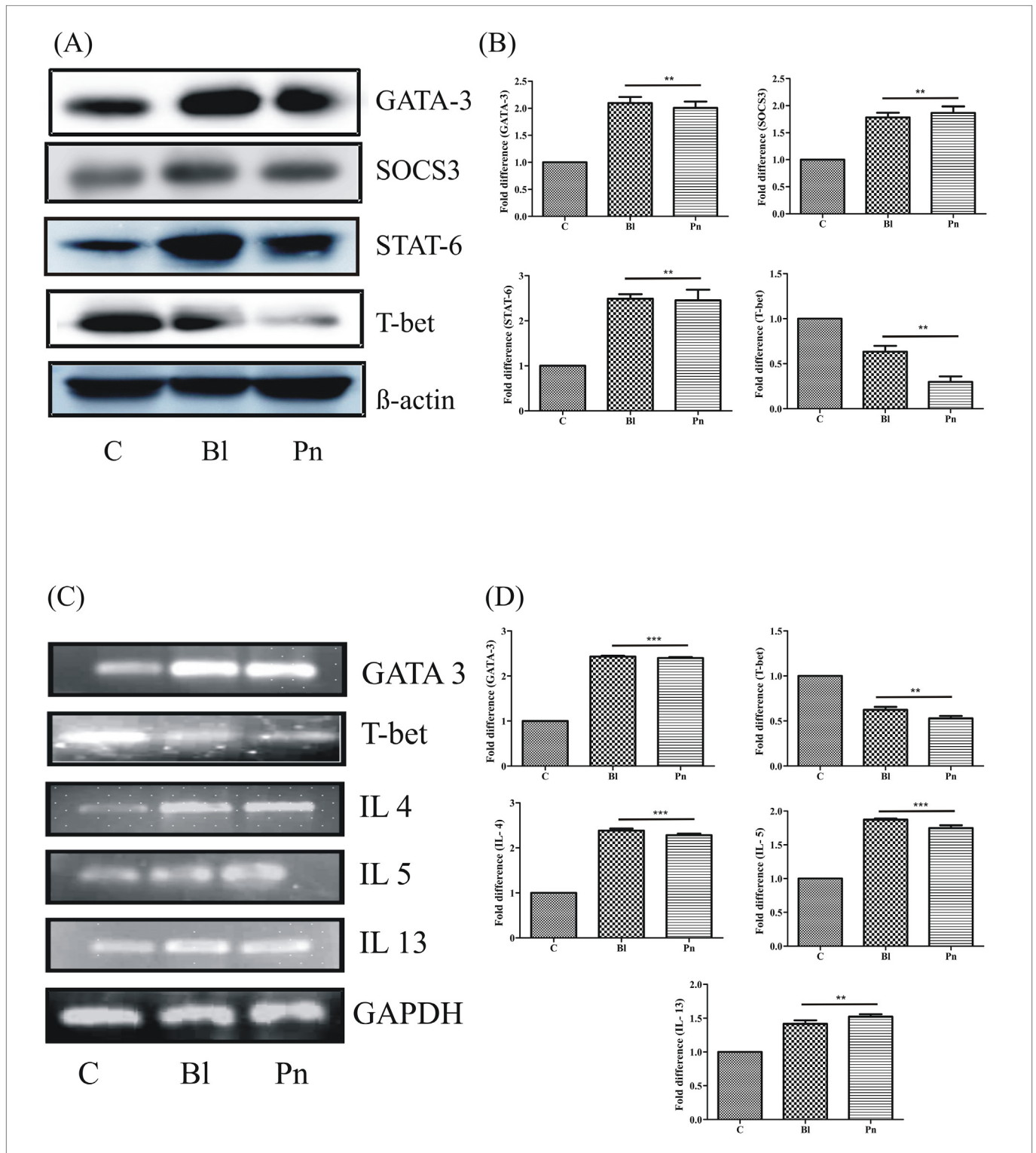


Fig. 6. (A) Western blotting for GATA-3, SOCS-3, STAT-6 and T-bet levels in the intestine of control, *Buchanania lanzan* (Bl) and Peanut (Pn) treated mice groups, where β -actin was taken as internal control. (B) Densitometric analysis of GATA-3, SOCS-3, STAT-6, and T-bet were performed and values were normalised with respect to β -actin and plotted in terms of fold difference. The data presented is from at least three independent experiments and expressed as the mean $\pm$ SD. ns = non-significant and significant difference (**p < 0.0061; **p < 0.01; **p < 0.009; **p < 0.0054 respectively). (C) Semi-quantitative PCR for gene expression. The mRNA expression of cytokines and transcription factors in the intestine of control, *Buchanania lanzan* (Bl) and Peanut (Pn) treated mice group and densitometry of (D) GATA-3, T-bet, IL-4, IL-5, and IL-13 were performed and values were normalised with respect to GAPDH and plotted in terms of fold difference, where GAPDH was taken as internal control. The data from at least three independent experiments are expressed as the mean $\pm$ SD. ns = nonsignificant and significant difference (***p < 0.0001; **p < 0.0017; ***p < 0.0001; ***p < 0.0003; **p < 0.0043 respectively).

