MATERIALS AND METHODS

3.1 PARASITE SPECIES/STRAINS

Two different strains of *Leishmania donovani* were employed for the study:
a. WHO reference, MHOM/IN/80/Dd8, sodium stibogluconate (SSG) sensitive strain procured from Post graduate Institute of Medical Education and Research (PGI), Chandigarh. b. P.B.-0014, SSG resistant strain procured from RMRI, Patna, Bihar.

3.2 CULTURE MEDIA

During the research work the promastigote stage was maintained and expanded in two different cultivation media; modified biphasic NNN (Novy-McNeal-Nicolle) and RPMI-1640.

To formulate NNN media 1.4 g bacto agar (Difco) and 0.6 g sodium chloride (NaCl) was dissolved in 90 mL double distilled water in a previously sterilized bottle. The solution formed was autoclaved at 15 p.s.i for 20 minutes which was then brought down to 50-55°C. It was then enriched with 10 mL defibrinated blood of rabbit and 2 units each of antibiotics viz. streptomycin and gentamycin. Approximately 2-3 mL of this final media was added to McCartney vials which were then placed in a slanting position till solidification of media. All these vials were kept at -20°C for a night and then kept at room temperature for 3-4 hours till the water of condensation appeared in the vials. The vials were then stocked at 4°C till further use.

3.2.1 Eagle’s Minimum Essential Medium (MEM)

1% of MEM was made and autoclaved at 15 p.s.i for 20 minutes. It was then kept at room temperature for a night to check any contamination and then was preserved at 4°C.

Sodium Bicarbonate (NaHCO₃)

Similarly 7.5% NaHCO₃ was autoclaved and kept at room temperature for a night to check for any contamination and then was preserved at 4°C.

At the time of culturing NaHCO₃ was added to MEM to set its pH to 7.2 which was indicated by the color change of MEM from yellow to orangish-red (Rao *et al.*, 1984).
3.2.2 Preparation of RPMI-1640 medium

1.64 g of RPMI-1640 and 0.2 g of NaHCO₃ were dissolved in DDW. To make complete media, 7% of heat inactivated fetal calf serum (FCS) was added to it and final volume of 100 mL was made. The medium was then treated with 2 units each of antibiotics viz. streptomycin and gentamycin (Marco et al., 2005).

3.2.3 Maintenance of promastigote culture

The promastigote culture was checked for any contamination after every 3-4 days and then sub-cultured by transferring 0.5-1 mL of previous culture suspension into fresh media.

3.3 PLANT MATERIALS

The roots of *R. imbricata* were procured from the hilly region of Leh-Ladakh, India. The plant material was identified and authenticated by Dr. O.P. Chaurasia, an Ethnobotanist at the Defence Institute of High Altitude Research (DIHAR), Leh-Ladakh. A voucher specimen of the same is available at the Field Research Laboratory (FRL), Leh. The plant material was washed thoroughly with water and shade dried and then crushed to powder.

3.4 PREPARATION OF THE EXTRACT

The extract of dried and powdered roots of *R. imbricata* was obtained by hot extraction using Soxhlet extractor in 70% ethanol. The solvent was evaporated in a rotary evaporator (Buchi) and then the lyophilisation was done and the extract was stored at -4°C.

3.5 REFERENCE DRUGS

Two standard drugs were employed in the present study, Sodium stibogluconate (SSG) (Wellcome Research Laboratories, U.K.) and Amphotericin B (AmB) (Sigma-Aldrich, A2942) which acted as positive controls.

3.6 PHYTOCHEMICAL SCREENING OF PLANT EXTRACT

The extract was assessed for the presence of various secondary phytochemical components by following qualitative standard methods (Tiwari et al., 2011).
Taxonomic Classification

Kingdom: Plantae
Class: Magnoliopsida
Order: Saxifragales
Family: Crassulaceae
Genus: Rhodiola
Species: imbricata

(Chaurasia et al., 2007)
3.6.1 Test for alkaloids

The plant extract was intermixed in 1% v/v hydrochloric acid (HCl), and filtered. The filtrate was then employed for following test:

3.6.1.1 Hager’s Test

Hager’s reagent i.e. saturated picric acid solution was prepared and few drops of it were added to the filtrate. Production of yellow precipitates marked the presence of alkaloids.

