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Evaluation of antileishmanial efficacy of Salidroside against the SSG-sensitive and resistant strain of *Leishmania donovani*

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ABSTRACT

The successful control and eradication of leishmaniasis are still challenging in view of the lack of adequate chemotherapy and potential prophylaxis. Research is going on for finding an appropriate anti-leishmanial drug which should be acceptable in terms of cost and safety. In view of this, the current study investigated the anti-leishmanial efficacy of salidroside (SAL) which is a phenylpropanoid glycoside. The leishmanicidal capacity of SAL was verified *in vitro* as well as *in vivo*. The SAL exhibited leishmanicidal activity against the promastigotes of *L. donovani* which was further validated by propidium iodide staining and its ability to arrest the promastigotes at the sub G_0/G_1 stage. SAL decreased and controlled the VL infection in mice as estimated by real-time PCR. Active immunomodulation was exhibited upon SAL treatment in BALB/c mice. The characteristic features like pronounced DTH reaction, polarization of immune status to Th1 type of immune response, increased the production of $CD4^+$ and $CD8^+$ T cells indicated the immune-stimulatory property of SAL. In addition to this the expression of NF-κB, iNOS genes along with the levels of leishmanicidal species, NO and ROS were found to be augmented in SAL treated infected animals. Moreover, SAL exhibited minimal toxicity to the THP-1 cells and it revealed no toxicity against liver and kidney. The capability of SAL in promoting the immune status in favor of host during VL infection without causing any side-effects may be used as an effective strategy to fight the disease.

1. Introduction

Visceral leishmaniasis (VL), is a critical problem worldwide mainly tormenting the developing countries and poor people. It is a serious neglected tropical disease caused by obligate intracellular parasites of the genus, *Leishmania* [1]. WHO has regarded leishmaniasis as one of the most deadly among the seven tropical diseases [2]. VL is the visceral form of leishmaniasis which is transmitted by sandfly vector of genus *Phlebotomus* in India. It is responsible for the inflammation of the spleen, liver, deranged hematological status with low total cell counts and loss of immunity [3].

The consequences of VL are potentially fatal if left untreated as without therapy victims develop severe immunosuppression. The latter makes the patients more susceptible to secondary microbial diseases and the patients succumb to their infections [4]. During VL the immune status gets adaptively modified with the switching of Th1 kind of immune response to the Th2 type. The latter is associated with increased production of IL-4, IL-10 and IL-6 and suppression of TNF-α, IFN-γ, IL-12 and NO [5]. However, the resistance to the disease is gained with the increments in the $CD4^+$ Th1 lymphocytes and activation of macrophages. In fact, both $CD4^+$ and $CD8^+$ T cells are required for checking the growth of parasite [6,7].

Pentavalent antimonials have been the main proposed drug for leishmaniasis for many years despite their severe side-effects against liver, heart [8], pancreas [9] *etc.* It requires a long duration of therapy and hospitalization. In addition, the cases of failure of antimonials from Bihar and reports of the development of drug resistance have further questioned the efficacy of antimonials and have lead to their abandonment [10]. In antimonial non-responsive regions, another drug amphotericin B (AmB) has been employed as second-line therapy for VL. However, the toxic effects against the kidney restricted its use and also encouraged its liposomal form. However, the requirement of low temperature for transport and storage of liposomal AmB has limited its use in the rural areas [11].

None of the available treatment modalities are ideal due to their high cost, toxic side-effects, and lengthy treatment regimens. This situation has prompted the search for alternative options for the treatment. Natural products provide a diverse variety of organic structures which are important ingredients in the vast group of medicines. The wide range of active pharmacological effects of the natural products tempted us to survey the antileishmanial activity of a natural compound, Salidroside. SAL is a phenylpropanoid glycoside found as an

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active component in many medicinal plants including many species of *Rhodiola*. It is an adaptogen which displays protective activity against liver [12], heart [13], nervous system [14] and many other skin disorders [15]. SAL also possesses the ability to modulate the immune functions by enhancing the population of the total $CD3^+$ and $CD\ 4^+$ T cells. It can counteract the immune-suppression and thus can rejuvenate the immune status [16].

The utmost requirement of framing a compound to be a potent antiparasitic drug is that it should solely target the parasite without affecting the host. The development of more effective and safe antileishmanials is prerequisite for the control of VL [17]. Therefore in the present study, we substantiated the activity of SAL against the life-threatening affliction of visceral leishmaniasis.

2. Material and methods

2.1. Parasite

The MHOM/IN/80/DD8 Sodium stibogluconate, (SSG)-sensitive strain and P.B.-0014, SSG-resistant strain of *L. donovani* was obtained from the Post Graduate Institute of Medical Education and Research (PGI), Chandigarh, and from RMRI, Patna, Bihar respectively. The promastigotes were cultured in modified NNN [18] and complete RPMI-1640 media [19] in the B.O.D. incubator at 22 ± 2 °C.

2.2. Animals

The 4-to 6-weeks old inbred BALB/c mice were obtained from the Central Animal House of Panjab University (PU), Chandigarh. The animals were fed with water and food *ad libitum* in the Central Animal House facility of PU, Chandigarh. All the animal experiments were approved by the Institutional Animal Ethics Committee of PU, Chandigarh, India which is registered under the CPCSEA (IAEC no. PU/IAEC/S/14/142).

2.3. Evaluation of anti-promastigote potential

Salidroside (SAL) was purchased from Sigma-Aldrich Co., USA (Cat. no. 43866). The anti-promastigote activity of SAL was appraised by trypan blue dye exclusion test. Briefly, it was calculated by incubating parasites (2×10^6/mL) with the different concentrations of SAL at 22 °C. DMSO (0.01%), which was used to solubilize SAL, served as solvent control at the equivalent concentration and the AmB, served as a positive control. The viable cells that excluded the dye were counted after 72 h under a light microscope in a neubauer's chamber. The percentage of growth inhibition was calculated as follows:

$$\text{Percentage viabiliy} = \frac{\text{No.of viable cells in treated well}}{\text{No.of viable cells in blank well}} \times 100$$

Percentage growth inhibition = 100–percentage viability.

IC_{50}, the concentration at which 50% of the parasite growth get reduced was enumerated by the graphical extrapolation [20].

2.4. Analysis of promastigotes in Sub G_0/G_1 phase by propidium iodide (PI) staining

Cell cycle analysis reveals the distribution of cells in different stages of the cell cycle according to the content of DNA. The promastigotes in the sub G_0/G_1 phase permits the identification of the parasitic cells with the fractional DNA content *i.e.* the cells showing apoptosis. In brief, the promastigotes (2×10^6/mL) were cultured in the presence of SAL (IC_{50} value) along with appropriate positive (AmB) and negative (untreated) controls for 72 h. The promastigotes were harvested from each well and then centrifuged (3000 ×*g*, 10 min, 4 °C), washed twice with PBS. The cells were fixed with chilled ethanol (70%) and kept at 4 °C for 1 h. The cells were again washed with PBS, and incubated with RNase (200 μg/mL). Further, the cells were incubated with PI (50 μg/mL) for 20 min in dark at 22–25 °C. The cells were acquired using a FACSCalibur flow cytometer and data analysis was done using BD FACS DIVA 8.0.1 software [21].

