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Original Research Article (Experimental)

Increased expression of co-stimulatory molecules and enhancement of the IgG response in rats orally administered with a polyherbal formulation

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ABSTRACT

Background: *Link Samahan*® (LS) is a standardized modern formulation of a polyherbal preparation used in the indigenous system of medicine in Sri Lanka.
Objective: Evaluation of the immunostimulatory activity of LS and the molecular mechanisms that modulate the humoral immune response.
Material and methods: Immunostimulatory activity of LS was tested in rats following oral administration on days 1-5 and 15-19 and immunization with bovine serum albumin (BSA) on day 1 and 15. Anti-BSA IgM and IgG response in rats treated with LS, water and sugar (as controls) were compared on days 0-35, using ELISA. The expression of co-stimulatory molecules on lymphocytes was assessed on days 0-8 and days 14-22 using RT-qPCR.
Results: IgM and IgG levels of LS-treated rats were increased significantly by day 7 and 21 respectively compared to controls ($p < 0.05$). IgG response of LS-treated group reached a higher magnitude compared to its IgM response. Gene expression of CD28 and CD40L on T cells (4.9-5.1 fold) and CD80, CD86 and CD40 on APCs (2.4-3.1 fold) were induced significantly by day 2 compared to their expression on day 0 ($p < 0.05$). The expression levels of CD28 and CD40L on day 2-4 and 16-18 were similar while the expression of CD80, CD86 and CD40 on day 16-18 was higher (3.7-5.1 folds) compared to their levels on day 2-4 (2.4-3.2).
Conclusions: These findings support an adjuvant effect of LS contributing to its immunostimulatory activity and increased expression of co-stimulatory molecules that contribute to boosting immune response.
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1. Introduction

Immunostimulators or immunostimulants are the substances or agents involved in the enhancement of the basal levels of immune response. Immunostimulators improve the natural resistance of the host against different types of infections, i.e., bacterial and viral infections, by activating the immune system [1]. Immunostimulants enhance the immune response either as specific immunostimulants that act as antigen for stimulation of the immune response such as vaccines or as non-specific immunostimulants without antigen properties enhancing immune response to other antigens such as adjuvants [2]. There are several types of stimulants with different mechanisms, chemical characteristics and functions as well as with different origins being either natural products or synthetic [1,2]. Immunostimulants induce activation or increase activity of different components of the immune system in humans and animals [3]. Immunostimulants can be classified into following classes; bacterial products, complex

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carbohydrates, vaccines, cytokines, immunoenhancing drugs, plant extracts, and animal extracts [1].

Several synthetic compounds have been developed which has immunostimulatory activity capable of inducing either humoral or innate immune responses or both to treat diseases. Levamisole (Ergamisol) is a synthetic drug which induces different immune cells as B and T lymphocytes, monocytes and macrophages, used to treat cancer patients [4]. Filgrastim (Neupogen) is another well-known immunostimulant which is used to treat neutropenia. It is a granulocyte colony-stimulating factor (G-CSF) and stimulates survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils to fight against infection [5]. Due to the adverse effects that occur through the usage of these chemically derived drugs, currently there is a vigorous search for potential natural substances as therapeutic agents. Plants have received much attention as natural therapeutic agents [6]. Traditional herbal medicines use plants or plant material as crude or processed products to treat diseases [7]. Herbal medicines have long recorded history worldwide and at present, there is rapid a development in designing innovative methods to investigate immunostimulatory activity of plants and plant derivatives.

Polyherbalism is a concept in Ayurveda in which more than one plant or plant derivatives are used to treat diseases [7]. Treatment of diseases using polyherbal formulations or combined extracts of plants is considered to achieve higher therapeutic effectiveness and safety, compared to using a single plant. *Link Samahan*® (LS) is a polyherbal formulation which has been available both locally in Sri Lanka and internationally during the past twenty five years. LS is commonly used for prophylaxis against common cold and common cold related symptoms by improving the immunity. LS is a standardized modern formulation of a polyherbal preparation used in the indigenous system of medicine (*Deshiya Chikitsa*) in Sri Lanka. It contains the extracts of fourteen medicinal plants; *Adhatoda vasica, Alpinia galangal, Carum copticum, Coriandrum sativum, Coscinium fenestratum, Cuminum cyminum, Evolvulus alsinoides, Glycyrrhiza glabra, Hedyotis corymbosa, Piper longum, Piper nigrum, Premna herbacea, Solanum xanthocarpum,* and *Zingiber officinale*, granulated with cane sugar. It is manufactured by Link Natural Products (Pvt) Ltd and presented in triple laminated sachets containing 4 g of the product. Each sachet contains 65 mg of active extract. LS is standardized using standard physicochemical and microbiological parameters and HPLC profile. Its HPLC profile is provided as supplementary material. LS has been shown to have potent clinical effect in reducing the incidence, incidence over time and severity of fifteen upper respiratory symptoms [8]. Our previous studies have shown that LS possesses potent anti-inflammatory activity as shown by the significant reduction in *in vivo* anti-inflammatory activity (rat paw-edema model) and in inducing several cellular immune mechanisms contributing to anti-inflammatory activity such as inhibition of rat peritoneal phagocytic cell migration, production of reactive oxygen species (O_2^- assessed using quantitative nitroblue tetrazolium (NBT) assay) and reactive nitrogen species (NO_2^- assessed using Griess assay) and inducible nitric oxide synthase gene expression (assessed using reverse transcription polymerase chain reaction (RT-PCR) in rat peritoneal cells (unpublished data). This previous study provided *in vivo* evidence for the involvement of LS in modulating the innate immune response.

Different medicinal plants and combinations have been shown to exhibit immunostimulatory activity including several formulations and concoctions which contain some of the ingredients in LS. A recent study on a Sri Lankan traditional concoction consisting two ingredients found in LS, ie, *Coriandrum sativum* and *Coscinium fenestratum* had demonstrated its immunostimulatory activity [9]. Other studies have demonstrated the effect of different *Ayurvedic* preparations/polyherbal formulations on immunomodulation/immunostimulation using animal models. *'Kanakasava'* (KNK) is a polyherbal formulation containing 11 plant materials which is for the treatment of respiratory tract diseases, such as asthmatic cough, chronic bronchitis, etc. The immunostimulating activity of KNK has been evaluated by measuring IgM production using BALB/c mice splenocytes [10]. This study has shown that KNK significantly enhanced polyclonal IgM production and lymphocyte proliferation. Another *Ayurvedic* preparation, '*Shirishavaleha*' has been tested for antibody formation against sheep red blood cells (SRBC). Following subcutaneous injection of SRBC into Wistar rats and oral administration of *Shirishavaleha*, the hemagglutination titer has increased significantly indicating immunostimulant activity [11].

The current study was designed to determine the effect of LS in modulating the adaptive immune response, ie. the immunostimulatory response. As the first step in showing the immunostimulatory activity of LS, the rat model was selected for the present study as it involves immunization with a protein antigen (bovine serum albumin - BSA), evaluating the humoral immune responses and the underlying immune mechanisms that facilitate or enhance the antibody production such as the expression on co-stimulatory molecules involved in T-cell dependent B-cell activation which is required to elicit the memory response and IgG secretion. Following oral administration of rats with LS and immunization with BSA, the effect on immunoglobulin M (IgM) and IgG responses were determined by anti-BSA enzyme linked immunosorbent assay (ELISA). Secondly the antigen and gene expression of co-stimulatory molecules on T cells and antigen presenting cells (APCs) were tested by immunofluorescence and Real-time quantitative polymerase chain reaction (RT-qPCR) respectively.