3.6.2 Test for Phenols

3.6.2.1 Ferric Chloride (FeCl₃) Test

3-4 drops of FeCl₃ solution was added to the plant extract. The appearance of bluish-black or green color indicated the presence of phenols.

3.6.3 Tests of flavonoids

3.6.3.1 Alkaline (NaOH) Reagent test

Extract was treated with 3-4 drops of Sodium hydroxide solution. Intense yellow coloration which disappeared after the addition of dilute acids like sulphuric acid, implied the existence of flavonoids.

3.6.3.2 Magnesium and hydrochloric acid reduction assay

Few fragments of magnesium ribbon and concentrated HCl were added to the ethanolic solution of extract. Establishment of pink to crimson color inferred the occurrence of flavonol glycosides.

3.6.4 Test for tannins

3.6.4.1 Gelatin test

Emergence of white precipitate confirmed the presence of tannins after introduction of 1% of gelatin solution comprising NaCl to the extract.

3.6.5 Tests for saponins

3.6.5.1 Froth forming test

Extract was taken in the graduated cylinder, 20 mL DW was added to it and the cylinder was shaken for about 15 minutes. Genesis of foam demonstrated the presence of saponins.
3.6.6 Tests for diterpenes

3.6.6.1 Copper acetate test

Aqueous solution of the extract was treated with 3-4 drops of copper acetate solution. Evolution of emerald green color suggested the presence of diterpenes.

3.6.7 Tests for triterpenes

Plant extract was dissolved and filtered in chloroform and then tested by following test:

3.6.7.1 Salkowski’s test

The mixture of the filtrate with concentrated sulphuric acid was shaken and then left undisturbed for few seconds. The appearance of golden yellow color detected the triterpenes in the extract.

3.7 QUANTITATIVE ANALYSIS OF DIFFERENT METABOLITES:

3.7.1 Estimation of total phenols:

Folin–Ciocalteu assay was used to determine the total phenolic content of the extract. 200 µL of crude extract (1 mg/mL) was made up to 3 mL with distilled water and then mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was incubated for 1 hour at 40°C in the dark, and absorbance was measured at 765 nm. Standard curve was made with different concentrations (25, 100, 300, 400, 500, 600 and 700 µg/mL) of gallic acid in methanol. Finally the results were expressed as mg of gallic acid equivalent per g dry weight (Agbor et al., 2014).

3.7.2 Estimation of total flavonoids:

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method. In the assay mixture of 200 µL of crude extract (1 mg/mL in methanol), 800 µL of DW and 60 µL of 5% sodium nitrite, another 60 µL of 10% AlCl₃ solution was added after 5 min of incubation. This final mixture was allowed to stand for 6 min. Then, 400 µL of 1M NaOH solution was added, and the volume of mixture was brought to 5 mL with DW. The mixture was again allowed to stand for 15 min, and optical density was measured at 510 nm. The total flavonoid content was calculated from a standard curve using quercitin in methanol at different
concentrations (25, 100, 300, 400, 500, 600 and 700 µg/mL), and the result was expressed as mg quercitin equivalent per g dry weight (Pekal and Pyrzynska, 2014).

3.7.3 Estimation of total terpenoids:

The total terpenoid content of crude extract was determined by the aluminium chloride (AlCl₃) colorimetric method. In this assay, 200 µL of crude extract (1 mg/mL in methanol) was mixed with 1.5 mL chloroform and then 100 µL of conc. sulfuric acid was added to each tube. The mixture was incubated for 2 hours in dark at room temperature. At the end of incubation supernatant was carefully and gently decanted without disturbing the reddish brown precipitates. The precipitates obtained were dissolved in methanol and absorbance was measured at 538 nm. The total terpenoid content was calculated from a standard curve using linalool in methanol at different concentrations (25, 100, 300, 400, 500, 600 and 700 µg/mL), and the result was expressed as mg linalool equivalent per g dry weight (Indumathi et al., 2014).