2.5. Cell toxicity assay

The toxicity of SAL was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on the differentiated THP-1 cells. The cells were maintained in complete RPMI-1640 in a humidified CO_2 incubator at 37 °C and 5% CO_2. The cell line was maintained by periodically changing the media after every 3–4 days. The THP-1 cells at about 95% viability were exposed to Phorbol 12-myristate 13-acetate (Sigma) for the differentiation of monocytes to macrophages. The conversion of the cells was inspected under the inverted microscope. Briefly, 3×10^5 differentiated cells were seeded in the 96-well plate. SAL was added at the different concentrations, and the plate was further incubated at 37 °C in 5% CO_2. After 72 h the media was changed with the fresh media and 10 μL MTT (1 mg/mL) solution was added in each well. After 4 h, 100 μL DMSO was added in each well to dissolve the formazan crystals. Finally, the absorbance was taken at 550 nm. Untreated cells were included as controls and the wells with only media were considered as blank. The percentage of cytotoxicity was calculated as [(OD of treated well – OD of blank well) / (OD of untreated well- OD of blank well)] × 100 respectively. The CC_{50} was calculated by regression analysis. Further, the degree of selectivity was expressed as SI and was obtained by calculating the ratio of CC_{50} for macrophage/IC_{50} for promastigotes [22].

2.6. In vivo studies

Two different strains were included in the study involving SSG-sensitive (S) strain and SSG-resistant (R) strain. Inbred BALB/c mice were infected with the promastigotes (1×10^7) through the tail vein injection. 1 month post-infection, the mice were distributed into a total of eight groups, each group included 6 mice. The two groups of infected animals *i.e.* S-strain and R-strain were treated with SAL (25 mg/kg b.wt./day, for 2 weeks, orally) after dissolving it in the standard suspension vehicle (SSV). The positive groups included S-strain and R-strain infected mice treated with the reference drug, AmB (2.5 mg/kg/b.wt./day, for 5 days, intraperitoneally). The negative controls involved S-strain and R-strain infected mice which received no treatment and the last two groups included the S-strain and R-strain infected mice treated with the SSV.

2.7. Assessment of infection

All mice from each group were sacrificed 14 days post infection. The splenic parasite burden was determined by RT-PCR. A SYBR green-based qPCR assay targeting a 229 bp fragment of microsatellite gene in the spleen was used to quantify *Leishmania* parasites. Briefly, 25 mg of spleen was processed for DNA isolation using phenol chloroform method [23]. The qPCRs were performed in a 20-μL volume consisting of 1 μL of DNA sample (150 ng), 1 μL of 10 μM (each) primer, 10 μL SYBR green Master Mix (Thermo scientific) and 8 μL of PCR grade water. The sequences of the primers were as forward *5′*-ACACGCAGA GAACTCGGTTT-*3′* and reverse *5′*-TGGAGCGAGAAAGGACAAGT-*3′*. Reactions were run on the system of Roche, LightCycler 480. The following thermal cycling conditions were used: 95 °C for 5 min, 35 cycles at 94 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. The amplicon specificity was checked by generating a melting curve with the melting curve program (65–95 °C and a continuous fluorescence measurement) and a cooling step at 40 °C at the end. Each run included a positive-control sample and negative control. Each sample was tested in triplicate. Standard curve: DNA of the promastigotes of *L. donovani* served as

the quantification standard for the qPCR assay. The standard curve was prepared from 10-fold serial dilutions (1×10^{10} parasites to 100 parasites/reaction) of DNA [24,25].

2.8. Evaluation of delayed-type hypersensitivity (DTH) responses

DTH is an inflammatory reaction which develops in response to antigen. It mainly involves cell-mediated immune cells. Briefly, leishmanin (40 μL) and PBS alone was intradermally injected in right and left hind footpads of mice respectively. The leishmanin was prepared from killed promastigotes (2×10^8/mL) with 0.5% phenol-PBS. The swelling of footpad was measured by vernier calipers after 48 h of antigen inoculation. Further, the DTH response was measured by finding the difference in the swelling of two footpads in mm [26].

2.9. Study of $CD4^+$ and $CD8^+$ T cell profile

The T cell populations including $CD4^+$ as well as $CD8^+$ T cells are crucial for the cure of leishmaniasis. The population of $CD4^+$ and $CD8^+$ T cells was delineated in splenocytes by immunophenotyping. Briefly, 2 weeks post-treatment; the spleen was homogenized and centrifuged (3000 rpm for 5 min). The obtained pellet was washed with PBS and the RBCs were lysed using NH_4Cl (1%). The cells were further centrifuged and washed with PBS. Finally, cells ($1X10^6$) were stained with anti-mouse-CD4-fluorescein isothiocyanate (FITC), antimouse-CD8-(APC) and antimouse-CD-19-(APC) antibodies (BD biosciences) for 20 min. Following staining, the cells were washed with PBS and finally, the cells were analyzed in the FACSCalibur flow cytometer. The required dot plots were made and quadrants were assigned to find the percentage of the population of different T cells [27].

2.10. Estimation of Th1 and Th2 cytokines

The concentration of different cytokines was estimated in the sera of animals on 14th p.t.d. The levels of different cytokines associated with Th1 type of immune response *i.e.* IFN-γ, TNF-α and IL-12 and those related to Th2 immune arm *viz.* IL-10 and IL-4 were measured. The levels of these Th1/Th2 cytokines were evaluated using commercially available Diaclone kits, France. The concentrations of these cytokines were calculated against the standard curve in picogram/ml [28].

2.11. RNA isolation and cDNA synthesis from spleen

The RNA was extracted from the spleen using TRIzol Reagent (Sigma). After homogenizing spleen in 1 mL TRIzol for 10 min, the sample was vortexed in chloroform (200 μL) for 10 min. The solution was centrifugated (13,000 rpm, 10 min, 4 °C) and to the upper layer obtained, 2-propanol (500 μL) was added. The sample was again centrifuged and the pellet was obtained. The pellet was washed with 80% ethanol and centrifuged. Finally, pellet was dried in air and dissolved in DEPC water.

First-strand cDNA was synthesized from RNA (5 μg) samples using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The gene expression of inducible nitric oxide synthase (iNOS) and the nuclear transcription factor kappa-B (NF-κB) was quantified in different groups of animals. The cycling conditions used for PCR were similar as followed during an assessment of infection. β-actin was selected as the housekeeping gene for the normalization of qPCR expression. The relative quantity values for the gene expression were obtained as fold change using formula $2^{-\Delta\Delta Ct}$, in relation to normal mice [29]. The sequences of forward and reverse primers are given in Table 1.

2.12. Detection of intracellular reactive oxygen species (ROS)

The generation of ROS by SAL in the spleen was monitored by H_2DCFDA (2′, 7′- dichlorodihydrofluorescein-diacetate) staining. The splenocytes (1×10^6) of treated or untreated mice were incubated with the dye (10 μM in DMSO) at 37 °C, 5% CO_2 in dark for 30 min. The cells were further washed with PBS to remove the extra dye. The intensity of the fluorescent signal was recorded at the excitation wavelength of 485 nm and emission, 535 nm. Controls included stained untreated cells and unstained untreated cells. The fold change of ROS was calculated as compared to normal control [30].

2.13. Determination of nitric oxide (NO)

The production of NO was analyzed in the spleen using Griess reagent assay. The spleen was isolated from the different groups of animals and homogenized in PBS. It was further cold centrifuged at 10,000 rpm for 15 min. A total of 50 μL of the obtained supernatant was incubated with a similar volume of Griess solution containing 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride (in 5% H_3PO_4) for 10 min. The absorbance was finally measured in the ELISA plate reader at 550 nm. The levels of nitrite were quantified with respect to the standard curve produced with sodium nitrite [31].

2.14. Estimation of in vivo toxicity on liver and kidney functions

To assess the *in vivo* toxicity of SAL (orally) and AmB (i.p), the sera of mice were obtained on the 14th p.t.d. The adverse effects on the liver were analyzed by estimating the levels of various enzymes of liver *viz.* serum glutamate pyruvate transaminase (SGPT), serum oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) using the commercially available kits (Fischer Scientific). Similarly, the side effects on the kidney were determined by evaluating the levels of kidney function tests *viz.* urea, blood urea nitrogen, uric acid and creatinine using the commercially available kits [28].