2. Materials and methods

2.1. Materials

All chemicals and consumables, unless otherwise stated, were purchase from Sigma Aldrich, USA. The syringes, needles, surgical blades, cannulas (18 G) were purchased from the State Pharmaceuticals Corporation of Sri Lanka and market samples of LS were used for the study. Pelleted rat food was purchased from SSN Sudalaimuthu Chetty & Co, Colombo 13, Sri Lanka. Reusable rat feeding needle was purchased from Orchid Scientifics, India. Tissue culture plates (24 wells and 96 wells round and flat bottom) were purchased from SPL Life Sciences Co., Ltd, Korea and ELISA plates were purchased from Nunc, USA.

Horseradish peroxidase (HRP) conjugated goat anti-rat IgM (μ chain specific) or IgG (γ chain specific) secondary antibodies were purchased from MyBioSource, Inc; USA. Phycoerythrin (PE) conjugated anti-rat CD3 and CD86 antibodies were purchased from BioLegend, San Diego, CA. Fluorescein isothiocyanate (FITC) conjugated CD40 and CD40L antibodies were purchased from Bioss Antibodies Inc; USA. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CD28, CD40L, CD80, CD86 and CD40 gene specific primers were obtained from Integrated DNA Technologies, USA. RT-PCR and PCR reagents including M-MLV reverse transcriptase, M-MLV reverse transcriptase buffer, RNase inhibitor (recombinant RNasin), deoxyneucleoside triphosphate (dNTPs), Go Taq Flexi buffer, magnesium chloride ($MgCl_2$), Taq polymerase and nuclease free water were purchased from Promega Cooperation, Madison, USA. RNA extraction kit and the abm BrightGreen qPCR MasterMix were purchased from Ceygen Biotech, Sri Lanka and abm, Canada respectively. DNA ladder (100 base pair) was obtained from New England Bio Labs Ltd, United Kingdom.

2.2. Experimental animals

Adult Wistar strain male and female rats weighing 150-250 g were purchased from Medical Research Institute, Colombo, Sri Lanka. They were acclimatized for the new environment around one week, grouped randomly (n = 6) and housed in clean polypropylene cages in the animal house by maintaining standard conditions (temperature of 28-31 °C, photoperiod approximately within a 12 h light/dark cycle and relative humidity of 55-60%) [12]. Rats were fed with pelleted food (SSN Sudalaimuthu Chetty & Co, Colombo 13, Sri Lanka) and water *ad libitum*.

Ethical clearance for this study was obtained (ERC IOBSL 149 08 16) from The Institute of Biology, Sri Lanka (IOBSL). All animal experiments were carried out in accordance with the internationally accepted concept of 3Rs (Replacement, Reduction and Refinement) laid on laboratory animal use and care [13]. Rats were mildly anesthetized using anesthetic ether to minimize the feeling of discomfort during all injections, tail bleeding and rat peritoneal cell collection.

2.3. Preparation of LS for oral administration to rats

The required dose of LS for oral treatment of rats corresponding to the human equivalent dose (HED) was calculated using the following formulae (1 and 2) [14].

$$\text{Human equivalent dose (HED) (mg / kg)} = [\text{Animal dose (mg / kg)}] \times [(\text{Rat Km / Human Km})] \quad (1)$$

$$\text{Volume of oral treatment} = \frac{\text{Animal weight (kg)} \times \text{Animal dose (mg/kg)}}{\text{Concentration (mg/ml)}} \quad (2)$$

where; the body weight of adult human was considered as 60 kg and K_m values for rat and adult human were considered as 6 and 37, respectively [14]. The weight of LS for the human dose was recommended as 4 g (one sachet, approved by Department of *Ayurveda*, Sri Lanka) dissolved in 240 ml (one cup) of boiled water (100 °C) and therefore human dose of LS is considered as 4 g/240 ml. Sugar solution used as one of the controls was prepared using the same procedure.

2.4. BSA immunization and oral administration of LS

BSA (1 mg/ml in 0.15 M NaCl) was used as the protein antigen to immunize rats during this study. Three groups of rats (n = 6) were immunized intraperitoneally with BSA (1 mg/ml; 3.6 mg/kg, body weight) on day 1 and 15 which were referred to as 1st and 2nd BSA immunization respectively. The test group was orally administered with HED of LS as calculated in the above section.

The other two groups treated with tap water or an aqueous solution of sugar were used as controls of this study. LS contains sugar as an excipient, hence a sugar-treated control group was used to exclude the possibility of any effect from sugar on the immune responses investigated. Oral administration of LS was carried out in two series of five consecutive days each from day 1 to day 5 and from day 15 to day 19 after 1st and 2nd BSA immunizations respectively. Pre-immune sera were collected on day 0 and immune sera were collected weekly from day 7 to 35 by tail bleeding. Serum was separated and stored at −20 °C until use.

2.5. Assessment of rat anti-BSA IgM and IgG response

The effect of LS on rat IgM and IgG antibody response against the protein antigen used (BSA) was determined using the in-house anti-BSA IgM and IgG enzyme-linked immunosorbent assays (ELISA). Two series of ELISA were carried out to determine the antibody response, initially using 1:100 serum dilutions and secondly using serial dilutions of rat sera to determine endpoint IgM and IgG titers. Two previously established in-house ELISA methods were used to measure rat anti-BSA IgM and IgG response. ELISA plates were coated with 100 μl of 2 μg/ml BSA in 0.13M phosphate buffered saline (PBS) and incubated overnight at room temperature (RT) in a moist chamber. On the following day, wells were washed six times with wash buffer (0.5 M PBS-Tween-20) and blocked using 200 μl of blocking buffer (0.5 M PBS-0.2% Tween-20 with 5% non-fat milk powder) for 1 hour at 37 °C. The plate was then washed six times with wash buffer and 100 μl of 1:100 dilutions of rat serum samples were added to duplicate wells. The plate was incubated for 2 hours at 37 °C. Plate was washed six times and 100 μl of 1:8000 dilutions of HRP conjugated goat anti-rat IgM (μ chain specific) or IgG (γ chain specific) secondary antibodies were added and incubated for 1 hour 37 °C. The plate was washed for six times and 100 μl 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added and incubated for 10 minutes in RT under dark condition. Reaction was terminated by adding 50 μl of 1 N HCl and the absorbance was measured at 450 nm using an ELISA plate reader (Synergy HT Multimode Microplate Reader, Biotek, USA).

The ELISAs were also carried using the same procedure described above using serial dilutions of rat immune sera ranging from 1:100 - 1:12,800 to determine the endpoint antibody titers of day 7, 14, 21, 28 and 35 as the reciprocal of the serum dilution giving an OD two-fold higher than the respective OD on day 0 of the same treated group.