3.8 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Liquid chromatography-mass spectrometry (LC/MS) integrated technique is a powerful method for the analysis of composite botanical extracts. Salidroside was identified in the plant extract by LC-MS/MS. The Waters, Micromass Q-Tof micro mass spectrometer used coupled the LC-MS/MS with exact mass measurement. The unisol YVR C18 column was equilibrated and eluted with uniform mixture of acetonitrile-water (1:9, v/v) at a flow rate of 0.8 mL/min with the injection volume of 20 µL. The ESI-MS/MS detection was performed under the following conditions: desolvation gas 550 Lts/hr, cone gas 30 Lts/hr, desolvation temperature 300°C, source temperature 110°C, capillary voltage 3000 V, cone voltage 30 V and collision energy 4ev (Guo et al., 2014).

3.9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

HPLC is a chromatographic practice to isolate, identify and quantify components in a crude extract or mixture of compounds. In the present study HPLC was carried out to identify rutin in the plant crude extract. The HPLC was performed using C18 column as stationary phase and acetic acid (5%) solution containing
methanol: acetonitrile: water (10:10:75) as the mobile phase. Total of 10 M extract solution (1 mg/mL) was taken as injection volume and flow rate of 1.0 mL/min was maintained. The presence of rutin in the extract was confirmed by comparing the retention time (RT) and UV spectrum with the standard RT and UV spectrum (Behnaz et al., 2013).

3.10 ANTI-PROMASTIGOTE ACTIVITY BY TRYPAN BLUE DYE EXCLUSION TEST

1. Stock solutions (10 mg/mL) of plant extract, salidroside, rutin and both reference drugs prepared separately in a vehicle mixture of 3:7 DMSO (dimethyl sulfoxide) and ethanol. The obtained solutions were filtered through 0.45 μm syringe filters.

2. Different concentrations (10, 20, 40, 60, 80, and 100 μg/mL in incomplete RPMI-1640) of each stock solution were made.

3. 2×10⁶/mL of the promastigotes were added in different wells of 24-well tissue culture plate. Further these promastigotes were incubated with different formulations. The reference drug treated wells served as positive control while DMSO as negative control.

4. The plate was incubated at 22±1°C. After 72 hours the numbers of live and dead promastigotes were counted using neubauer’s chamber under a light microscope after adding trypan blue stain.

5. The percentage growth inhibition was calculated as follows:

\[
\text{Percentage viability} = \frac{\text{No. of viable cells in treated well}}{\text{No. of viable cells in blank well}} \times 100
\]

Percentage growth inhibition = 100- percentage viability

IC₅₀ was calculated with the help of SPSS software (Singh et al., 2011).

3.11 CYTOTOXICITY ASSAY

3.11.1 Maintainence of cell line

The human monocytic cell line, THP-1 was obtained from PGI, Chandigarh and maintained in complete RPMI-1640 in humidified CO₂ incubator at 37°C and 5% CO₂. The cell line was maintained by periodically changing the media after every 3-4 days.
3.11.2 MTT Assay

The cytotoxic concentration (CC<sub>50</sub>) of the extract was determined on THP-1 leukemia cell line in 96-well plate. The THP-1 cells at about 95% viability were exposed to Phorbol 12-myristate 13-acetate (Sigma) for the differentiation of monocytes to macrophages and then this cell suspension was seeded at 3X10<sup>5</sup> cells/well in the plate and incubated for 24 hours at 37°C in 5% CO<sub>2</sub>. The conversion of cells was inspected under inverted microscope. Non-adherent cells were removed by discarding the medium and plate was then complemented with fresh complete RPMI-1640 (Daigeneault et al., 2010). The cytotoxicity of the HERERI, SAL, RTN, SSG and AmB was tested by supplementing the wells with 6 concentrations (20, 50, 100, 200, 500 and 1000 µg/mL) of each of them. The plate was incubated under the same conditions for 72 hours. Untreated macrophages were included as controls and the wells with only media were considered as blank. Cells viability was determined using the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Sigma). 10 µL of 1 mg/mL of MTT was added to each well and incubated at 37°C in 5% CO<sub>2</sub>. After 4 hours the formazan crystals were dissolved by adding 100 µL DMSO in each well. Optical density (OD) was measured at 550 nm using an ELISA reader. The percentage of cytotoxicity was calculated as \[ \frac{(A-B)}{(C-B)} \times 100 \], where A, B and C were the absorbances of treated, blank and untreated cells, respectively. The 50% cytotoxic concentration (CC<sub>50</sub>) was defined as the compound’s concentration (µg/mL) required for the reduction of cell viability by 50%, which was calculated by regression analysis (Mehta et al., 2011).