2.15. Statistical analysis

The *in vivo* study included 6 mice per group and the results were expressed as mean ± SEM. Comparisons among the groups of animals were done by one-way ANOVA followed by Tukey's test. The difference of $p < .05$ was implied statistically significant.

3. Results

3.1. Determination of IC_{50}

The growth inhibitory effect of SAL was assessed against the promastigotes of SSG sensitive and resistant strains of *L. donovani*. After 72 h the viability of parasites was found to be significantly ($p < .05$) decreased in the case of SAL treatment as compared to control untreated groups. IC_{50} of 15.54 ± 1.53 μg/mL and 38.16 ± 0.86 μg/mL was observed after SAL treatment in case of sensitive and resistant strain respectively. The reference drug AmB rapidly eliminated both strains of *Leishmania* parasites *in vitro* with IC_{50}–0.037 ± 0.003 μg/mL against the sensitive and IC_{50}–0.048 ± 0.003 μg/mL against the resistant strain.

3.2. Cell cycle analysis

The appearance of cells in the sub G_0/G_1 phase of cell cycle depicts the occurrence of apoptosis. SAL triggered significant ($p < .05$) cell cycle arrest at sub G_0/G_1 phase in sensitive (34.07 ± 1.76%) and resistant (25.53 ± 1.63%) strain of *L. donovani* in comparison to the respective untreated parasite controls. AmB treatment resulted in maximum ($p < .05$) apoptosis with 55.43 ± 0.99% cells in the sub G_0-G_1 phase in case of sensitive strain and 51.57 ± 1.99% in case of resistant strain as compared to the untreated parasite (Fig. 1).

Table 1
Primers for gene expression studies.

Primers	Primer sequence	Annealing temp.	Melting temp.
β-actin	Forward 'GCATTGCTGACAGGATGCAG' Reverse 'CCTGCTTGCTGATCCACATC'	57	63
NF-kB	forward 'CTGGTGGACACATACAGGAAGAC' reverse 'ATAGGCACTGTCTTCTTTCACCTC'	57	63
(iNOS)	forward 'GATCCGATTTAGAGTCTTGGTG' reverse 'TCCCTGGCTAGTGCTTCAG'	60	75

3.3. Toxicity profile and selectivity against THP-1 cell line

The cytotoxicity of SAL and AmB was evaluated against THP-1 cells using MTT assay. The intensity of the color of dissolved formazan crystals depicted the cell viability. Further, the safety profile was assessed by calculating the selectivity index. With SAL, the CC_{50} obtained was 1108.01 ± 12.5 μg/mL. However, in the case of AmB CC_{50} of 23.70 ± 12.5 μg/mL was obtained. SI of > 10 was found with SAL as well as AmB.

3.4. SAL reduces splenic parasite burden

To assess the *in vivo* antileishmanial capacity of SAL, 4 weeks-infected BALB/c mice with either strain were treated daily up to 2 weeks with SAL (25 mg/kg b.wt.). The antileishmanial efficacy was assessed by quantifying the parasite in the spleen on the 14th p.t.d. by RT-PCR. As shown in Fig. 2, SAL conferred significant ($p < .05$) protection to mice infected with either sensitive or resistant strain as compared to the infected controls. Similarly, the AmB treatment also endowed significant ($p < .05$) protection to the mice infected with either strain as compared to the only infected animals. The mice receiving only vehicle exhibited similar parasite load as those of the infected control (data not shown).

3.5. Induction of DTH response

The positive DTH reaction marks the acquisition of cell sustained immune response and thus implies successful treatment. SAL treatment exhibited pronounced DTH response ($p < .05$) in case of sensitive and resistant strain infected mice as compared to the untreated infected controls. However the strongest ($p < .05$) DTH response was found after AmB treatment of mice infected with either strain as compared to the infected controls (Fig. 3).

3.6. Estimation of percentage of $CD4^+$, $CD8^+$ T and CD19 B cells

The frequency of $CD4^+$, $CD8^+$ T and CD19 B cells in the spleen was examined in the infected and the treated animals. The population of these cells was detected by flow cytometry after staining these cells with specific monoclonal fluorescent antibodies. A significant ($p < .05$) incline in the population of $CD4^+$ T and CD19 B cells was found in the SAL treated sensitive as well as resistant strain infected animals as compared to the only infected control animals. The population of $CD8^+$ T cells was found to be increased after SAL treatment of animals infected with either S- or R-strain. Statistically, the difference was found to be non-significant ($p > .05$) among SAL treated S-strain and S-strain infected animals. However, the difference was significant ($p < .05$) in case of SAL treated R-strain and only R-strain infected animals. The magnitude of these cells was found to be significantly ($p < .05$) higher in the AmB treated animals infected with either strain as compared to the infected controls (Fig. 4).

3.7. Generation of Th1/Th2 type of cytokine profile

Cytokines play an important role in governing the outcome of treatment. The cure of VL depends mainly on the effect of the drug on the immune status so in the present study the Th1 and Th2 cytokine profile was studied in the sera of animals. Notably, we observed significantly ($p < .05$) high levels of IFN-γ in SAL treated animals infected with either strain than infected animals. Similar pattern was noticed in the levels of TNF-α and IL-12 after treatment of sensitive or resistant strain infected animals with SAL. The levels of Th1 cytokines were found to be significantly ($p < .05$) higher in all AmB treated groups as compared to the infected controls.

A significant ($p < .05$) decline in the levels of IL-10 was found after SAL treatment in case of sensitive and resistant strain as compared to the infected animals. Likewise the levels of another Th2 cytokine, IL-4 was significantly ($p < .05$) found to be reduced in the both SAL treated groups as compared to the untreated infected animals. The infected groups receiving AmB also showed significant ($p < .05$) diminished levels of these Th2 cytokines as compared to the infected controls (Fig. 5).

3.8. Gene expression

NO is an important agent toxic to leishmanial parasite which is transcribed by the iNOS gene. The expression of iNOS mainly relies on the activation of another crucial gene, NF-κB. To confirm the NO producing property of SAL its effect on the expression of iNOS and NF-κB genes in the spleen was analyzed by real-time PCR. The SAL treatment resulted in the up-regulation of expression of NF-κB gene by 6.14 ± 0.18 fold in the mice infected with S-strain and by 6.29 ± 2.52 fold in mice infected with R-strain. However, the difference was found to be non-significant ($p > .05$) when compared with the respective infected controls (Fig. 6A).

Similarly, the expression of iNOS genes was found to be up-regulated after SAL treatment by 5.71 ± 0.035 fold and by 5.07 ± 1.54 fold in the animals infected with S- and R-strain respectively. However, the difference was found to be non-significant ($p > .05$) when compared with the respective infected controls. The AmB treatment of animals infected with either strain resulted in significant ($p < .05$) up-regulation of expression of both these genes as compared to the respective infected controls (Fig. 6B).

3.9. Induction of ROS generation

To examine the generation of ROS post treatment with SAL in the spleen, H_2DCFDA was used. A significant ($p < .05$) fold increase in ROS in case of SAL treated sensitive (1.49 ± 0.14) and resistant (1.48 ± 0.06) strain in comparison to untreated infected controls was found. However, maximum ROS production was observed following AmB treatment in case of animals infected with either of the strain as compared to infected controls (Fig. 7).

3.10. NO production

The influence of the SAL treatment on *L. donovani* was also determined in terms of NO production in the spleen. SAL treatment led to a significant ($p < .05$) elevation of nitrite levels (4.42 ± 2.42 μM) in sensitive strain infected animals as compared to only infected animals.