2.6. Assay for infiltration of rat peritoneal cells

Rat peritoneal cell preparations containing lymphocytes were obtained on day 0 and on day 2, 4, 6, 8 after the 1st BSA immunization and on day 14 and on day 16, 18, 20, 22 after the 2nd BSA immunization. Briefly rats were intraperitoneally injected with 40 ml of 0.13 M PBS and 35 ml of fluid was drained from the peritoneal cavity. Drained peritoneal cells were centrifuged at 500 *g* for 10 minutes at 4 °C. The cells were resuspended in complete RPMI-1640 medium containing, 0.02 M $NaHCO_3$ and 1% BSA. Ten μl of cell suspension was mixed with 10 μl of 1% Neutral Red stain. Total and differential counts were taken by loading 10 μl of this mixture on Neubauer's haemocytometer (Neubauer, Germany) and visualized under light microscope (x40 magnification). Total cell, phagocytic cell (macrophage and neutrophil) and lymphocyte counts were made using a hemocytometer.

2.7. Immunofluorescence assay for detection of co-stimulatory molecules

The collected peritoneal cells were then plated in 24-well tissue culture plate at a concentration of 1×10^6 cells per well and incubated overnight at 37 °C in 5% CO_2 incubator. After incubation, the supernatant of each well was aspirated into a sterile 1.5 ml eppendorf tube and centrifuged at 10,000 *g* for 10 minutes at RT. The supernatant obtained after centrifugation was discarded and the pellet obtained was resuspended in 50 μl of sterile 0.13M PBS containing 0.1% BSA (0.13M PBS; 0.1% BSA) and used for immunofluorescence assay (IFA).

Cells were double stained with phycoerythrin (PE) and fluorescein isothiocyanate (FITC) conjugated antibodies specific for APC's and T cell's surface receptors (CD86 and CD3 respectively) and co-stimulatory molecules (CD40 and CD40L). Antibody staining was performed by adding PE conjugated antibody (against either APCS or T lymphocyte marker) and FITC conjugated antibody (against one of the co-stimulatory molecules) at a final concentration of 1:200. Cells were incubated for 30 minutes at 4 °C in dark, centrifuged at 400 *g* for 5 minutes. The supernatant was discarded and the pellet was washed twice with 0.13M PBS; 0.1% BSA by centrifuging at 400 *g* for 5 minutes. After discarding the supernatant final cell pellet was re-suspended in 20 μl of 0.13M PBS; 0.1% BSA. Cell suspension were observed under fluorescence microscope (x400 magnification). One hundred stained cells were counted from each sample and the percentage of B and T cells expressing co-stimulatory molecules were calculated.

2.8. Detection of the gene expression for co-stimulatory molecules by RT-qPCR

The effect of LS on the gene expression of co-stimulatory molecules [CD28, CD40, CD40L, CD80 (B7-1) and CD86 (B7-2)] involved in T-cell dependent B-cell activation was determined using RT-qPCR. Rat peritoneal cells were obtained from the three groups of rats on day 2, 4, 6 and 8 after the1st BSA immunization and on day 16, 18, 20 and 22 after the 2nd BSA immunization. Cells collected on day 0 and 14 were used as controls for baseline gene expression. Collected cells at a concentration of 1×10^6 cells/ml were incubated *in vitro* for overnight at 37 °C in 5% CO_2 in 24-well tissue culture plates. Total RNA was extracted using Genospin™ total RNA extraction kit (Ceygen Biotech, Sri Lanka) according to the manufacturer's instructions. RNA was then reverse-transcribed using random primers and resultant cDNA samples were amplified using abm Bright Green qPCR Master Mix (abm, Canada) and Mx3000P real time thermal cycler (Stratagene, La Jolla, CA, USA). Rat glyceraldehyde-3- phosphate dehydrogenase (GAPDH) gene was used as the internal reference gene to normalize the mRNA levels. Relative gene expression was assessed using the Ct method ($2^{-\Delta\Delta Ct}$) [15]. Specific primer sequences used to amplify the target genes during RT-qPCR are listed in Table 1.

2.9. Statistical analysis

Statistical analysis was performed using IBM Statistical Package for Social Sciences (SPSS) Version 20 software package. Initially Shapiro–Wilk test was carried out to assess the distribution normality of the data. Parametric and non-parametric tests were carried out according to the distribution of data. Differences were considered as statistically significant at $P \leq 0.05$.

3. Results

3.1. Anti-BSA IgM response in LS-treated rats

As depicted in Fig. 1a, IgM levels of pre-immune sera on day 0 (before the 1st BSA immunization and oral treatment of LS) of the three groups of rats were comparable and by day 7, the LS-treated group reached a significantly higher level of IgM ($OD_{(450nm)} = 0.610 \pm 0.09$) compared to the two control groups (water: $OD_{(450nm)} = 0.186 \pm 0.03$; $p = 0.003$ and sugar: $OD_{(450nm)} = 0.245 \pm 0.04$; $p = 0.008$). After day 7, the IgM response of three groups showed a reduction up to day 35, however the IgM levels were maintained at significantly higher levels compared to the two controls during the period from day 7-35 ($p < 0.05$). Further, there was no significant difference in the IgM levels produced by the two control groups.

In addition to OD at 1:100 serum dilutions, endpoint antibody titers were also assessed and are depicted in Fig. 2. All three groups showed the highest endpoint IgM titers on day 7. However, the LS-treated group had a significantly higher endpoint IgM titer of 356 compared to the two control groups (112 in water control; $p = 0.011$ and 126 in sugar control; $p = 0.016$). From day 14 up to day 35, endpoint IgM titers showed a decrease in all three groups, however the LS-treated group maintained significantly higher endpoint IgM titers compared to controls ($p < 0.05$). The endpoint IgM titers of water and sugar control groups were comparable ($p > 0.05$).

3.2. Enhancement of anti-BSA IgG response in LS-treated rats

IgG levels of all three groups of rats were comparable on day 0, prior to the BSA immunization and oral treatment with LS. The IgG levels of 1:100 dilutions of rat sera of the LS-treated group increased to a significantly higher level by day 14 ($OD_{(450nm)} = 0.246 \pm 0.09$) compared that of both water and sugar controls ($OD_{(450nm)} = 0.054 \pm 0.03$; $p = 0.027$ and $OD_{(450nm)} = 0.071 \pm 0.02$; $p = 0.032$) (Fig. 1b). By day 21, the 7th day after the 2nd BSA immunization given on day 15, all three groups of rats reached their highest IgG level. However, on day 21, the LS-treated group showed a significantly higher IgG level ($OD_{(450nm)} = 1.135 \pm 0.09$) compared to water and sugar control groups ($OD_{(450nm)} = 0.297 \pm 0.12$; $p = 0.003$ and $OD_{(450nm)} = 0.465 \pm 0.11$; p = 0.008 respectively). By day 28, IgG levels started declining till day 35, but the LS-treated group maintained a significantly higher IgG levels ($OD_{(450nm)} = 0.471 \pm 0.11$ - 0.378 ± 0.11) compared to that of the two controls ($OD_{(450nm)} = 0.068 \pm 0.05$ - 0.052 ± 0.03 and $OD_{(450nm)} = 0.118 \pm 0.05$ - 0.062 ± 0.03 respectively).