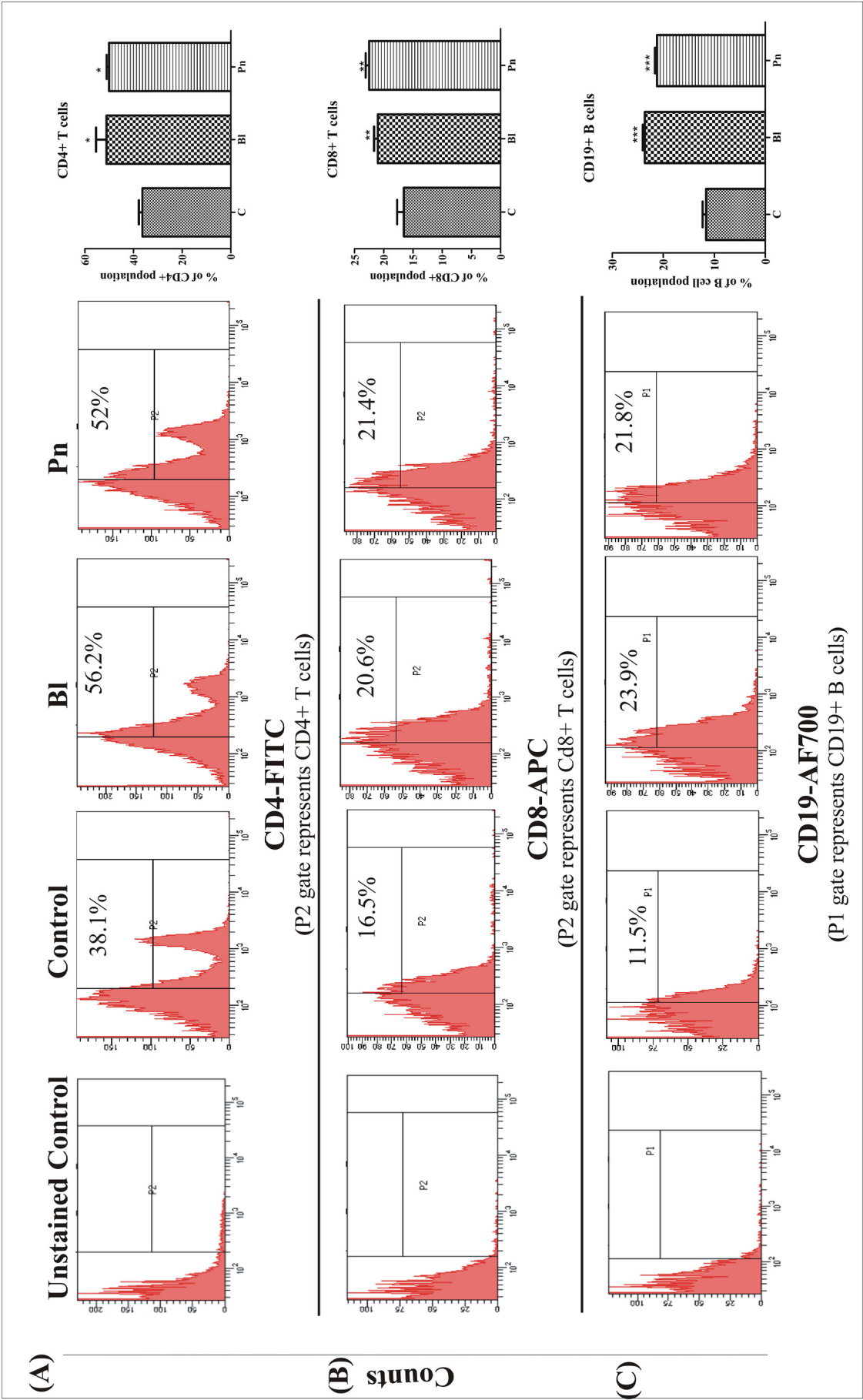


Fig. 7. Effect of *Buchanania lanzan* (BI) on the relative distribution of CD4+ T cells, B cells (mice n = 5/group). Relative percentage of (A) CD4+ T cells; (B) CD8+ T cells; (C) B cells following the exposure of *Buchanania lanzan* (BI) and Peanut (Pn). Histogram from a typical experiment is shown while values represent mean $\pm$ SD of three independent experiments and corresponding graph representing the relative percentage (*p < 0.0138; **p < 0.0060; ***p < 0.0001 respectively significant with respect to control).