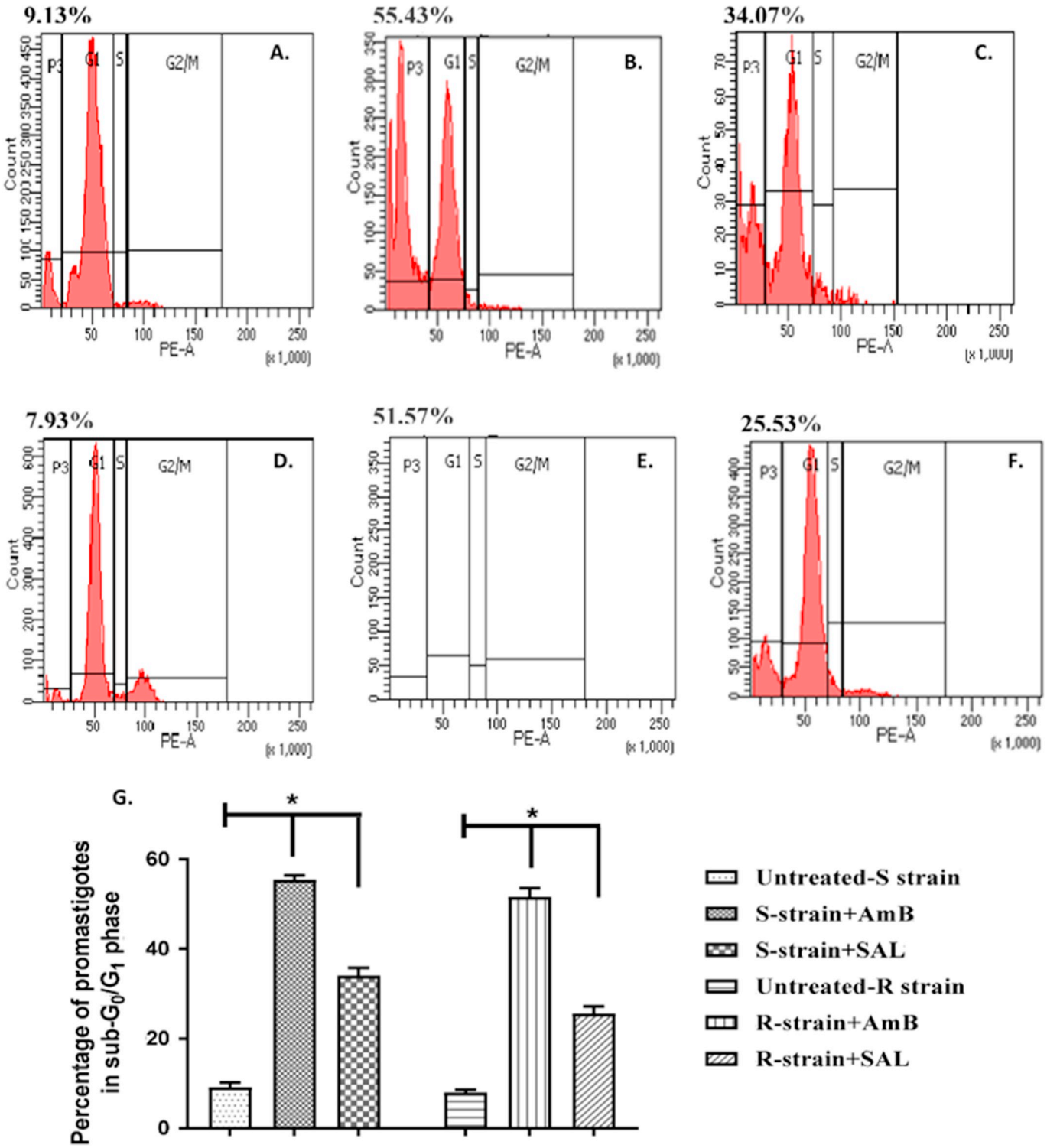

Fig. 1. Analysis of cells in sub G_0/G_1 phase by flow cytometry. The promastigotes of two different strains of *L. donovani* were incubated with the SAL and reference drug, AmB for 72 h. The PI staining revealed the cells in the different phases of cell cycle. The P_3 quadrant in the histograms show the promastigotes in sub G_0/G_1 phase. A. Untreated S-strain promastigotes (9.13 ± 1.09%), B. AmB treated S-strain (55.43 ± 0.99%), C. SAL treated S-strain (34.07 ± 1.76%), D. Untreated R-strain promastigotes (7.93 ± 0.67%), E. AmB treated R-strain (51.57 ± 1.99%), F. SAL treated R-strain (25.53 ± 1.63%). G. The bar diagram depicts the percentage of cells in the sub G_0/G_1 phase. *$p < .05$ represents a significant difference with respect to untreated controls.

Similarly, significant ($p < .05$) levels of NO of about 4.51 ± 1.43 μM were noted in SAL treated animals infected with resistant strain as compared to only infected controls. AmB treatment also resulted in a significant ($p < .05$) increase in the NO production in animals infected with sensitive strain and in resistant strain infected mice as compared to untreated infected animals (Fig. 8).

3.11. Biomarkers

The treatment-related side-effects against the liver and kidney were assessed by estimating the levels of biomarkers characterizing the functions of liver and kidney. The levels of SGOT, SGPT and LDH were found to be in the normal range in all SAL and AmB treated groups as

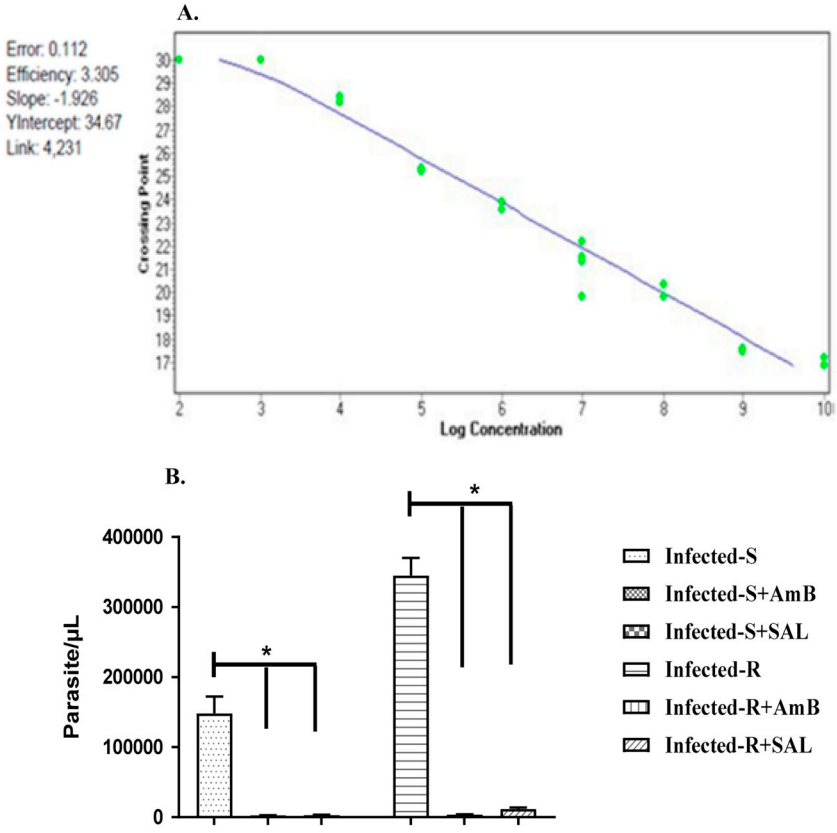

Fig. 2. Determination of anti-leishmanial efficacy of SAL in inbred BALB/c mice. The splenic parasite burden was estimated by real-time PCR. After treatment of infected animals with the SAL (25 mg/kg b.wt.) and AmB (2.5 mg/kg b.wt.), the animals were sacrificed and spleen was removed to determine the parasite load. A. Standard curve. It was generated from 10-fold serial dilutions of DNA of promastigotes, corresponding to 1×10^{10} to 100 parasites/reaction. The average cycle threshold (CT) of duplicates in each dilution was plotted against the different dilutions of the parasites. B. The graph depicts the concentration of parasite/µL. *$p < .05$ represents a significant difference of treated groups with respect to the untreated controls.

