Research Paper

In vitro and *in vivo* Immunomodulatory Effect of *Lavatera cashmeriana* Protein Concentrate

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This study investigated the immunomodulatory effect of *Lavatera cashmeriana* protein concentrate. Ammonium sulphate method was used to obtain crude protein concentrate from mature seeds. Splenocytes were collected from BALB/c mice and the effect of *Lavatera cashmeriana* protein concentrate on cell viability was investigated using MTT assay. Splenocyte proliferation was triggered by lipopolysaccharide and Con-A and absorbance was recorded using enzyme-linked immunosorbent assay. Lipopolysaccharide was used to induce endotoxic shock in BALB/c mice. Blood samples were analysed for TNF-α and IL-6 levels using enzyme-linked immunosorbent assay. T and B cell surface markers were evaluated using flowcytometry. Primary and secondary antibody titre was determined by haemagglutination technique. Delayed type hypersensitivity model was used to check the ability of *Lavatera cashmeriana* protein concentrate in blocking SRBC antigen-induced paw oedema. *Lavatera cashmeriana* protein concentrate showed no toxic symptoms in mice up to the concentration of 500 mg/kg. *Lavatera cashmeriana* protein concentrate caused dose-dependent decrease in B and T cell proliferation. Maximum inhibition was observed at 800 µg/ml. *Lavatera cashmeriana* protein concentrate reduced lipopolysaccharide-induced production of TNF-α and IL-6. *Lavatera cashmeriana* protein concentrate decreased the levels of T cells (12.5 % CD4+ and 9.65 % CD8+ T cells) in BALB/c mice receiving 50 mg/kg compared to control (22.16 % CD4+ and 16.44 % CD8+ T cells). Mice administered with 200 mg/kg of *Lavatera cashmeriana* protein concentrate exhibited lowest % of CD19+ B cells (10.44 %) compared to control (20.16 %). Dose-dependent reduction was observed in antibody titre and delayed type hypersensitivity response. *Lavatera cashmeriana* protein concentrate played a potential role in modulating immune response and could serve as an effective antiinflammatory for treating inflammation.

Key words: Cytokines, immunophenotyping, lipopolysaccharide, lymphocytes, splenocyte

Besides providing energy to the body, proteins also influence biological activities such as inflammation and oxidative stress. Normally, inflammation plays an imperative role in host defence, but when uncontrolled, can lead to several human pathologies, such as allergic reactions, ischemia-reperfusion injury and tumorigenesis[1,2]. The lack of specific therapeutics has greatly impaired the successful treatment of inflammation related disorders. A better perceptive of the inflammatory mechanisms may highlight novel therapeutic targets. The natural antiinflammatory agents derived from plant sources have received considerable attention due to their potential therapeutic properties[3,4]. Parts of the plants such as their roots, stem, seeds, leaves and flowers contain proteins that potentially modulate the immune system. Overwhelming studies indicate that the plant derived proteins may contain biologically active peptides in their primary sequences with immunomodulating properties. The strategy to utilize plant derived peptides for targeting immune system may prove quite beneficial from therapeutic point of view. Ingesting food proteins and peptides could serve as a safe mode of treatment for preventing human ailments. As compared to most small-molecule drugs, peptides possess strong specificity for targets, low toxicity and good penetration of tissues[5]. This

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clearly shows reason for the increasing interest of scientific community in exploring bioactive peptides with health-promoting effect on the immune system. Inflammation is an early protective mechanism mediated via immune cells and cytokines which helps to protect our body during infection or injury. The bacterial lipopolysaccharide (LPS) has the property to stimulate immune cells like monocyte and macrophage to induce the release inflammatory cytokines from them. Certain peptides such as LL-37 (antimicrobial peptide human of the cathelicidin family) and defensins are known to neutralize bacterial debris like LPS and lipotechoic acid, resulting in reduced levels of proinflammatory cytokines[2]. One of the key mediators of inflammation, the tumour necrosis factor receptor plays an important role in activating neutrophils, promoting adhesion molecule expression on vascular endothelial cells and enhancing the production of other proinflammatory cytokines. Thus targeting this receptor could prove quite productive in taming inflammation and peptides could play an imperative role in this regard. Recent studies have shown that crude protein extracts of Zingiberaceae show significant antiinflammatory activity by inhibiting nitric oxide production in LPS-stimulated RAW 264.7 murine cell line. Recently, it has been reported in many studies that oral administration of lactoferin or its fragment peptides enhances host protection against infection, cancer and inflammation in adults as well as in infants[6,7]. Since the ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions, the inhibition of the cellular reactions is therefore one of the targets that are generally used as an *in vitro* model for antiinflammatory testing have recorded suppression of T-cell mitogenesis by crude whey preparations[8-10]. There have been limited studies to investigate the protein drugs for oral administration purposes as protein drugs are readily degraded in the low pH environment of the stomach. The absorption of protein drugs is also challenging due to their high molecular weight, hydrophilicity, and susceptibility to enzymatic inactivation. Various proteins have been purified from plants capable of retaining the immunomodulatory even at constrains of pH and proteolytic action, permitting significant amounts to reach large intestines in active form. Various studies have demonstrated that the biological activities of some proteins are unaffected by the metabolic/proteolytic activities of faecal microbiota, thereby retaining activity potentially linked to antiinflammatory and anticancer properties. Here the ongoing research effort was to elaborate the plant-derived natural molecules showing promising immunosuppressive potentiality and extensively be applied for inflammation and autoimmune diseases. In this endeavour, the focus of the present investigation was to evaluate the *in vitro* and *in vivo* immunosuppressive activity of protein concentrate isolated from *Lavatera cashmeriana* seeds.