Further, there was no significant difference observed between the IgG levels produced by water and sugar control groups ($p > 0.05$).

As depicted in Fig. 2, in the LS-treated group the IgG response increased to significantly high endpoint antibody titers by day 14 (2263; $p < 0.05$) and reached the highest endpoint IgG peak titer of 5702 on day 21 which was significantly higher compared to that of two controls; water (800; $p = 0.007$) and sugar (1008; $p = 0.008$). Endpoint IgG titers of the LS-treated group decreased thereafter but maintained significantly high endpoint antibody titers compared control groups up to day 35 ($p < 0.05$). As in all the above cases,

Table 1
Primer sequences used in RT-qPCR.

Gene	Sequence (5′–3′)	Reference
Rat GAPDH	F - GTA TTG GGC GCC TGG TCA CC R - CGC TCC TGG AAG ATG GTG ATG G	[16]
Rat CD28	F - GTA TTC CTA CAA CCT TCT CGC AA R - GGG GCT GAT AGG TAA AAT TCC CA	[17]
Rat CD40L	F - CAG ATG ATT GGG TCG GTG C R - CAC TGA AGA ACA GAT GCT GCA TT	[18]
Rat CD80	F - CCA AGT GTC CAA GTC GGT GAG R - CCC GGA AGC AAA GCA GGT AAT	[19]
Rat CD86	F - AGG ACA CGG GCT TGT ATG ATT G R - GGT TTC GGG TAT CCT TGC TTA G	[19]
Rat CD40	F - CTC TGG GGC TGC TTG TTG A R - TTG ATT GAG TTC GCA GTG TCG	[19]

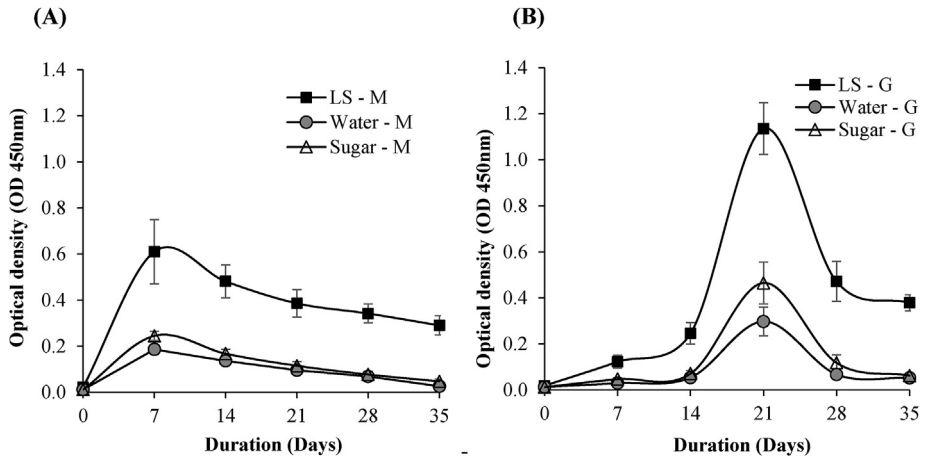

Fig. 1. Anti-BSA (A) IgM and (B) IgG antibody response measured in 1:100 dilutions of rat anti-sera following BSA immunizations on day 1 and 15. Three groups of rats were orally administered with LS, sugar or water from day 1–5 and day 15–19. Antibody response is presented as OD (450 nm) values of 1:100 serum dilutions as mean ± SEM of all rats in the group; n = 6 rats per group; each serum was tested in duplicates in two independent ELISA experiments. M– IgM; G - IgG.

endpoint IgG titers observed between the two control groups were not significantly different ($p > 0.05$).

LS-treated group showed a greater magnitude of IgG level and endpoint titer on day 21 compared to IgM level and endpoint titer produced by the same group on day 7.

3.3. Increase in lymphocyte counts in peritoneal cavity of LS-treated rats

There was no significant difference observed in the differential cell counts of three groups of rats treated with LS, sugar and water on day 0 ($p > 0.05$). In contrast by day 2, the LS-treated group showed a significant increase in their lymphocyte count (2.5×10^5 cells/ml) compared to both the its respective level on day 0 and to the two controls (sugar and water) ($p < 0.05$). Lymphocyte count of LS-treated group was decreased to 1.7×10^5 cells/ml and 1.6×10^5 cells/ml by by day 6 and 14 respectively. After the 2nd immunization, the lymphocyte count of LS-treated group showed a significant increase up to 3.0×10^5 cells/ml by day 16 compared to the count of day 14 and also compared to the two controls ($p < 0.05$). By day 22, lmphocyte count of LS-treated group dropped down to 1.9×10^5 cells/ml.

When the macrophage and neutrphil counts of LS-treated group were considered, by day 2 and 16 both cell counts were increased significantly compared to their respective counts on day 0 and also compared to their levels of controls. However, those counts of the LS-treated group were significantly low compared its lymphocyte count observed on day 2 and 16 ($p < 0.05$). By day 6 and 22, the counts of macrophages and neutrophils were decreased to very low levels (Fig. 3).

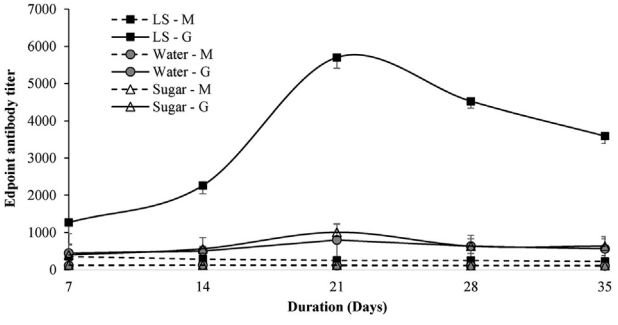

Fig. 2. Endpoint IgM and IgG titers of rat anti-sera following BSA immunizations on day 1 and 15. Three groups of rats were orally administered with LS, sugar or water from day 1–5 and day 15–19. Data is presented as Geometric mean */SEM for each group; n = 6 rats per group; each serum was tested in duplicate in two independent ELISA experiments. M– IgM; G - IgG.

3.4. Higher and early appearance of APCs and T lymphocytes in LS-treated group detected by immunofluorescence assay

In all three treated groups, the percentage of CD86+CD40+ APCs were very low on day 0 (Fig. 4a) whereas there were a small percentage (<10%) of CD3+CD40L+ T cells (Fig. 4b), before the BSA immunization and oral administration. In the LS-treated group, the percentage of CD86+CD40+ APCs and CD3+CD40L+ T cells increased significantly by day 2 after one dose of oral administration of LS (17% and 66% of – APCs and T cells respectively; $P < 0.05$) compared to day 0 which had no detectable APCs and 7% of T cells (Fig. 4a and b). Although the control groups also had some APCs and T cells by day 2, their percentages were significantly lower than those of the LS-treated group. Interestingly, the highest percentages of CD86+CD40+ APCs (18%) and CD3+CD40L+ T cells (50%) cells were observed only by day 6 whereas these percentages of APCs and T cells were observed in the LS-treated group 4 days earlier, ie., by day 2. Similarly, after the second LS administration and BSA immunization when controls achieved the highest percentages of APCs and T cells, the LS-treated group has reached those levels again 4 days earlier, ie., by day 16 (Fig. 4a and b). However, Fig. 4b also indicates an increase in the percentage of CD3+CD40L+ T cells in the control group (water & sugar) on day 6 and day 20 which represents the expected response following immunization with a protein antigen.