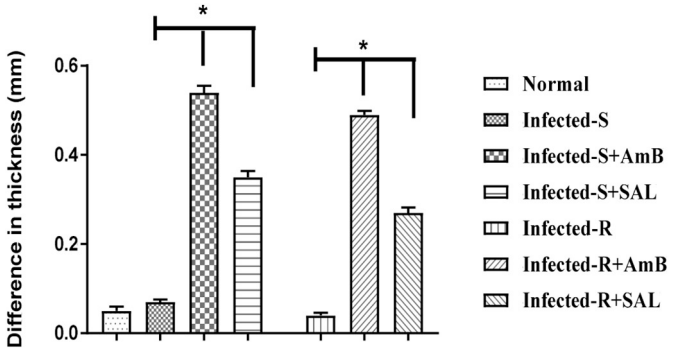

Fig. 3. Evaluation of DTH response as CMI marker. The response was calculated as the difference (in mm) in the right (leishmanin injected) and left (PBS injected) hind footpad after 48 h. *$p < .05$ represents a significant difference of treated groups with respect to the untreated controls.

compared to the increased abnormal levels of these enzymes in case of infected controls (Table. 2).

The levels of kidney function markers *viz.* uric acid, urea, blood urea nitrogen and creatinine were found within the normal range in case of SAL treated infected groups with respect to only infected animals where the levels of these metabolites were found to be deranged. The infected animals treated with AmB displayed abnormal levels of these molecules. The results revealed the adverse toxic effects of AmB on the kidney while nephro-protective nature of the SAL was demonstrated (Table. 3).

4. Discussion

Visceral leishmaniasis is a fatal systemic parasitic disease which accounts for high morbidity and mortality by compromising the immune status of the host. To combat the problem of VL it is important to mitigate the suppression of the immune system of the host [32]. In many anti-parasitic therapies augmentation of the immune system is known to synergistically enhance the effectiveness of the drugs [33]. Moreover, the co-therapy of immunomodulators along with the conventional antileishmanial drugs decreases the dose regimen of the therapy and hence the side-effects. Thus the antileishmanial agents which could rejuvenate the dampened immunity of the VL infected host are most favorable [34]. In a similar direction, the antileishmanial therapeutic efficacy of the SAL, a natural phenylpropanoid was investigated in adjunct to its immunomodulatory capacity. This study explores the therapeutic and immunomodulatory efficacy of SAL against *L. donovani.* The present study revealed the antileishmanial efficacy of SAL, substantiated by immunopotentiation through Th1 immune response with no adverse side-effects. The SAL attenuated the depressed CMI response and also generated leishmanicidal agents which complemented its therapeutic potential against visceral leishmaniasis.

The antileishmanial potential of SAL was assessed *in vitro* against the promastigote forms by trypan blue exclusion assay and by PI staining. The light microscopy revealed effective IC_{50} of SAL against both strains of *L. donovani*. The efficacy of AmB in inhibiting the growth of promastigotes of either strain was more pronounced than SAL. However in terms of safety as studied by the 50% cell cytotoxicity assay, SAL was found to be more superior than AmB. The cell cycle analysis further displayed the ability of SAL treatment to drive the promastigotes of *L. donovani* towards apoptosis. The incubation of promastigotes with SAL halted the cells at the sub G_0/G_1 stage of cell cycle which signifies the DNA fragmentation. Wang et al., examined the apoptotic activity of SAL against carcinoma cells of lung, A549 and observed that SAL arrested the cell cycle at the G_0/G_1 phase. SAL prevented the multiplication and metastasis of cancer by obstructing the transforming growth factor-β and by repressing the manifestation of protein Snail in A549 cells [35]. In the study of Sen et al., parasite killing efficacy of Artemisinin (sesquiterpene lactone) was found to be

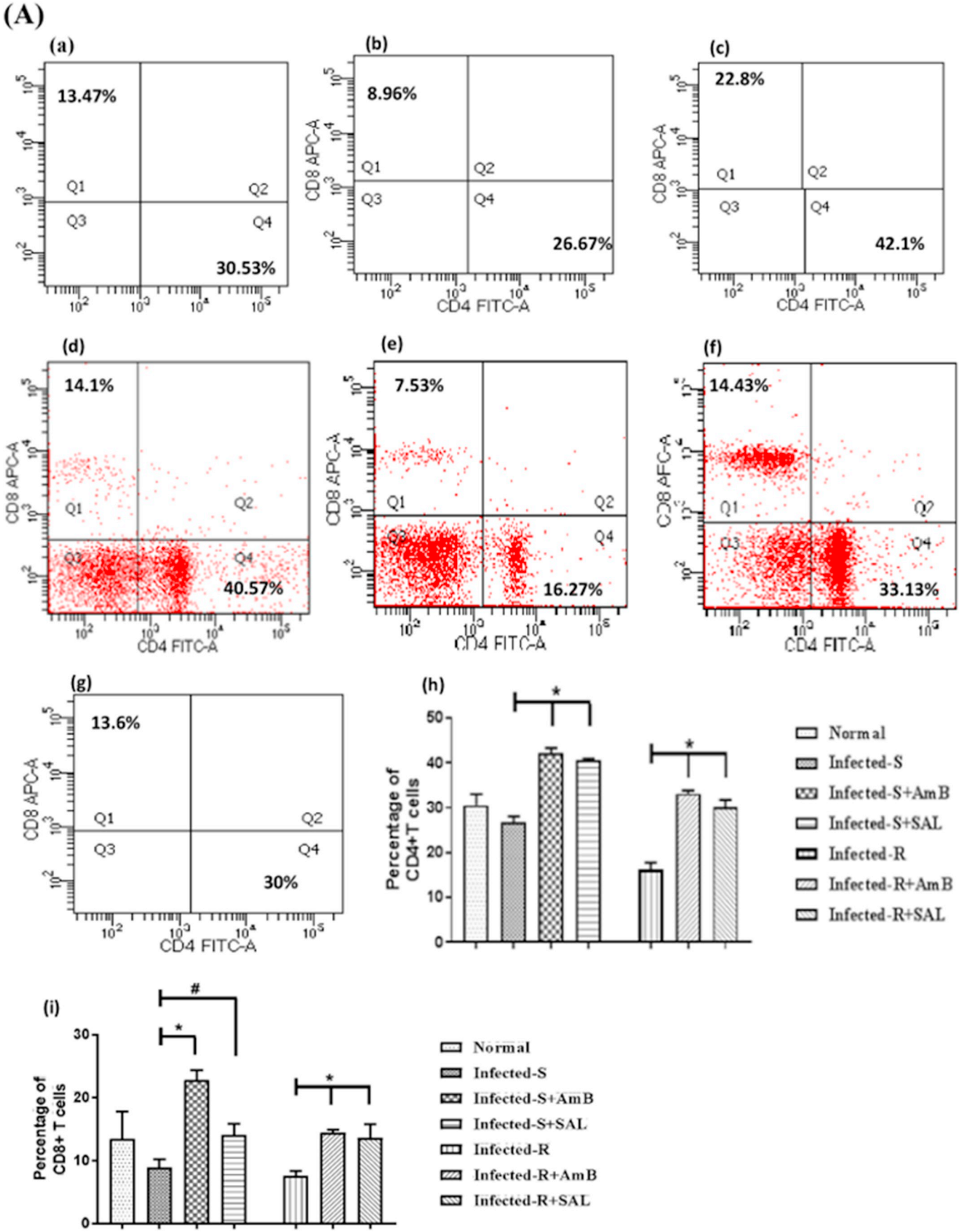

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Fig. 4. Analysis of different T- lymphocytes and CD19 B cells in the splenocytes. 4A. In the dot plots the Q4 and Q1 quadrant indicates the percentage of $CD4^+$ and $CD8^+$ T cells. (a) Normal mice (b) Infected-S strain mice (c) Infected-S + AmB treated mice (d) Infected-S + SAL treated mice (e) Infected-R strain mice (f) Infected-R + AmB treated mice (g) Infected-R + SAL treated mice. (h) The bar diagram depicts the percentage of $CD4^+$ and (i) $CD8^+$ T cells in different groups of animals. 4B. The P3 quadrant in the histograms represents the CD19 B cells. (a) Normal mice (b) Infected-S strain mice (c) Infected-S + AmB treated mice (d) Infected-S + SAL treated mice (e) Infected-R strain mice (f) Infected-R + AmB treated mice (g) Infected-R + SAL treated mice. (h) The bar diagram depicts the percentage of CD19 B cells in different groups of animals. $^*p < .05$ represents significant difference of treated groups with respect to the untreated controls. $^{\#}p > .05$ represents non-significant difference of treated groups with respect to the untreated controls.

mediated by its ability to bring apoptotic like changes. Artemisinin increased the proportion of promastigotes in the sub G_0/G_1 phase and thus resulted in cell death [36].