MATERIALS AND METHODS

Medium RPMI 1640 (Sigma), 96 V-wells microtitre plates and micro-tissue culture plates (96 U wells) from Tarsons, trypan blue, fetal calf serum (FCS), concanavalin-A (Con-A), LPS (*Escherichia coli* 055 B5), dimethylsulphoxide (DMSO), Hank's balanced salt solution (HBSS), HEPES, 2-mercaptoethanol, penicillin, streptomycin and MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2,5-dimethyltetrazolium bromide) were purchased from Sigma. Assay kits for all cytokines IL-6 and TNF-α were purchased from R&D Systems, USA.

Sample preparation:

Mature seeds of *L. cashmeriana* Cambess were procured from Department of Botany, University of Kashmir**.** Seeds were crushed in liquid nitrogen by using pestle and motor and the fine powder was made. Seed powder was dissolved in ice cold saline Tris buffer (20 mM, pH 8.0) containing 1 mM sodium meta-bisulphate for 10 min. The homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 12 000 g for 20 min. From the supernatant, the proteins were precipitated by ammonium sulphate at 90 % saturation and the crude protein solution was dialysed against the distilled water, concentrated and lyophilized.

Isolation of splenocytes and determination of cell viability:

Spleen collected under aseptic conditions in incomplete RPMI, was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogenous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8 % w/v). After centrifugation (380×g at 4° for 10 min), the pellet was washed three times with phosphate buffered saline (PBS) and resuspended in complete medium RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10 % FBS. The cell number was counted with a haemocytometer by the trypan blue dye exclusion technique and the absorbance was evaluated in an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm after 15 min. Cell viability

should exceed 95 %. The measurement of cell viability of *L. cashmeriana* protein concentrate was performed via MTT assay. Cells were plated at a density of (1×10^7 cells/ml) in a 96-well plate and incubated at 37° for 24 h. The cells were treated with different concentrations of *L. cashmeriana* protein concentrate (50, 100, 200, 400, 800 and 1600 µg/ml) or vehicle alone. *L. cashmeriana* protein concentrate was first dissolved in DMSO to make 2 mg/ml stock concentration and further diluted with DMSO for working concentration. Final DMSO concentrations on the cells were <0.25 % and were shown not to interfere with the assay. After 20 h of incubation at 37°, 20 ml of the MTT (5 mg/ml in PBS) solution was added to each well and incubated under the same conditions for another 4 h. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 100 µl of DMSO working solution (192 µl of DMSO with 8 µl 1M HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

LPS and Con-A-induced splenocyte proliferation:

To evaluate the effect of *L. cashmeriana* protein concentrate on LPS and Con-A-induced lymphocte proliferation, the spleen cells suspension (1×10^7 cells/ml) was pipetted into 96 well plates (200 µl/well) and cultured at 37° for 72 h in a humid saturated atmosphere containing 5 % CO_2 in the presence of Con-A (5 µg/ml) and LPS (1 µg/ml) and increasing doses of *L. cashmeriana* protein concentrate (25-400 µg/ml). After 72 h 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4 h. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well 100 µl of DMSO working solution (192 µl of DMSO with 8 µl 1M HCl) was added and the absorbance was evaluated in ELISA reader at 570 nm after 15 min.

Experimental animals:

Male Balb/C mice (*Mus musculus*) 8-10 w old and weighing 18-22 g, in groups of six, were used for the study. The protocol for acute toxicity and *ex vivo* studies on immunomodulatory activity was approved by Institutional Animal Ethics Committee (IAEC) of Indian Institute of Integrative Medicine (CSIR), Canal Road Jammu (CPCSEA registration No. 67/CPCSEA/99). The animals were maintained under standard laboratory conditions: temperature (25±2°) and a photoperiod of 12 h fed with standard pellet diet and received water *ad libitum*.