3.5. Increased mRNA expression encoding for genes of co-stimulatory molecules involved in T-cell dependent B-cell activation in the LS-treated rats

On day 0 (before BSA immunization and oral administration of LS), the expression of all five tested co-stimulatory molecules were low and there was no significant difference ($p > 0.05$) between the expression of CD28, CD86, CD40, CD40L and CD80. Further, the expression levels of co-stimulatory molecules in all three groups (LS-, water- and sugar-treated) were comparable and not significantly different on day 0 (Figs. 5 and 6). By day 2 after oral

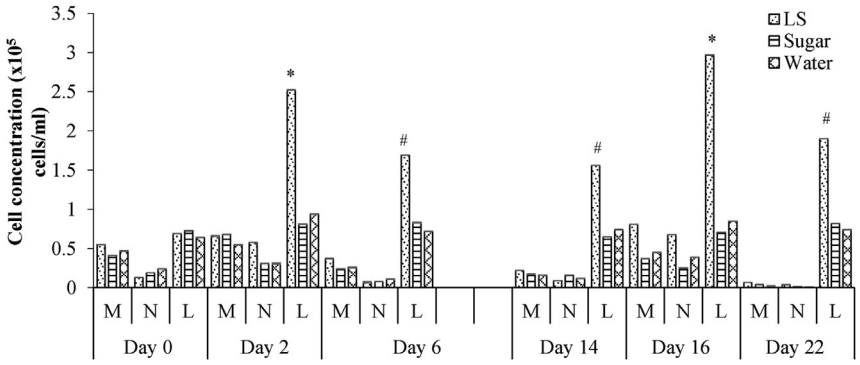

Fig. 3. Differential peritoneal cell count of LS-, sugar- and water-treated rats. L – Lymphocytes, M– Macrophages, N- Neutrophils. Percentage was expressed as mean ± SEM of all rats in the group; n = 6 rats per group; Cell concentration of LS-treated group is significant compared to controls (Significance level - * $p \leq 0.001$; # $p < 0.05$).

administration of one dose of LS, the expression of all the co-stimulatory molecules increased significantly ($p < 0.05$) in LS-treated group compared to the two controls (Figs. 5 and 6). Fold change in the gene expression of CD28, CD40L, CD80, CD86 and CD40 by LS ranged from 2.4 to 5.1 folds compared to the both control groups. The expression levels in the LS-treated group remained high in the same fold change (3.0-5.2) until day 4 compared to the two controls. Interestingly on both day 2 and 4, the expression levels of CD28 and CD40L increased (4.9-5.2) folds compared to the expression levels of CD80, CD86 and CD40 (2.4-3.2) (Figs. 5 and 6).

From day 6-8, CD28 and CD40L expression started decreasing (Fig. 5a and b) whereas the expression of the other tested co-stimulatory molecules, ie., CD80, CD86 and CD40 did not show a significant change (Fig. 6a-c). When comparing the gene expression levels from day 8 up to day 14 (the day before 2nd immunization and 2nd series of oral administration of LS), the expression of CD28, CD40L and CD80 by day 14 had reached significantly lower levels compared to day 8 ($p < 0.05$) (Figs. 5 and 6a) whereas the expression of CD86 and CD40 had remained at similar levels from day 8 to day 14 (Fig. 6b and c).

Similar pattern of gene expression was observed after the 2nd immunization and initiation of the 2nd series of oral administration of LS. By day 16-18, the expression of all five co-stimulatory molecules reached their highest levels and remained at significantly higher levels until day 18.

There was no significant difference in the expression of CD28 and CD40L when comparing their respective expression levels on day 2 with day 16 and on day 4 with day 18 ($p > 0.05$) (Fig. 5). CD80, CD86 and CD40 expression levels on day 16 and 18 (3.7-5.1 folds) were higher than their respective expression levels observed on day 2 and 4 (2.4-3.2) (Fig. 6). From day 20 up to 22, the expression of co-stimulatory molecules started decreasing and by day 22, the expression of all tested co-stimulatory molecules reached their lower levels compared to that of other days (Figs. 5 and 6). In contrast, the expression of all five co-stimulatory molecules tested after 2nd immunization was in comparable levels and not significantly different in two control groups showing a similar result as observed after 1st immunization.

4. Discussion

This study has confirmed that oral treatment with LS has resulted in an enhanced and rapid development of an IgG response suggesting that it has potent immunostimulatory activity against a protein antigen (BSA) used in this study. The IgM response made initially was also significant, however findings support an effective boosting of the IgG response (Fig. 1). The analysis of the cells obtained after the initial dose of LS from the peritoneal cavity which is the site of immunization also indicate that there is a significant flux of lymphocytes including T cells and APCs. Further as early as day 2, these lymphocytes have shown an increased gene expression of the co-stimulatory molecules, namely CD28, CD40L, CD80, CD86 and CD40. Our findings support that the increased gene expression of these co-stimulatory molecules may have contributed to an enhanced T-cell dependent B-cell activation leading to a significantly higher IgG response in the LS-treated rats. Our findings on enhanced immunostimulatory effect of LS, specifically on inducing

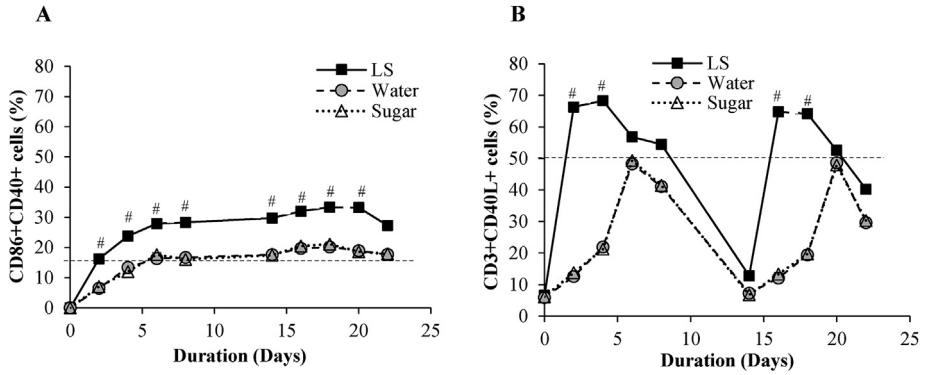

Fig. 4. The percentage of (A) The percentage of CD86+CD40+ APCs (B) CD3+CD40L+ T cells. Percentage was expressed as mean ± SEM of all rats in the group; n = 6 rats per group. # - Percentage of LS-treated group is significant compared to controls. Significance level (P value < 0.05). Horizontal bar indicates the highest percentage achieved by the control groups.