3.11.3 Selectivity Index (SI)

The degree of selectivity was expressed as SI and was obtained by calculating the ratio of CC<sub>50</sub> for macrophage/IC<sub>50</sub> for promastigotes.

3.12 PROMASTIGOTE CELL-CYCLE ANALYSIS

1. Cultured promastigotes were centrifuged at 3000 rpm for 5 min and then washed with PBS three times.
2. 2X10<sup>6</sup>/mL of parasite cells were suspended in 24-well culture plate and were incubated with IC<sub>50</sub> dose of HERERI, SAL, RTN and reference drugs (control wells).
3. After 72 hrs parasites from each well were taken and centrifuged. The cells were washed with PBS and resuspended in 500 µL of PBS.
4. The cells were fixed and permeabilized by adding 70% (v/v) ice cold ethanol drop by drop and incubated at 4°C for 1 h.
5. After 1 h cells were again centrifuged and washed with PBS.
6. The resultant pellet was incubated for 1 h in 500 μL DNase-free RNase (200 μg/mL).
7. Finally cells were stained with 50 μg/mL of propidium iodide (PI) and incubated in dark for 20 min.
8. The proportion of cells in each stage of cell cycle was assayed within 2 hrs by using a FACSCalibur flow cytometer.
9. BD FACSDiva 8.0.1 software was used for data analysis and then the percentage of cells in each phase was determined (Sarkar et al., 2008).

3.13 ETHICS

The ethical approval for conducting experiments on inbred BALB/c mice was acquired from the Institutional Animal Ethics Committee of Panjab University, Chandigarh, India (IAEC no. PU/IAEC/S/14/142). Highly susceptible inbred BALB/c mice of age group 4-6 weeks were maintained in the conventional housing in the Central Animal House of Panjab University, Chandigarh. They were provided food and water ad libitum.

3.14 PREPARATION OF DOSE FOR IN VIVO ADMINISTRATION

1. The plant extract, SAL and RTN were dissolved in the standard suspension vehicle which was formulated by mixing 5 g of carboxy methyl cellulose, 5 mL of benzyl alcohol, 4 mL of Tween 80 and 0.9% sodium chloride in 1000 mL of DW.
2. The two reference drugs, SSG and AmB were dissolved in water.

3.15 DETERMINATION OF LETHAL TOXIC DOSE

Oral lethal dosage was found using method of Lorke, 1983 by administering maximum dose of 5 g/kg body weight to a group involving 5 fasting mice. Acute toxicity was analysed by measuring its poisoning potential in different organs like liver, kidney and spleen, by investigating any negative behavioural changes and by observing any mortality upto 2 weeks.
3.16 **EXPERIMENTAL LEISHMANIASIS**

Promastigotes in the logarithmic growth phase were amassed from the culture and centrifuged at 3000 rpm for 10 minutes. The recovered pellet was washed three times with autoclaved PBS. Infection was given to animals by injecting $1 \times 10^7$ promastigotes intravenously (tail vein) in mice (Kaur and Kaur, 2018).

3.17 **GROUPS OF ANIMALS**

In the present study two sets of experiments were carried out. Each group comprised of 6 mice.