General behavior and acute toxicity test:

Acute oral toxicity studies were carried out following OECD guidelines no. 423 after approval from the Institutional Animal Ethics Committee. Three male Balb/C mice, fasted 3-4 h prior to the test, were used for each step and observed individually after dosing at least once during the first 30 min, and periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter, for a total of 14 d. Simultaneously, general behaviour and any toxic symptoms produced by the crude protein concentrate dissolved in normal saline were observed for 14 d for routine pharmacological parameters such as cyanosis, tremors, convulsions, ataxia, body tone, muscle tone, piloerection, salivation, tail flick, drowsiness, alertness, spontaneity, diarrhoea, pupil size, ptosis, breathing rate, urination.

LPS-induced endotoxin shock:

LPS-induced endotoxin shock model is an established models for systemic inflammation in which macrophages are the major source of the proinflammatory cytokines responsible for the development of the shock. For the determination of the 50 % lethal dose (LD50), groups composed of five mice were received intraperitoneal (ip) injections of 1.25, 2.5, 5.0, 7.5 and 10 mg/kg of body weight of Salmonella-derived LPS/mouse dissolved in PBS. Survival of animals was monitored for a period of 7 d. To determine the effect of *L. cashmeriana* protein concentrate on the survival of mice injected with LPS, 35 mice were divided in 7 different groups, group I (control) and group II received normal saline, group III received *L. cashmeriana* protein concentrate (50 mg/kg), group IV received *L. cashmeriana* protein concentrate (100 mg/kgt), group V received *L. cashmeriana* protein concentrate (200 mg/kg), group VI received *L. cashmeriana* protein concentrate (400 mg/kg) and group VII received *L. cashmeriana* protein concentrate (800 mg/kg) for 6 d via oral gavage. On day 7, LPS 5 mg/kg (LD_{50} dose) was given intraperitoneally to all groups except group I. Survival of animals was monitored for a period of 7 d.

ELISA:

Blood from retro-orbital plexus was collected for the determination of TNF-α 3 h after LPS administration and at 8 h for the determination of IL-6 levels. Each time point and treatment group was composed of 5 animals per experiment. Sera were collected and

frozen until used for cytokine determination according to the instructions of the manufacturer (R and D).

Evaluation of T and B cell surface markers by flow cytometry analysis:

The animals were divided into 5 groups of 6 animals each. Group I (control) received normal saline, group II received β-methasone, a standard immunosuppressant (5 mg/kg); group III received *L. cashmeriana* protein concentrate (50 mg/kg); group IV received *L. cashmeriana* protein concentrate (100 mg/kg) and group V received *L. cashmeriana* protein concentrate (200 mg/kg). *L. cashmeriana* protein concentrate and standard drug was given to mice for 6 d via oral gavage. On day 7, LPS 5 mg/kg was given intraperitoneally 12 h before blood sampling. Each animal received 100 µl of this concentration dissolved in PBS. Animals were bled retro-orbitally and blood was collected in EDTA-coated tubes for $CD4^+/CD8^+$ T cell surface markers and $CD19^+$ B cell surface marker estimations. Fluorescence isothiocynate-labelled $CD4^+$ and phycoerytherin (PE)-labelled $CD8^+$ monoclonal antibodies were added to 100 µl of whole blood. Tubes were incubated in dark for 30 min at room temperature. Subsequently, 2 ml of 1 X FACS lysing solution (BD Biosciences) was added at room temperature with gentle mixing followed by incubation for 10 min. Samples were centrifuged (300-400×g), the supernatant was aspirated and samples were given 3 washings of PBS (pH 7.4). The resulting stained cell pellet was resuspended in 500 µl of PBS and was run on a flow cytometer. The forward and side scatter gating applied for data acquisition on 10 000 events and fraction of cell population representing different phenotypes analysed using cell quest software.

Immunization schedule and antiSRBC antibody titre:

SRBC collected in Alsever's solution were washed 3 times in large volumes of pyrogen-free 0.9 % normal saline and adjusted to a concentration of 5×10^9 cells/ml for immunization and challenge. The animals were divided into 5 groups of 6 animals each. Group I (control) received 1 % gum acacia; group II received β-methasone (5 mg/kg), group III received *L. cashmeriana* protein concentrate (100 mg/kg), group IV received *L. cashmeriana* protein concentrate (200 mg/kg) and group V received *L. cashmeriana* protein concentrate (400 mg/kg). *L. cashmeriana* protein concentrate was dissolved in 1 % gum acacia and was administered orally for 14 d. The dose volume was 0.2 ml. Mice were immunized by injecting 0.2 ml of 10 % of fresh SRBC suspension intraperitoneally on day zero. Blood samples were collected in micro centrifuge tubes from each experimental mice via retro-orbital plexus on d 7 for primary antibody titre and d 14 for secondary antibody titre. Serum was separated and antibody levels were determined by the haemagglutination technique[11]. Briefly, equal volumes of individual serum samples of all the groups were pooled. Two fold dilutions of pooled serum samples were made in 25 µl volumes of normal saline in a micro titration plate to which were added 25 µl of 1 % suspension of SRBC in saline. After mixing, the plates were kept for 1 h at room temperature and examined for haemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