The degree of cure after treatment was assessed by examining the parasite burden in the spleen by RT-PCR. SAL (25 mg/kg b.wt.) treatment considerably decreased the degree of parasite load in the spleen in

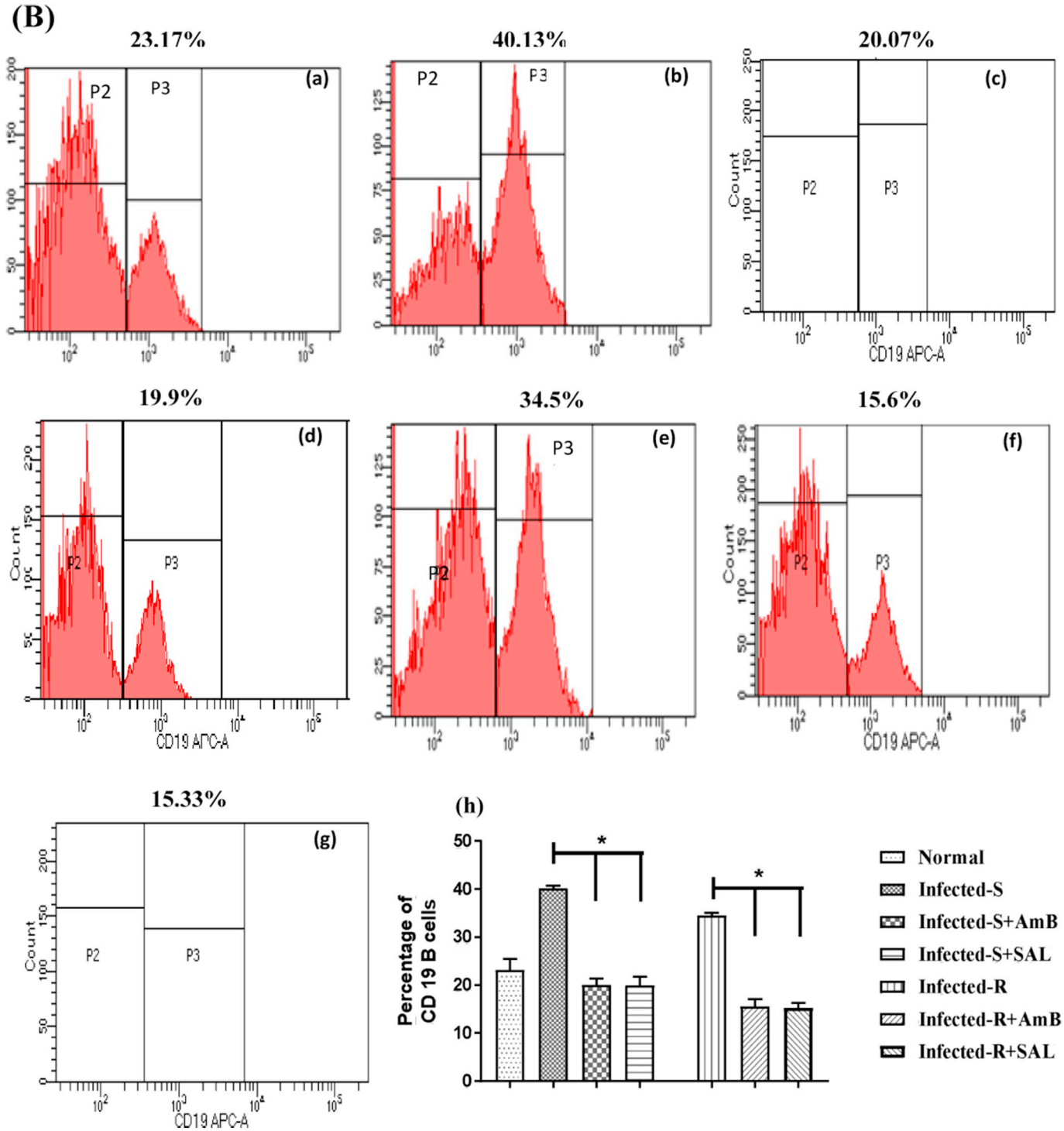

Fig. 4. (*continued*)