**3.17.1 Groups of first set of experiment:**

Group I: Normal control
Group II: Infected with SSG sensitive strain of parasites
Group III: Infected and treated with Standard suspension vehicle (SSV)
Group IV: Infected and treated with Sodium stibogluconate (SSG) (40 mg/kg b.wt.)
Group V: Infected and treated with Amphotericin B (AmB) (2.5 mg/kg b.wt.)
Group VI: Infected and treated with HERERI at the dose of 500 mg/kg b.wt.
Group VI: Infected and treated with HERERI at the dose of 1000 mg/kg b.wt.
Group VII: Infected and treated with 25 mg/kg b.wt. Salidroside (SAL)
Group VIII: Infected and treated with 25 mg/kg b.wt. Rutin (RTN)

**3.17.2 Groups of second set of experiment:**

Group I: Normal control
Group II: Infected with SSG resistant strain of parasite
Group III: Infected and treated with SSV
Group IV: Infected and treated with SSG (40 mg/kg b.wt.)
Group V: Infected and treated with AmB (2.5 mg/kg b.wt.)
Group VI: Infected and treated with HERERI at the dose of 500 mg/kg b.wt.
Group VI: Infected and treated with HERERI at the dose of 1000 mg/kg b.wt.
Group VII: Infected and treated with 25 mg/kg b.wt. SAL
Group VIII: Infected and treated with 25 mg/kg b.wt. RTN

3.18 **TREATMENT PLAN**

The infection was allowed to progress for 30 days, after which treatment was initiated. The treatment regimen included:
• Oral administration of HERERI (both doses), SAL and RTN once daily for 14 days.
• Intraperitoneal administration of reference drugs, SSG and AmB once daily for 5 days.

3.19 SACRIFICE OF ANIMALS

Mice were sacrificed on 7 and 14 post treatment days with anesthetic diethyl ether. Different organs viz. liver, spleen and kidney were obtained for different tests. Blood samples were also collected and sera was separated and stored at -4°C until their use in immunoassays.

3.20 ASSESSMENT OF PARASITE LOAD

3.20.1 By Light microscopy

Visceral infection was monitored in liver and spleen. Both these organs were washed with PBS, weighed and their imprints were formed on clean slides. The slides were dried, fixed in methanol and then stained in classic Giemsa blood film stain. The parasite load was measured by counting the amastigotes per 100 cell nuclei and multiplying it with respective organ weight. Thus parasite burden was computed in terms of Leishman Donovan Units (LDU) (Bradley and Kirkley, 1977):

\[
LDU = \frac{\text{No. of amastigotes}}{\text{No. of cell nuclei}} \times \text{Weight of organ (in mg)}
\]

3.20.2 By Real time-quantitative Polymerase Chain Reaction (RT-qPCR):

3.20.2.1 DNA extraction by phenol–chloroform method (Sambrook et al., 1989):

Reagents preparation

- **Tris-EDTA Buffer**: 1.214 g of tris-chloride was dissolved in 10 mL DW constituting 1 M solution. Similarly 0.5 M of EDTA was prepared by adding 1.861 g of EDTA in 10 mL DW. pH of each solution was set to 8 using HCl and NaOH respectively. Both the reagents were autoclaved at 15 p.s.i for 20 min. Finally the working solution was formulated by mixing 1 mL of tris and 0.2 mL of EDTA in 98.8 mL DW.

- **Sodium dodecylsulphate (SDS)**: 5% of SDS was made by dissolving 0.5 g of it in 10 mL DW.
Materials and Methods

- **Proteinase K**: 20 mg of Proteinase K was dissolved in 1 mL DDW.
- **Lysis buffer**: 50 μL Tris, 10 μL EDTA and 100 μL NaCl in 4840 μL of DW.
- **Phenol:Chloroform:Isoamyl alcohol**: The mixture was prepared by adding the reagents in the ratio (25:24:1).
- **Alcohol**: 70% of Absolute alcohol was made.
- **Isopropanol**

Procedure for DNA Extraction:

1. 25 mg of spleen tissue was coalesced in 2 mL micro-centrifuge tube containing 500 μL of Tris-EDTA buffer (TE).
2. The amalgamated matter obtained was centrifuged in a cooling centrifuge at 4°C at 10,000 rpm for the short period of 10 minutes.
3. After discarding the supernatant the pellet was dissolved in 400 μL of lysis buffer and 100 μL of SDS ensued by 6 μL of proteinase K for digesting proteins and other components of cells except nucleic acids. The mixture was incubated at 65°C for about one and a half hour.
4. After incubation 120 μL of phenol:chloroform:isoamyl alcohol mixture was added to the tubes and were vortexed for 30 seconds. This separates out the lipids, cell debris and DNA into different phases.
5. The solution was then centrifuged at 10,000 rpm at 4°C for 10 min to partition the aqueous layer of DNA.
6. Following centrifugation this upper aqueous layer of DNA was transferred to clean tube without disrupting the protein layer at the interphase.
7. To the aqueous layer 500 μL of isopropanol was added and it was stored at 4°C for night and then cool centrifuged at 7000 rpm for 10 minutes next day.
8. Precipitated DNA was washed with 500 μL of 70% ethanol and was centrifuged at 7000 rpm for only 5 minutes.
9. The pellet was dried till transparent and then redissolved in 30 μL TE.
10. The purity of DNA was checked at NanoDrop spectrophotometer and the DNA samples were stored at –20°C.
3.2.2.2 Agarose Gel Electrophoresis

The integrity of obtained DNA was monitored by this method.

1. To 0.2 g of agarose 500 µL of TAE buffer and 25 mL of DW were added to it.
2. The agarose was dissolved by heating and after adding 2 µL of ethidium bromide, it was poured in the gel tray and the combs were placed.
3. The solution was permitted to settle for 30 minutes.
4. The gel was placed to electrophoresis chamber after carefully removing the combs and then TAE buffer was added to it.
5. Mixture of 6 µL DNA and 2 µL bromophenol blue was loaded in the wells.
6. The current was passed at 100 V for 30 minutes.

3.2.2.3 Quantification of parasites by RT-qPCR

The SYBR Green based real-time PCR assay was performed to amplify a 229bp fragment of microsatellite gene in the spleen of different groups of BALB/c mice for quantifying the number of parasites. The sequences of the primers were as forward 5'-ACACGCAGAGAACTCGGTTT-3' and reverse 5'-TGGAGCGAGAAAGGACAAGT-3'. Real-time PCR System® was run at the following thermal profile: denaturation at 95°C for 5 min, next amplification and quantification program (94°C for 10 s, 58°C for 20 s, 72°C for 30 s and 72°C for 1 min) with 35 cycles, melting curve program (60-95°C and a continuous fluorescence measurement) and finally a cooling step at 40°C.

Each PCR run included test samples, negative control consisting DNA free water plus PCR mix and positive control of known concentration in triplicates. The cycle threshold (CT) which is a point at which the fluorescence of reaction rises markedly above the background fluorescence was noted for each reaction. The data was analysed using LightCycler software 3.3 (Roche Diagnostics).

3.2.2.4 Standard curve analysis

Standard curve was generated using DNA extracted from the promastigotes growing in vitro. 10-fold serial dilutions of DNA of promastigotes, corresponding to 1x10^10 parasites to 100 parasites per reaction were prepared and the average CT of each dilution was plotted against the different dilutions of the parasites (Hossain et
al., 2017; Margaroni et al., 2017). The specificity of qPCR reaction was confirmed by analyzing the melting curves.

3.21 ASSESSMENT OF DELAYED TYPE HYPERSENSITIVITY (DTH) RESPONSES

DTH reactivity was determined by skin test in the foot pad of mice. To perform leishmanin test the antigen was prepared as follows:

1. Promastigotes were pooled from the growing culture after 3-4 days and washed three times with sterile PBS.
2. The obtained pellet of parasite was killed with 0.5% (wt/v) phenol-PBS.
3. The killed parasite was again washed with PBS three times to remove the phenol and the final concentration of parasite was set to 2x10^8/mL.