Delayed type hypersensitivity (DTH):

This model is based upon the ability of test drug to inhibit the oedema produced in the hind paw of mice after injection of SRBC antigen[12]. *L. cashmeriana* protein concentrate was administered 2 h after SRBC intraperitoneal injection and once daily on consecutive days; six days later, the thickness of the left hind footpad was measured with a Vernier caliper (pitch, 0.01 mm) The mice were then challenged by injecting 20 µl of 5×10^9 SRBC/ml intradermally into the left hind footpad. The foot thickness was measured again after 24 and 48 h. The animals were divided into 5 groups of 6 animals each. Group I (control) received 1 % gum acacia; group II received β-methasone (5 mg/kg), group III received *L. cashmeriana* protein concentrate (100 mg/kg), group IV received *L. cashmeriana* protein concentrate (200 mg/kg) and group V received *L. cashmeriana* protein concentrate (400 mg/kg). *L. cashmeriana* protein concentrate was dissolved in 1 % gum acacia and was administered orally for 14 d. The dose volume was 0.2 ml.

Statistical analysis:

Data were expressed as mean±SEM and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test). Dunnett's test was used to analyze the different variables in the same subject and P values less than 0.05 were being taken as statistically significant.

RESULTS AND DISCUSSION

To determine the effect of *L. cashmeriana* protein concentrate on cell viability, the concentrate was

tested in the MTT cell viability assay using Balb/C mice splenocytes. The cytotoxic effect was tested to establish the appropriate concentration ranges of *L. cashmeriana* protein concentrate for the analysis of ongoing experiments. Concentrations of 50, 100, 200, 400, 800 and 1600 µg/ml were tested. No cytotoxic effect was observed up to 800 µg/ml as shown in fig. 1A. At a higher concentration of the protein (1600 µg/ml), about 60 % of cytotoxic effect was seen on mice splenocytes.

To characterize the effect of *L. cashmeriana* protein concentrate on LPS and Con-A-induced splenocyte proliferation, splenocytes cultured in presence of LPS (a B cell stimulator) and Con-A (a T cell stimulator) with increasing doses of *L. cashmeriana* protein concentrate. The protein concentrate caused a dose-dependent decrease in the proliferation of B and T cells as observed by reduction of MTT. During this study, it was observed that B-cells are more sensitive to *L. cashmeriana* protein concentrate as compared to T cells. Maximum inhibition was observed at the dose of 800 µg/ml as depicted in fig. 1B.

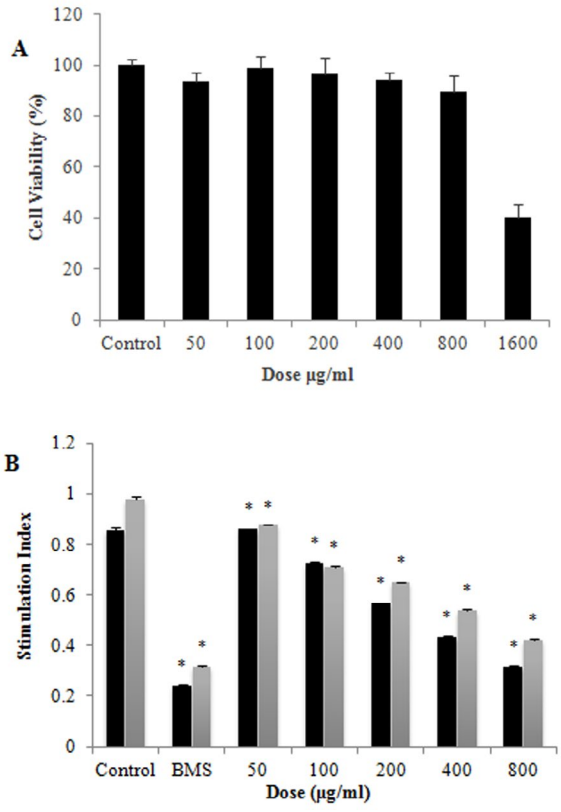

Fig. 1: Effects of *Lavatera cashmeriana* seed protein concentrate on cell viability and LPS-induced B cell and Con-A-induced T cell proliferation

A. Effects of *Lavatera cashmeriana* seed protein concentrate on cell viability of Balb/C mice splenocytes, B. dose-dependent decrease in LPS-induced B cell and Con-A-induced T cell proliferation. Values are means±SE; (■) B cell, (□) T cell

No effect on general behaviour or any toxic symptoms was observed in mice up to a dose of 500 mg/kg. Evaluation of the test material was initiated by giving the mice 1/10th of this dose (50 mg/kg) as well as a lower (12.5 mg/kg) and higher (400 mg/kg) doses. Overall no behavioural changes or any toxicity were observed in the mice treated with the protein concentrate up to a dose of 500 mg/kg.