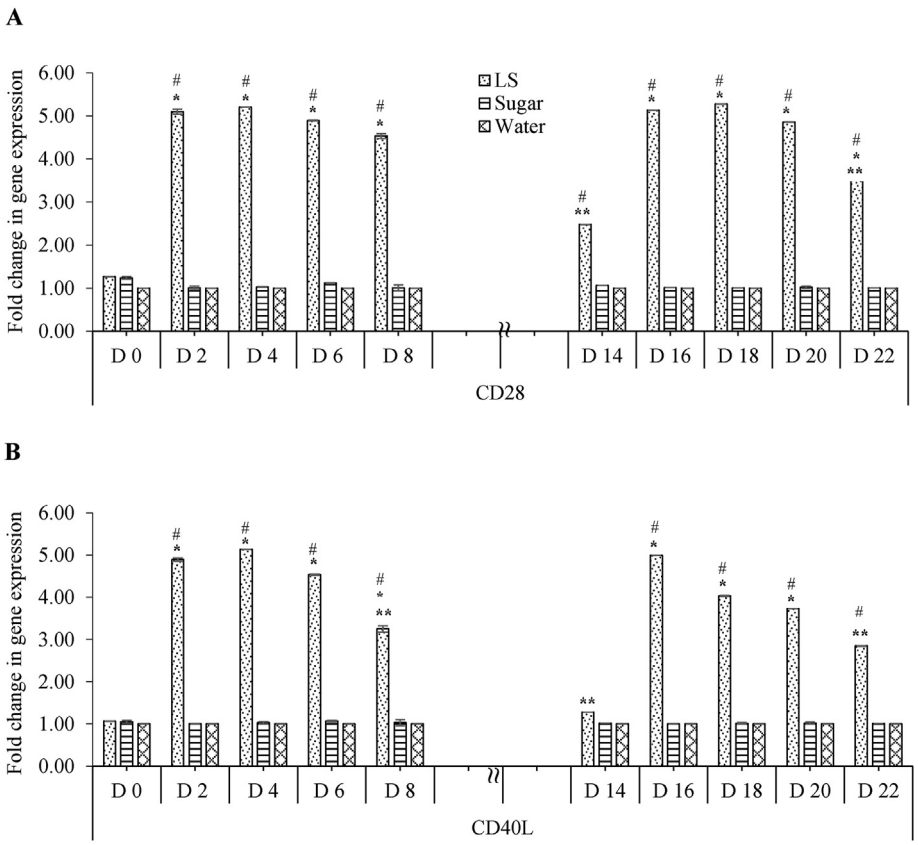

Fig. 5. Effect of LS treatment on the mRNA expression of (A) CD28 and (B) CD40L on rat peritoneal lymphocytes compared to sugar and water control. Expression of each target gene was calculated using $2^{-\Delta\Delta Ct}$ method and expressed as a fold change normalized to the internal reference gene, GAPDH. # - Fold change in gene expression of LS-treated group is significant compared to controls. * - Fold change in gene expression of LS-treated group is significant compared to LS-treated group's gene expression on day 0. ** - Fold change in gene expression of LS-treated group is significant compared to LS-treated group's gene expression on day 2. (Significance level (P value < 0.05).

an effective antibody response is consistent with findings from previous studies in medicinal plants inducing enhanced antibody response [10,11]. To the best of our knowledge, this is the first report of an enhanced IgG response involving an increased expression of co-stimulatory molecules with a polyherbal formulation acting as an immunostimulator.

LS has been previously shown to have protection against upper respiratory symptoms and hence it is currently widely used as a prophylactic agent [8]. Findings of the present study on rapid and enhanced gene expression of the co-stimulatory molecules leading to T-cell dependent B cell activation resulting in an effective IgG response supports the previous findings on the prophylactic effect of LS. It was interesting to note that, with the initiation of oral treatment with LS and only after one dose of LS was administered, ie. on either day 2 or day 16, the gene expression of co-stimulatory molecules were rapidly induced to significantly high levels as well as to the highest lymphocyte counts observed at the site of immunization whereas in the controls it took a longer duration (by day 6 or day 20 respectively) to reach the equivalent levels of co-stimulatory molecules expression and lymphocyte counts (Figs. 4–6). This rapid increase in co-stimulatory molecules with the oral administration of LS may provide a key mechanism for its effective prophylactic effect.

It is noteworthy that the commercial preparation LS contains sugar as an excipient. Sugar is one of the most widely used excipient in manufacturing of commercial drugs [20]. Hence, two types of control groups were included in this study, namely sugar and water. Our findings indicate that in all the activities tested, ie., the IgM and IgG response, the lymphocyte counts and the gene expression of co-stimulatory molecules where both controls showed the similar activity. Further, both groups were significantly different from the LS-treated group while having no significant differences between the water and sugar control groups. These findings allow a clear exclusion of any possible contribution by sugar to the immunostimulatory activity observed with LS.

Different hematological and serological parameters have been assessed in previous studies to report immunostimulatory activity of medicinal plants and formulations in murine models [21]. The hemagglutination assay is used to detect antibodies raised against sheep red blood cell (SRBC) antigens. However, ELISAs to determine the specific IgM and IgG antibody concentrations raised against immunogens such as keyhole limpet hemocyanin (KLH) [22] or BSA [23] have became the preferred assays to measure the humoral immunity [24]. Our previous studies have also used the hemagglutination assay [9] or ELISA (unpublished data) for the detection of antibodies against SRBC to describe the immunostimulatory activity of the concoction of *C. sativum* and *C. fenestratum*. The present study deployed the use of ELISA to determine anti-BSA specific IgM and IgG responses to demonstrate the immunostumulatory activity of LS.

This study had used a protein antigen, bovine serum albumin (BSA) as the immunogen, injected to rats intraperitoneally and LS was administered orally to assess immunostimulatory activity on rat IgM and IgG response. This study was also designed to determine the specific immune cell subpopulations that were either present or migrated to the site of antigen injection and would be directly involved in antigen uptake, antigen presentation and activation. Our preliminary studies had shown that the lymphocyte