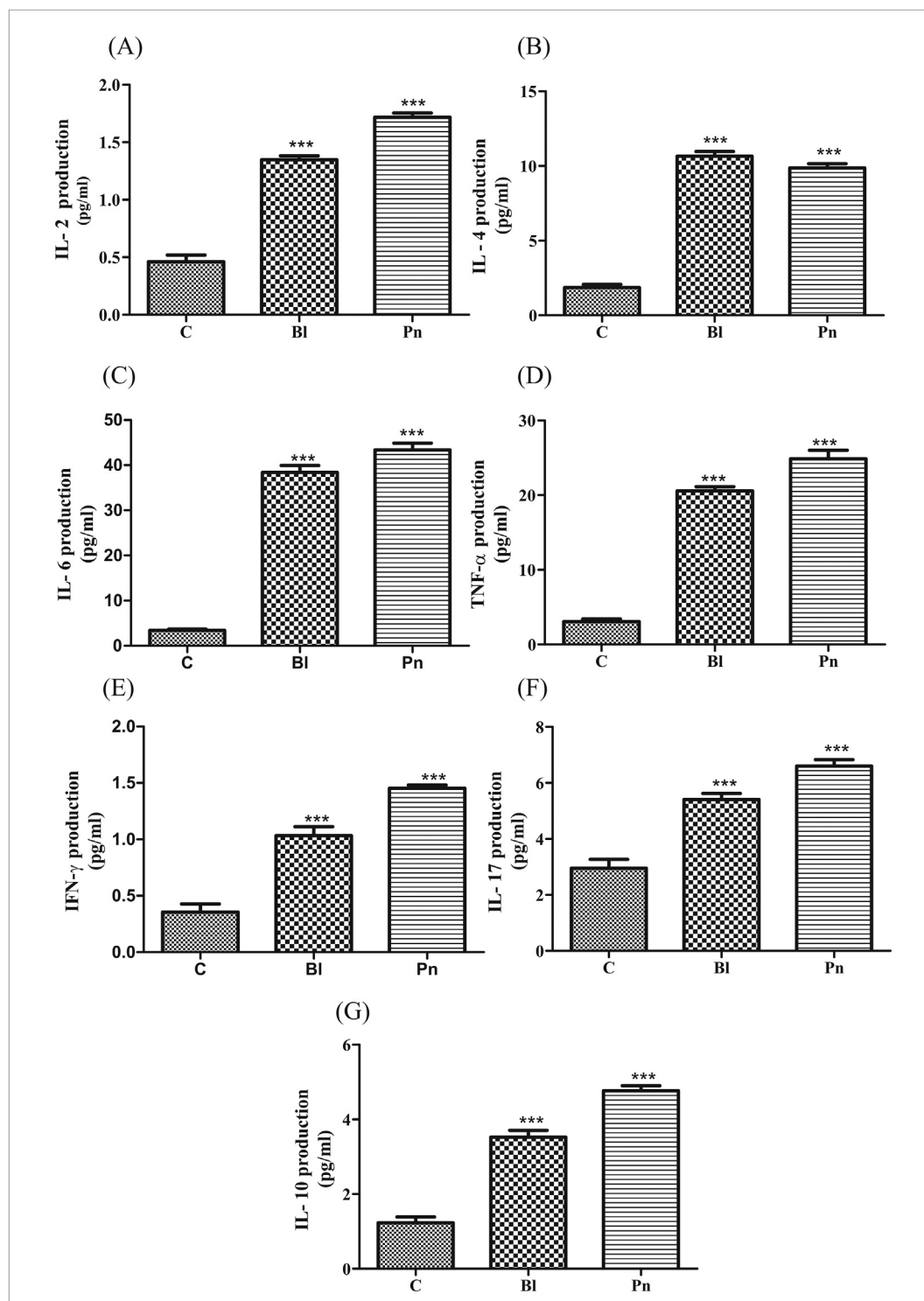


Fig. 8. Cytokine levels in the pooled sera from all treated groups (control, *Buchanania lanzan* (Bl) and Peanut (Pn), $n = 15$ /groups). Th1/Th2 cytokines (A) IL-2; (B) IL-4; (C) IL-6; (D) TNF- α ; (E) IFN- γ ; (F) IL-17 and (G) IL-10, were assessed by cytometric bead array kit with a flow cytometer. Results are expressed in terms of mean $\pm$ SD of three independent experiments and corresponding graph representing the relative percentage (*** $p < 0.0001$; *** $p < 0.0001$; *** $p < 0.0001$; *** $p < 0.0001$; *** $p < 0.0001$; *** $p < 0.0001$; *** $p < 0.0001$ significant with respect to control).

[34]. The activated mast cells or basophils experience degranulation and release various allergic mediators like histamine, cysteinyl leukotriene, and PGD₂ [35]. All these effects contribute to development of anaphylactic symptoms [36]. In the sera of Bl-CPE increased levels of all these allergic mediators were found suggesting its role in cross linking with IgE as well as degranulation.