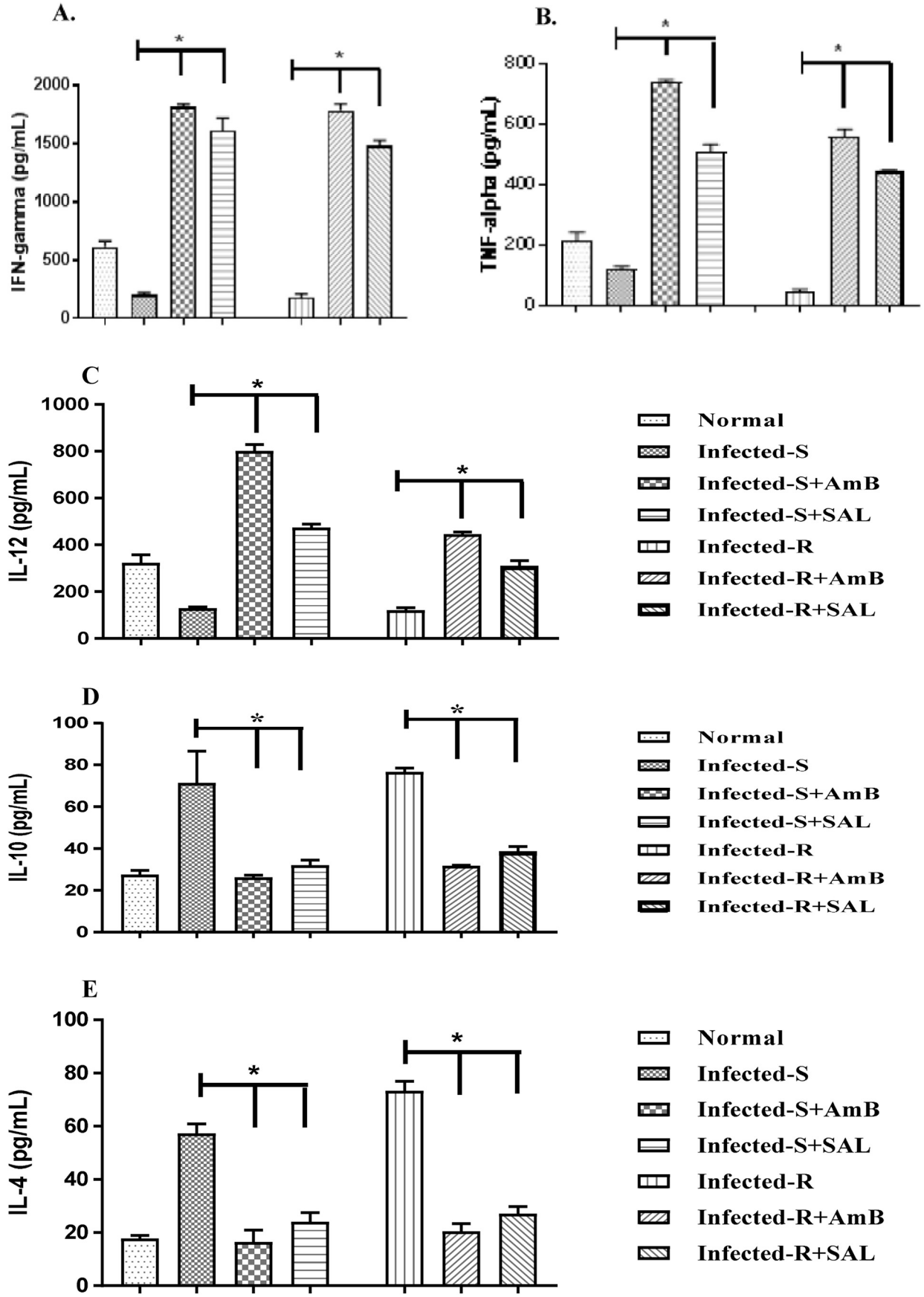

Fig. 5. Assessment of Th1/Th2 immunomodulatory efficacy of SAL. Serum levels of Th1 and Th2 favored cytokines were appraised by cytokine ELISA kit. A. IFN-γ B. TNF-α C. IL-12: SAL increased the levels of IFN-γ, TNF-α and IL-12 in both S-strain and R-strain infected mice. D. IL-10 and E. IL-4: These Th2 types of cytokines were observed to decrease in SAL treated S- and R-strain infected mice as compared to infected controls. *$p < .05$ represents a significant difference of treated groups with respect to the untreated controls.

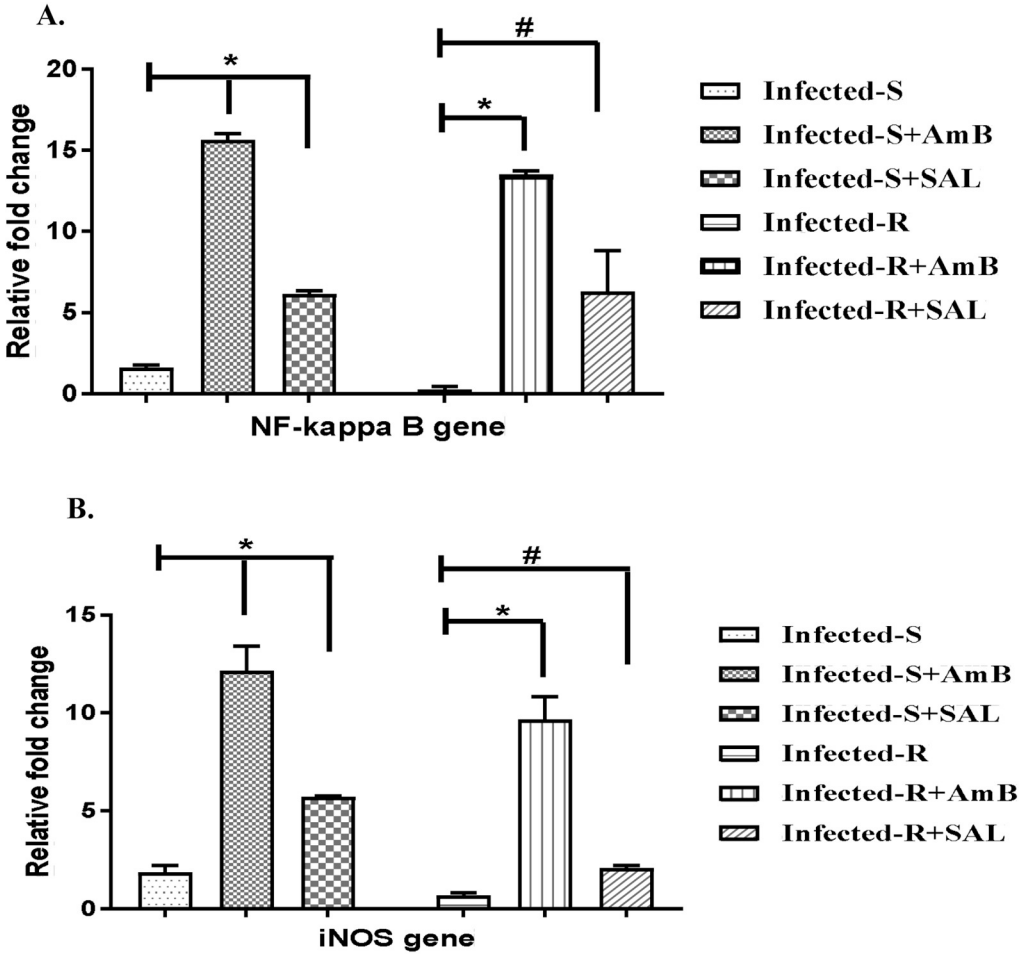

Fig. 6. Effect of SAL at the expression of iNOS and NF-κB gene. Relative gene expression of A. NF-κB and B. iNOS was determined by real-time PCR. $^{*}p < .05$ represents a significant difference of treated groups with respect to the untreated controls. $^{\#}p > .05$ represents a non-significant difference of treated groups with respect to the untreated controls.

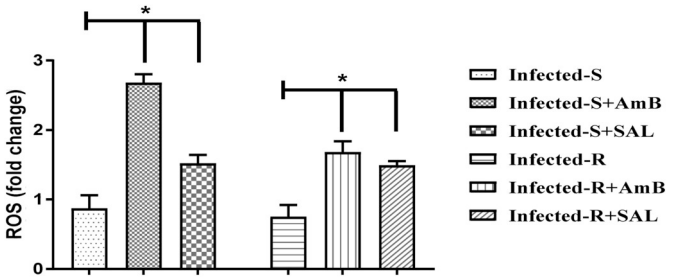

Fig. 7. Generation of reactive oxygen species (ROS) in the spleen. ROS was monitored by H_2DCFDA staining in the splenocytes. SAL treatment induced ROS production in both S-strain as well as R-strain infected mice. $^{*}p < .05$ represents a significant difference of treated groups with respect to the untreated controls.

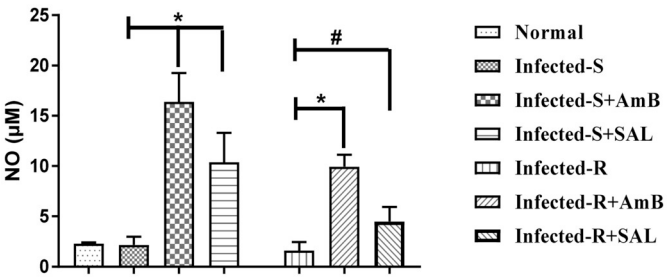

Fig. 8. Estimation of nitric oxide (NO) in the spleen. It was quantified in the form of nitrite in the splenocytes by incubating the cells with the Griess reagent. $^{*}p < .05$ represents a significant difference of treated groups with respect to the untreated controls. $^{\#}p > .05$ represents a non-significant difference of treated groups with respect to the untreated controls.

comparison to the untreated control animals. The results showed that the SAL possesses therapeutic ability against the experimental VL. The cure of VL mainly relies on the refurbishment of the functional cellular immune response (CMI) [37]. The development of DTH reaction indicates the immunogenicity due to the specific cellular immune responses [38]. SAL treated infected mice showed pronounced DTH response to the leishmanin antigen of *L. donovani*. The result indicated that SAL has the ability to restore the depleted CMI. The SAL has also been known to help in the development of DTH against anti-keyhole limpet hemocyanin in aged mice after 48 h [16].