LPS was administered ip at concentrations of 1.25, 2.5, 5.0, 7.5 and 5.0 mg/kg and 80 % percent of the animals treated with LPS at 0.5 mg/kg survived and only 69 % survived at 1 mg/kg, compared to 51 % of the animals survived once treated at a dose level of 5 mg/kg, 37 % at 7.5 mg/kg and only 10 % of animals survived at 10 mg/kg as shown in fig. 2A. The LD_{50} was estimated at 5 mg/kg ($p<0.05$).

Male Balb/c mice were grouped and maximum 5 animals were housed in a polyvinyl cage. Each mouse was administered an LD_{50} dose of LPS 5 mg/kg, i.p. with or without *L. cashmeriana* protein concentrate. A dose-dependent increase in animal survival was observed from the concentration of 50-400 mg/kg after *L. cashmeriana* protein concentrate administration. At the concentration of 400 mg/kg, *L. cashmeriana* protein concentrate about 70 % of animals survived from the septic shock induced as shown in fig. 2B. Cells present in peritoneal cavity are the first immune cell population exposed to LPS in this model. LPS rapidly access the circulation, where it triggers a global inflammatory response by activating immune cells in different organs such as the spleen, lungs to produce proinflammatory mediators. Significant decrease of TNF-α and IL-6 was observed in animals treated with *L. cashmeriana* protein concentrate as compared to LPS alone ($n=5$, $p<0.05$). Maximum decrease in cytokine level was observed at the higher concentration of *L. cashmeriana* protein concentrate depicted in fig. 3A and B.

Furthermore, the effect of *L. cashmeriana* protein concentrate was observed on the population of T and B cell surface markers like $CD4^+$, $CD8^+$ and $CD19^+$ in the whole of mice treated with variable doses of *L. cashmeriana* protein concentrate. *L. cashmeriana* protein concentrate supplement showed a dose-dependent decrease in $CD4^+$ and $CD8^+$ T cell proliferation in peripheral blood lymphocytes of mice challenged with intraperitoneal injection of 5 mg/kg of LPS. The maximum inhibition of $CD4^+$ and $CD8^+$ T cell proliferation was observed in mice received 50 mg/kg of *L. cashmeriana* protein concentrate and showing 12.5 % $CD4^+$ and 9.65 % $CD8^+$ T cells

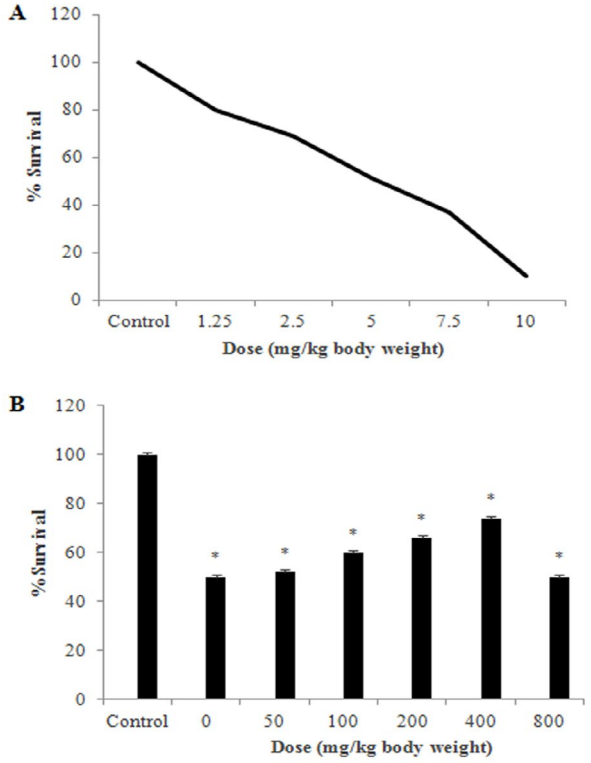

Fig. 2: LD_{50} of LPS in Balb/C mice
Percentage decrease in survival rate at higher doses of LPS

as compared to control showing 22.16 % $CD4^+$ and 16.44 % $CD8^+$ T cells as shown in fig. 4A and Table 1. Similarly a dose-dependent decrease in $CD19^+$ B cell population was observed in this study. At a higher concentration of *L. cashmeriana* protein concentrate (200 mg/kg) about 10.44 % of $CD19^+$ B cell markers were observed as compared to control having 20.16 % of $CD19^+$ B cells as shown in fig. 4B and Table 1.