Development of anaphylactic symptoms by the sensitization from

peanut (Pn)-CPE was reported earlier [20]. To examine such anaphylactic symptoms induced by Bl-sensitization of mice were administered with a booster dose of 5 mg/ml of Bl-CPE on day 60 that resulted in a marked drop in range of 3–4 °C from normal body temperature indicating the onset of systemic anaphylaxis. MCPT-1 also known as β -chymase, is largely present in the mucosal mast cell of intestine and tends to enhance mucosal permeability of the intestine when some

allergic reaction occurs within intestine [14]. MCPT-1 level was significantly upregulated in Bl-CPE treated mice sera (~2 fold) that marked the presence of hypersensitive response in intestine. Mast cell activation, inflammation and Th2 differentiation is facilitated by TSLP during allergic manifestation and its increased level (~3.2 fold) in the sera of Bl-CPE treated mice support serious allergic hypersensitivity reactions.

The histopathological studies of spleen showed lymphoid hyperplasia. Large-sized megakaryocytes were present in Bl-CPE administered mice that can be attributed to T-lymphocytes's immense proliferation and differentiation, indicating chronic inflammation within lymphoid organs. The histological sections of lung and intestine of Bl-CPE treated mice showed infiltration of inflammatory cells that caused airway blockade similar to the cases of asthma.

Mast cells are widely distributed in whole body, but mainly seen in action on the epithelial surface of the respiratory tract, gastrointestinal tract, and genitourinary tract [34, 37]. Excessive infiltration of MCs play a crucial role (by producing chemical mediators) in the progression of allergic manifestations via food derived proteins as found in various studies related to food allergy [18]. Enormous infiltration of MCs was found in spleen, lungs and intestine of the Bl and Pn treated mice indicating the severity of allergy in these experimental groups.

Immunophenotyping gives information about the relative distribution of the T-cells and B-cells population within splenocyte. T-cells are known to play a crucial role in mediating allergic responses [18] and B-cells are well known to produce specific IgE upon antigenic exposure. The significantly increased population of the T-cells and B-cells was observed contributing to Bl-mediated allergy. Cytokines and transcription factors together regulate the Th1/Th2 responses. Cytokines, especially Th2 cytokines like IL-4, IL-5 and IL-13, along with transcription factors play a pivotal role in allergic manifestations. Transcription factors T-bet/GATA3 are involved in deciding Th1/Th2 balance. In the present study, upregulation of Th2 mediated cytokines namely, IL-4, IL-5 and IL-13 was observed at both mRNA as well as protein level suggesting that Bl may be involved in eliciting Th2 mediated allergic response. Further, the enhanced level of transcription factors GATA-3, SOCS3 and STAT-6 together with decreased expression of T-bet confirmed the inclination towards of Bl induced Th-2 mediated allergic reaction.

5. Conclusion

The present study confirms the allergenic potential of Bl-CPE, as it elicits allergic responses like induction of specific IgE, IgG1, histamine, PGD₂, CysL, MCPT-1, prominent histopathological changes, increased immune effectors, heightened Th2 response and anaphylactic shocks. Seven major proteins with molecular weight of 11, 20, 23, 25, 48, 54, and 65 kDa components from the crude protein extract were found to be IgE reactive. Conclusively the study brings out evidences about the allergic manifestations of Bl-CPE which may be similar in the human subjects as well, with identification of seven clinically significant protein components with allergic potential. Therefore extensive studies are required for further characterization of the identified protein components and to understand the mechanism underneath responsible for Bl-CPE mediated allergic manifestations. The present study confirms the allergenic potential of Bl-CPE and identifies seven major proteins in mice as well as in human to be involved in induction of IgE reaction. Bl-CPE significantly induced specific IgE, IgG1 production, increased immune effectors (CD4+ T cells, CD8+ T cells) and the release of other allergic mediators (histamine, PGD₂, CysL, and MCPT-1, etc.) in the murine model, along with the heightened Th2 cytokine responses. Conclusively, outcome from the study helps to understand the Bl mediated allergic response in mice that could be possibly similar in human subject, with identification of responsible proteins having capacity to elicit allergy. This is a step forward for characterization of individual protein and determining underlying

mechanism by which they exert allergic responses further studies are required.

Acknowledgment

We gratefully acknowledge Professor Alok Dhawan, Director of the Institute (CSIR-IITR), India for the support and interest to carry out this study. This work was financially supported by the network project In Depth (BSC 0111) of Council of Scientific and Industrial Research (CSIR), India. SK is thankful to University Grants Commission (UGC), New Delhi, India, for the award of Senior Research Fellowship. This is a CSIR-IITR manuscript number 3521.

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