The successful treatment is dependent on the mounting of the Th1 type of immune response over the Th2 with the restoration of levels of various pro-inflammatory chemokines [39]. In the present study, the cytokine profile was found to be skewed towards Th1 after treatment of animals with SAL. The reduction in parasite burden after the administration of SAL was supported by an increase in the levels of IFN-γ, TNF-α and IL-12. IFN-γ is a crucial chemokine which suppresses the parasite growth by activating the macrophages. Alongwith TNF-α, IFN-γ results in the induction of an important leishmanicidal effector agent, NO [40]. In the current study, the decreased parasite load after SAL treatment was found to be accompanied by enhanced Th1 and reduced Th2

Table 2
Levels of different liver function enzymes in the sera of different groups of animals.

	Normal	Inf-S	Inf-S + AmB	Inf-S + SAL	Inf-R	Inf-R + AmB	Inf-R + SAL
SGPT (U/L)	28.29 ± 2.04	63.69 ± 0.65	36.03 ± 0.97	23.97 ± 1.41	65.26 ± 0.8	32.18 ± 1.74	29.11 ± 1.39
SGOT (U/L)	26.75 ± 1.05	67.58 ± 1.71	33.79 ± 1.04	30.25 ± 1.04	62.59 ± 1.34	34.22 ± 0.68	29.47 ± 1.36
LDH (IU/L)	185.03 ± 1.2	260.18 ± 1.96	244.64 ± 2.04	172.4 ± 1.96	265.58 ± 1.37	232.49 ± 2	139.47 ± 3.46

The levels of SGPT, SGOT and LDH were found to be increased in both S- and R- strain infected animals. However, the levels of these enzymes were within the normal range in all treatment groups.

Table 3
Levels of kidney function markers in the sera of different groups of animals.

	Normal	Inf-S	Inf-S + AmB	Inf-S + SAL	Inf-R	Inf-R + AmB	Inf-R + SAL
Urea (mg/dL)	30.92 ± 0.58	69.01 ± 0.69	54.57 ± 0.63	23.65 ± 1.17	66.74 ± 1.03	53.25 ± 1.03	28.41 ± 0.96
BUN (mg/dL)	14.44 ± 0.27	32.23 ± 0.32	25.17 ± 0.26	11.05 ± 0.55	31.17 ± 0.48	24.87 ± 0.48	13.27 ± 0.45
Uric acid (mg/dL)	4.73 ± 0.13	8.02 ± 0.15	8.62 ± 0.31	4.6 ± 1.4	9.03 ± 0.48	8.79 ± 0.26	4.65 ± 0.5
Creatinine (mg/dL)	0.68 ± 0.10	1.82 ± 0.09	1.64 ± 0.18	1.24 ± 0.06	1.85 ± 0.08	1.52 ± 0.05	0.86 ± 0.03

The levels of urea, BUN, uric acid and creatinine were found to be abnormally increased in S- or R-strain infected animals and in AmB treated infected animals. This depicted nephro-toxic behavior of AmB. However, the examination of these biochemical markers of kidney in SAL treated animals reveals no nephro-toxicity of SAL.

response. The remarkably high levels of IFN-γ, TNF-α and IL-12 and low levels of IL-10, IL-4 after SAL treatment revealed the immunostimulatory capacity of SAL. It has been used as an immunological adjuvant as it favored the Th1 skewed immune response by increasing the levels of IFN-γ and IL-2 cytokines [41]. The fractions of *Azdirachta indica* [42] and cystatin [43] have also been shown to exert its therapeutic potential against *L. donovani* by the expansion of Th1 immune response by augmenting the levels of cytokines like IFN-γ, TNF-α and IL-2.

The cytotoxic $CD8^+$ lymphocytic response and the helper $CD4^+$ T lymphocytes response play a crucial role in the outcome of VL. The determination of these cells represents the dynamics of the immune status of the host. $CD4^+$ and $CD8^+$ T cells have been implicated in curbing the growth of the parasite. $CD4^+$ T cells are the key sources of Th1 cytokines primarily IFN-γ and thus plays a crucial role in limiting the parasite growth [44]. Similarly, the exhaustion of the $CD8^+$ T cells further impairs the secretion of the various Th1 cytokines like IFN-γ, IL-12 and TNF-α during infection [45]. $CD4^+$ and $CD8^+$ T-cell count was found to increase in infected mice after SAL treatment as compared to the untreated infected controls. This indicates that SAL eliminated the parasite by boosting the weakened immune response. The immunomodulatory property of SAL is well documented in the previous studies too however its role as an antileishmanial agent has not been studied. The supplementation of SAL (24 mg/kg b.wt.) in the feed of aged rats resulted in the augmentation of the number of $CD3^+$ cells and $CD4^+$ T helper cells [16]. The bioactive fraction of *Piper nigrum* resulted in the abrogation of the parasite by enhancing the proportion of $CD4^+$ and $CD8^+$ T-cell population [31]. The polyclonal B cell activation is an important characteristic feature of VL. CD19 B cells are responsible for the exacerbation of VL disease [46]. In the present study too dramatic increase in the population of the CD19 B cells was observed in the animals infected with S or R-strain of *L. donovani*. However after SAL treatment, the population of these cells was found to be decreased indicating that SAL potentially contributed towards tilting the immune response in the favor of host during VL.

Leishmania is known to inhibit the NO generating capacity of macrophages. NO, a principle leishmanicidal molecule frames an environment unsuitable for the growth and multiplication of the parasite [47]. In the present study, SAL was found to trigger the production of this crucial effector molecule in the spleen. The finding could be correlated to the resolution of the parasite burden in SAL treated animals. Further, the production of ROS is another means to kill intracellular parasites as inhibition of ROS compromises the control of parasite growth [48]. In the present study, reactive oxygen species were found to increase after SAL treatment in the infected animals. The result clearly depicted that the therapeutic capability of SAL is also substantiated by the production of ROS. However, this finding was contradictory to the previous study of Li et al., where SAL was found to protect the PC12 cells from the apoptotic effects of 1-methyl-4-phenylpyridinium by decreasing the levels of ROS and NO [49].

The activation of NF-κB is common during many infections like VL. It is known to induce the innate arm of the immune system by expressing the different chemokines which in turn produce secondary mediators like iNOS [50,51]. We studied the mRNA expression levels of NF-κB and iNOS genes by RT-PCR. SAL was found to enhance the expression of both these genes which implies that SAL can modulate the transcriptional potential of the cells for NF-κB and iNOS genes. The up-regulation of the expression of these genes is responsible for the development of the Th1 arm of immunity and NO following SAL treatment.

In vivo toxicity of the SAL was examined by assessing the levels of liver and kidney function tests. The serum levels of SGOT, SGPT, and LDH were not found altered in any of the treatment groups. Similarly, no toxic effects of SAL were found on the kidney function tests as the levels were in the normal range in the mice after SAL treatment. This data indicates that SAL does not cause renal or hepatotoxicity. SAL has been shown to protect hepatic tissue from the deteriorating effects of hepato-toxin,lipopolysaccharide. It repaired the micro-architecture of the liver and also restored the levels of liver function tests [12]. SAL treatment to the diabetic mice markedly reduced the elevated levels of creatinine and helped in the restoration of other important molecules related to the functions of kidney in the normal range. It also ameliorated the complication of nephropathy, an important feature of the diabetic mellitus [52].

Taken together, our study illustrates the antileishmanial capacity of SAL which is mainly potentiated by its immunostimulatory ability and by its potential to induce the secretion of NO and ROS through upregulation of the genes like NF-κB and iNOS. It was noteworthy that SAL generated collateral immunostimulation and production of NO and ROS which could be used as a potential therapy for VL. However, the study recommends further confirmation of the data in the higher models of VL.

Declaration of competing interests

The authors declare no conflict of interest.

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