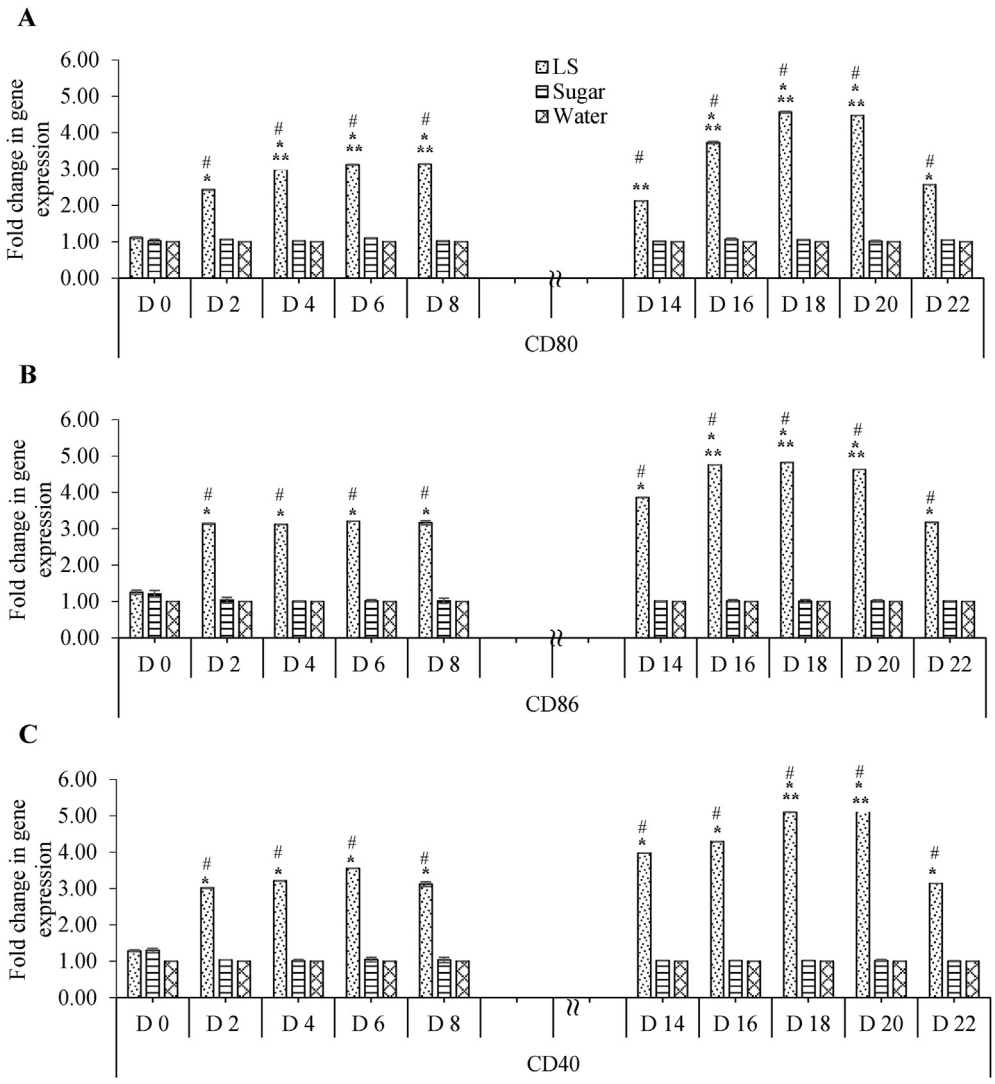

Fig. 6. Effect of LS on the mRNA expression of (A) CD80, (B) CD86 and (C) CD40 on rat peritoneal lymphocytes compared to sugar and water control. Expression of each target gene was calculated using $2^{-\Delta\Delta Ct}$ method and expressed as a fold change normalized to the internal reference gene, GAPDH. # - Fold change in gene expression of LS-treated group is significant compared to controls. * - Fold change in gene expression of LS-treated group is significant compared to LS-treated group's gene expression on day 0. ** - Fold change in gene expression of LS-treated group is significant compared to LS-treated group's gene expression on day 2. (Significance level (p value < 0.05).

subpopulation increases at the site of injection after antigen (BSA) injection (data not shown). Therefore, it was considered more appropriate to determine the expression of co-stimulatory molecules on these immune cell subpopulations that were present at the site antigen injection compared to the mononuclear cells circulating in the peripheral blood (PBMC) which represents a distant locality.

Numerous studies have shown that medicinal plants act as immunostimulants especially on APC, macrophages and dendritic cells. Certain plant metabolites and herbal formulations are known to be immunomodulatory in various diseases [25]. A review had identified 20 plants belonging to 15 botanical families that have shown immunostimulatory effects *in vitro* and out of which 7 plants have been tested in *in vivo* models [26]. Polysaccharides isolated from *Astragalus membranaceus*, *Ganoderma lucidum* and *Radix ophiopogonis* have decreased CD40 expression but not CD80/CD86 in dendritic cells [27]. Further, a review based on the immune modulation of plants targeting dendritic cells [28] has identified that *Azadirachta indica* upregulated CD40, CD83, CD80 and CD86 [29], Astragalus increased the expression of CD40, CD80 and CD86 [30], garlic elevated CD40 levels but not CD86 [31], curcumin inhibited the expression of CD80 and CD86 [32], *Plantago asiatica* increased levels of CD80 and CD86, *Corydalis bungeana* decreased the expression of CD40 & CD86 and Licorice root significantly suppressed the expression of CD80 and CD86 in Dendritic cells [28]. Though there are multiple studies available on the effect of medicinal plants on the expression of co-stimulatory molecules such as CD80, CD83, CD86 and CD40 on dendritic cells, studies on cell type such as lymphocytes are limited. Hence, the present study may represent the first to show the effect of medicinal plant extracts or a polyherbal formulation on the expression of co-stimulatory molecules such as CD80, CD86 and CD40 including CD28 and CD40L on APCs and T lymphocytes respectively.

Naïve T cells are known to require a two-step signaling process to complete their activation [33]. The first signal is provided by the interaction between T-cell receptor, the antigen and the major histocompatibility complex on antigen presenting cell (APCs) including B cells and the second signal is mediated through the interaction between co-stimulatory molecules present on the surfaces of B and T cells [34]. The co-stimulatory signals are crucial for

T cell activation for T-dependent antigens that would lead to an immune response which would have some of the essential criteria such as isotype switching and eliciting an effective memory response. In this study, BSA which is a known immunogenic protein that act as a thymus-dependent antigen was used to induce an immune response in rats. Further, we have selected the components of B7 complex (CD80 and CD86), CD28, CD40 and CD40L [33] which are the best defined molecules co-stimulatory pathways to determine the molecular mechanism that lead to boosting of the IgG response to a higher magnitude and was a rapid response. The rapid response in the LS group was clearly evident by the increased presence of $CD86^{+}CD40^{+}$ APCs and $CD3^{+}CD40L^{+}$ T cells detected by immunofluorescence by day 2 after 1st immunization whereas the controls reached to the highest percentages of respective cells much later (by day 6) (Fig. 4). This was further corroborated by the increased gene expression of the co-stimulatory molecules (CD80 and CD86), CD28, CD40 and CD40L) by day 2 in the LS groups compared to the controls (Figs. 5 and 6). The higher expression of the co-stimulatory molecules would lead to more efficient completion of the second signal being completed for an enhanced T-dependent B cell activation.

Previous studies have shown that the co-stimulatory molecules in the B7 complex (CD80 and CD86) expressed on APC play a major role in T-cell activation [34]. Of the two co-stimulatory molecules in the B7 complex, studies performed with knock-out mice have shown that CD86 plays an important role than CD80 in initiating an immune response [35]. CD86 is constitutively expressed in low amounts on professional APC while CD80 is expressed on activated APC. Though the CD86+CD40+ APCs were absent on day 0 according to IFA result (Fig. 4), there was a lower number of macrophages present on day 0 as indicated in differential cell count. These macrophages might be acting as professional APC and express CD86 on day 0. Previous studies also revealed that the upregulation of CD86 occurs rapidly after activation and reaches to its maximum level by 18–24 h after stimulation whereas CD80 upregulation is delayed and reaches to its maximum after 48–72 h [36]. LS has upregulated both CD80 and CD86 expression required for T-cell activation, but it is interesting to note that in the present study, the upregulation of CD86 has increased to its maximum level rapidly by day 2 in the LS group compared to the controls whereas CD80 has taken a longer time to reach its maximum expression level. CD80 and CD86 interact with the co-receptor protein, CD28 on the surface of T cell and CD28:B7 signaling is crucial for the naïve T cell activation [34]. The significant upregulation in the expression of co-receptor, CD28 (Fig. 5a) by LS at the same time with the increase in expression of its ligands, CD80 and CD86 (Fig. 6a and b) from day 2–4 and 16–18 indicate that the interaction mediated by CD80 and CD86 with CD28 could occur in the LS group providing a crucial signal for T-cell activation.