Male Balb/C mice challenged with SRBC antigen and administered with *L. cashmeriana* crude protein showed a dose-dependent decrease in both primary and secondary antibody titre ($p<0.05$). After 7 d post immunization *L. cashmeriana* protein concentrate (400 mg/kg) dissolved in 1 % gum acacia caused the maximum decrease in IgM titre. After 14 d IgG titre was also decreased at the maximum dose i.e. 400 mg/kg. The results clearly demonstrated that *L. cashmeriana* protein concentrate played a role in humoral immunity as shown in fig. 5. Male Balb/C mice were challenged with SRBC antigen into the sub-plantar side of left hind paw and were fed with *L. cashmeriana* protein concentrate. The results clearly demonstrated that there was a dose-dependent decrease in DTH response as evident by the decrease in footpad thickness at 24 and 48 h (Table 2). Maximum decrease was observed at 400 mg/kg ($p<0.05$).

L. cashmeriana Cambess belongs to family Malvaceae and is endemic to Kashmir valley. It has great medical importance. Its parts are used for treating inflammation and asthma in herbal medicine. To date, there is no information on the antiinflammatory activity of protein extract from the seeds of *L. cashmeriana* Cambess[13]. The present study was aimed to investigate the immunosuppressive property of protein fraction from *L. cashmeriana* Cambess seeds. *L. cashmeriana* protein concentrate exerted antiinflammatory effect directly on immune cells both *in vitro* and *in vivo*. *L. cashmeriana* protein concentrate caused a dose-dependent decrease in the LPS-induced proliferation of B and T cells *in vitro*. Since *L. cashmeriana* protein concentrate exerted an effect on splenocyte proliferation *in vitro*, it was hypothesized that *L. cashmeriana* protein concentrate might play an important role in systemic inflammation *in vivo*, including activation of the cascade of events following exposure to LPS and leading to the endotoxin shock syndrome. LPS, a group of bacterial endotoxins, stimulates monocytes/macrophages, to produce the

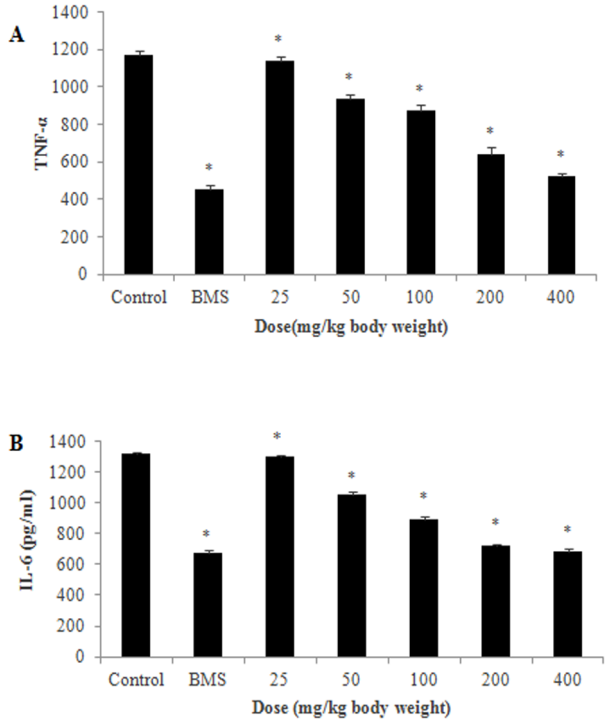

Fig. 3: Decrease in LPS-induced cytokine expression by *L. cashmeriana* protein concentrate
Blood was collected from retro-orbital plexus for the determination of TNF-α at 3 h after LPS administration and at 8 h for the determination of IL-6 levels. Each time point and treatment group was composed of five animals per experiment. Sera were used for cytokine determination by enzyme-linked immunosorbent assay. Values are means±SE (n=5); *p<0.05, (control vs. treated groups). Error bars represent standard errors and asterisk represents statistical significance (control vs. treated groups)

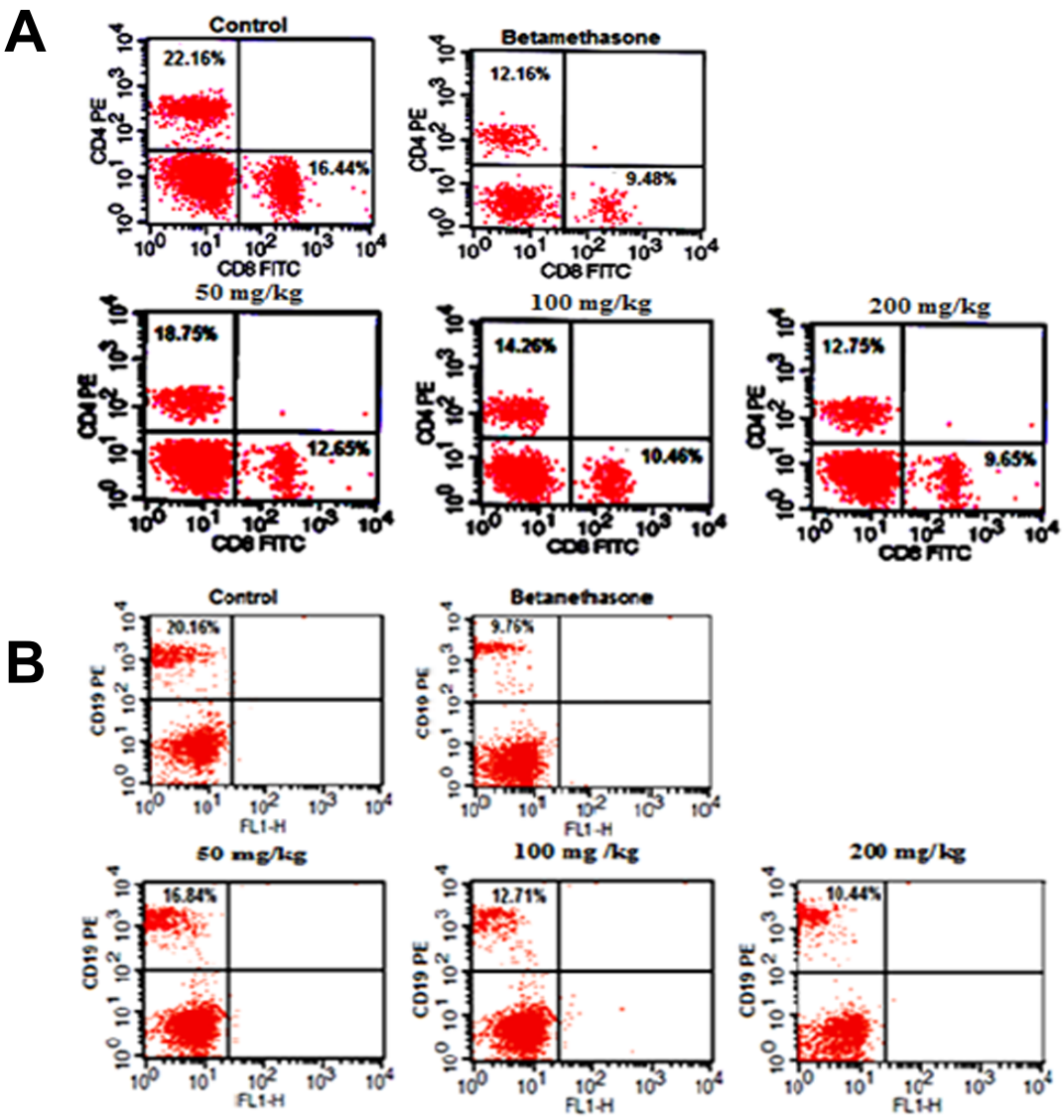

Fig. 4: Effect of *L. cashmeriana* protein concentrate on (A) $CD4^+$, $CD8^+$, (B) $CD19^+$ populations

TABLE 1: EFFECT OF *L. CASHMERIANA* PROTEIN CONCENTRATE ON $CD4^+$, $CD8^+$ AND $CD19^+$ POPULATION IN WHOLE BLOOD

Treatment	Dose mg/kg	%$CD8^+$	%$CD19^+$	% $CD4^+$
Control	-	16.44±0.5	20.16±0.9	22.16±1.24
BMS	5	9.48±0.22*	9.76±0.4*	12.16±0.3*
L. cashmeriana protein concentrate	50	12.65±0.16*	16.84±0.15*	18.75±0.5*
L. cashmeriana protein concentrate	100	10.46±0.4*	12.71±0.35*	14.26±0.15*
L. cashmeriana protein concentrate	200	9.75±0.29*	10.44±0.6*	12.65±0.25*

Values are means±SE (n=5); *p< 0.05 (control vs. treated groups)

proinflammatory cytokines TNF-α and IL-6, which in turn are the principal initiators of the endotoxin shock syndrome[14]. It was found that *L. cashmeriana* protein concentrate suppressed endotoxin-induced proinflammatory cytokines like TNF-α and IL-6 in plasma. TNF-α and IL6 was significantly reduced in mice pre-treated with *L. cashmeriana* protein concentrate compared to LPS only. This study concluded that *L. cashmeriana* protein concentrate could protect against the systemic inflammatory response and subsequent organ injury induced by LPS, at least partly, through the inhibition of proinflammatory cytokine and chemokine expression. T cells expressing $CD4^+$ are increased when the physiological systems of the body are stimulated due to the activation of the non-specific immune system and inhibition of this phenomenon indicates immunosuppression. LPS from Gram-negative bacteria causes polyclonal activation of B cells and stimulation