CD28 is known to be expressed as a homodimer and it is engaged by the two ligands, CD80 and CD86. Of the two ligands, CD86 is expressed as a monomer whereas CD80 is expressed as a dimer [37]. Though CD80 is expressed as a dimer, previous studies have shown that the optimal co-stimulatory function of B7-1 (CD80) requires the initial engagement of CD28 by dimers of B7-1 followed by dissociation to monomer. Hence, the dissociation of non-covalent dimers of B7-1 play an important role in the optimal activation and function of T cells [38]. In our study, CD28 was expressed at a higher level (~5.1–5.2 fold) compared to CD80 and CD86 at the transcriptomic level (~2.4–3.1 fold) on day 2–4 which supports to the existence of monomer-dimer structures of these co-stimulatory molecules. Similarly, with regard to the CD40:CD40L interaction, previous studies on the CD40-CD40L complex have reported that two CD40 receptor monomers bind to subunit grooves of the CD40L trimer [39]. The present study revealed that expression of CD40L was higher (~4.9–5.1 fold) at transcriptomic level compared to its receptor, CD40 (~3.0–3.2 fold). Although the RNA expression is concordant with structural existence of these co-stimulatory molecules as monomer and trimer as published in previous literature [39], further studies on quantitative expression at protein level is required to proven this hypothesis.

The interaction between CD40 and CD40L involves many different immunomodulatory mechanisms such as increased survival of APC, production of cytokines and upregulation of B7 complex (CD80 and CD86). Finally the engagement of CD40 with CD40L is crucial for B cell activation and differentiation, antibody production, immunoglobulin class switching and germinal centre formation of B cells [40]. The importance of this CD40:CD40L signaling pathway in mediating humoral response has been illustrated using $CD40^{-/-}$ and $CD40L^{-/-}$ mice, who were unable to form germinal centres, generate memory B cells or to produce high affinity, isotype switched antibodies to T-cell dependent antigens [41]. In the present study, LS group showed significant increase in both CD40 (Fig. 6c) and CD40L (Fig. 5b) expression by day 2–4 and 16–18, indicating its effect in mediating the second crucial co-stimulatory pathway required to complete the T-cell activation.

The present study shows that during the period from day 2–4 and 16–18, LS has induced an upregulated expression of the co-stimulatory molecules necessary for T cell activation which may subsequently lead to T-dependent B cell activation. Further, the antibody response of the LS group showed a higher IgM (on day 7) and IgG response (on day 21) compared to the two control groups (Figs. 1 and 2). The significantly high IgG response in the LS group is indicative of an effective memory response as well as an isotype switching that had occurred. The day 21 response is also indicative of a secondary response with the boosting of IgG levels which also suggest the LS may have an adjuvant effect. There is increasing awareness of the scientific community appreciating the biological properties of plant-derived medicines as herbal immunoadjuvants [7,25]. Our previous studies and others have shown that a concoction of two ingredients (*Coriandrum sativum* and *Coscnium fenestratum*) which are present in LS [9] and *Vitex negundo* [42,43] has immunostimulatory effects in enhancing humoral antibody response and also suggesting adjuvant effects.

The RT-qPCR and ELISA results of the current study collectively indicate that LS has played a major role in upregulating the expression of the key co-stimulatory molecules (CD28, CD40L, CD80, CD86 and CD40) for T-cell dependent B-cell activation. Medicinal plant components in LS may act as immunostimulants that are capable of stimulating specific immune response towards an infection [44]. B7 direct signaling pathway has been identified as an extremely potent inducer of IgG antibody secretion by previously activated B cells. The present study was focused on assessing the humoral response and the increased expression of the co-stimulatory molecules and our findings indicate that LS may have been an essential contributor for B cell activation, isotype switching and an effective memory response. *In vivo* and *in vitro* studies also indicates that CD40, CD70, CD80 and CD86 are key molecules in the signaling for the regulation of antibody production, particularly the IgG [45]. Results of the present study are consistent with such findings where LS has shown significant upregulation of gene expression three key molecules among four (CD40, CD80 and CD86) required for the regulation of IgG antibody, thus accounting for a higher production of IgG. Further, the blockade of CD28:B7 interaction has been shown to cause a reduction in overall antiviral IgG production following influenza virus infection [46]. Conversely, this indicates that the CD28:B7 interaction was essential for the antiviral (influenza) IgG production. It is also noteworthy that simultaneous exposure to the immunogen and the enhancement of the co-stimulatory molecules may be crucial for T cell activation

which would be required to eliminate or control intracellular viruses [47]. This indicates that upregulating CD28:B7 co-stimulation may be an important feature of immunostimulation by LS.

Although, this study has shown an increased production of boosting of IgG response against the protein antigen following oral administration with LS, there is no information available on the sub-class of IgG responsible for this increased production. The enhanced expression of co-stimulatory molecules on B and T lymphocytes following LS treatment showed in this study could be further studied using cytokine profiling. The future steps of this study will be directed towards studying the isotype switching and the sub-classes of IgG responsible for generation of antibodies. Another aspect that will be looked at in the future is the expression of cytokines like IL-6, IL-21 and transcription factors like Bcl-6 will be further studied as it is instrumental in elucidating the differentiation of Follicular helper T cells, which play a crucial role in germinal centers to generate antigen specific long lasting memory B cells. Understanding the molecular aspects of co-stimulatory molecule signaling and isotype switching for generation of antibodies can advance the understanding of the medicinal effects of LS and its mechanism.

5. Conclusions

This study has shown that the increased production and boosting of IgG response against the protein antigen following oral administration with Link Samahan®. Further, an enhanced expression of co-stimulatory molecules on B and T lymphocytes was also shown with LS treatment. These findings suggest that this polyherbal formulation exerts immunostimulatory and adjuvant effects.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical approval

Ethical clearance for animal work was obtained (ERC IOBSL 149 08 16) from The Institute of Biology, Sri Lanka (IOBSL).

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Conflict of interest

Ajita Mahendra Abeysekera is an advisor to Link Natural Products (Pvt) Ltd. Other authors declare that they have no conflicts of interest.

Author contributions

BVLRR, DE, AMA, OVDSJW and SMH designed the experiments. BVLRR analyzed the data and wrote the paper, which was improved by AMA, OVDSJW and SMH supervised the research. All authors contributed to the final version of the manuscript. The manuscript has been read and approved by all the authors and that each author believes that the manuscript represents honest work.

Abbreviations

LS	Link Samahan®
BSA	Bovine serum albumin
RT-qPCR	Real-time quantitative polymerase chain reaction
ELISA	Enzyme linked immunosorbent assay

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaim.2021.09.006.

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