TABLE 2: DOSE-DEPENDENT DECREASE IN FOOTPAD THICKNESS AT 24 AND 48 HOURS AS COMPARED WITH THE CONTROL GROUP

Treatment	Dose mg/kg	24 h	48 h
Control	-	2.5±0.03	2.50±0.02
BMS	50	0.80±0.05*	0.58±0.06*
L. cashmeriana protein concentrate	100	1.75±0.01*	1.54±0.05*
L. cashmeriana protein concentrate	200	1.20±0.04*	0.97±0.06*
L. cashmeriana protein concentrate	400	0.83±0.21*	0.66±0.02*

Values are means±SE (n=5) *p< 0.05, (control vs. treated groups)

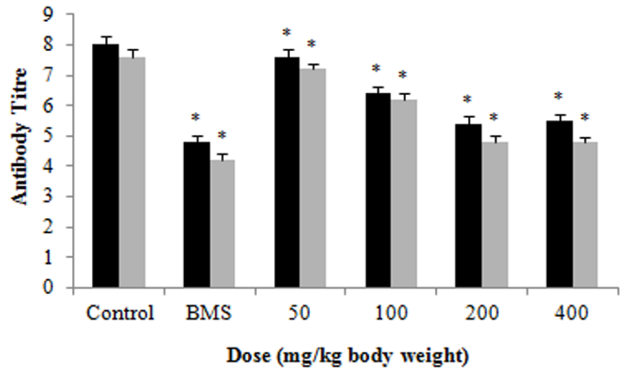

Fig. 5: Decrease in primary and secondary antibody titre by *L. cashmeriana* protein concentrate
***Values are means±SE (n=5); *p<0.05, (control vs. treated groups), (■) primary antibody titre, (■) secondary antibody titre**

of macrophages and other antigen presenting cells[15,16]. LPS also induces strong stimulation of T cells under *in vivo* conditions. It was observed that a significant decrease in $CD4^+$, $CD8^+$ and $CD19^+$ population in whole blood of mice challenged with intraperitoneal injection of LPS and *L. cashmeriana* crude protein.

Many of the disorders today are based on the imbalances of immunological processes like DTH (cell-mediated) reactions and humoral response[17]. DTH is a part of the process of graft rejection, tumour immunity and most important immunity to many intracellular infectious micro-organisms, especially those causing chronic diseases *viz* tuberculosis[18]. Further, DTH requires the specific recognitions of a given antigen by activated T-lymphocytes, which subsequently proliferate and release cytokines. These in turn, increase vascular permeability, induce vasodilation, macrophage accumulation[9], and activations, promoting increased phagocytic activity and increased concentration of lytic enzymes for more effective killing[19]. Significant dose-dependent decrease in DTH response as evident by the decrease in footpad thickness at 24 and 48 h was observed in mice challenged with SRBC antigen into the sub-plantar side of left hind paw. This inhibition of DTH response indicated that *L. cashmeriana* protein concentrate has an inhibitory effect on lymphocytes and accessory cell types required for the expression of the reaction and thus decreases cell-mediated immunity. The humoral immunity involves interaction of B-cells with the antigen and their subsequent proliferations and differentiations into antibody secreting plasma cells[20]. Further, antibody functions as the effector of the humoral response by binding to antigen by neutralizing it or facilitating its eliminations by cross-linking to form clusters that are more readily ingested by phagocytic cells. *L. cashmeriana* protein concentrate showed a significant dose-dependent decrease in antibody titre in mice challenged with SRBC antigen. Minimum antibody titre was observed at 400 mg/kg. After 7 d post-immunization *L. cashmeriana* protein concentrate caused the maximum decrease in IgM titre. After 14 d IgG titre was also decreased. This inhibition of the humoral response to SRBC antigen by decrease in haemagglutination antibody titre indicated the lack of response of macrophages and T and B lymphocyte subsets involved in antibody synthesis[21]. In conclusion, oral administration of *L. cashmeriana* protein concentrate modulated the expression of immunity-related genes specifically or non-specifically in the small intestines of mice. It was speculated that systemic circulation of immune cells would transmit the immunomodulation by *L. cashmeriana* protein concentrate in the intestinal compartment to other systemic compartments involving B and T cells. The identity of the individual components of the *L. cashmeriana* protein concentrate that induced suppression of murine lymphocyte function remains uncertain. As current understanding of the efficacy and mechanism of antiinflammatory proteins and peptides increases, so will the growing interest in their prophylactic preventive and therapeutic use. In conclusion, *L. cashmeriana* protein concentrate has a strong antiinflammatory activity. The component or components of *L. cashmeriana* protein concentrate capable of immunomodulatory response are still unknown need further isolation and characterization. The present study suggests that *L. cashmeriana* seeds would serve as a source for the discovery of novel antiinflammatory proteins.

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Conflict of interests:

Authors declare that they have no conflict of interest.

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