BALB/c mice were inoculated intradermally with 40 µL of antigen in the right hind foot pad. The left foot was inoculated with the same amount of PBS. After 48 hrs the thickness of both feet was measured via vernier callipers. The DTH response was calculated by finding the difference of thickness of right and left hind foot pad (Banerjee et al., 2008; Chouhan et al., 2015).

3.22 QUANTIFICATION OF Th1/Th2 CYTOKINES

The levels of different key cytokines (IL-12, IL-4, IL-10, TNF-α and IFN-γ) important during VL were assessed in the sera of different groups of animals by using commercially available Diaclone cytokine ELISA detection kits based on the biotin-avidin system. The manufacturers’ instructions were followed and the results were expressed in pg/mL. Absorbance was measured with ELISA reader and the concentration of the cytokines was computed against the standard curve (Kaur and Kaur, 2018).

3.23 IMMUNOPHENOTYPING OF LYMPHOCYTES BY FLOW CYTOMETRY

The flow cytometric assay was performed to quantify the percentage of total T cells (CD3+), T helper cells (CD4+, CD8-), T cytotoxic cells (CD8+), and total B cells (CD19+) in different groups of mice. Antimouse-CD4-fluorescein isothiocyanate (FITC), antimouse-CD8-allophycocyanin (APC), antimouse-CD3-phycocerythrin (PE) and antimouse-CD-19-(APC) antibodies (BD Biosciences) were used. Required dot plots were constructed and quadrants were applied to them. Quadrant statistics
were generated and used to deduce the percentage of each population. The cells were prepared as follows:

1. The animals of different groups were sacrificed and spleen was removed and washed with the PBS.
2. The spleen was crushed in cell strainer and the cell suspension was collected.
3. The cell suspension was centrifuged at 3000 rpm for 5 minutes.
4. The pellet obtained was washed three times with the PBS.
5. Then NH₄Cl (1% in distilled water) was added to the pellet to remove the RBCs. The cells were incubated for 15-20 minutes and during incubation intermittent shaking was performed.
6. The cells were then centrifuged at 3000 rpm for 5 minutes.
7. The white pellet of cells was obtained which was then washed with the PBS twice.
8. The cells were finally suspended in the PBS and counted in the neubaer’s chamber.
9. About 1X10⁶ cells were added to the falcon tubes. For each group 3 tubes were prepared. The first tube was used as unstained sample, to the second tube 1 μL of the APC conjugated CD19 antibody was added and to the third tube PE conjugated CD3⁺, FITC conjugated CD4⁺ and APC conjugated CD8⁺ were added.
10. The tubes were incubated at 4°C for 20 minutes.
11. 200 µL PBS was added to each tube and it was centrifuged at 3000 rpm for 5 minutes.
12. The supernatant was discarded and 200 µL PBS was added to each tube and the tubes were analyzed in the FACS (Pathak and Khandelwal, 2009).

3.24 RNA ISOLATION AND TRANSCRIPTIONAL ANALYSES BY QUANTITATIVE PCR

3.24.1 RNA isolation by TRIzol or TRI reagent

TRIzol reagent solubilizes biological cell substances and denatures the protein as well. It separates out organic protein at the bottom, DNA at the middle and RNA at the upper layer (Rio et al., 2010).

1. Spleen was removed after sacrifice of mice of different groups of animals.
2. It was placed on the cavity block on ice and then severed and homogenized in about 1 mL of TRIzol (Sigma).

3. The homogenized tissue was transferred to micro-centrifuge tubes after 10 min.

4. To the same 200 µL of chloroform was added and the solution was vortexed briefly.

5. After 10 min it was centrifuged at 13,000 rpm for 10 min at 4°C.

6. It resulted in the segregation of solution into three layers with upper layer containing RNA.

7. The upper layer was carefully isolated into fresh micro-centrifuge tube.

8. To this 500 µL of 2-propanol was added.

9. After 10 min of incubation, tube was again centrifuged at 13,000 rpm for 10 min at 4°C.

10. The pellet was collected and washed with ethanol (80%) by pouring 400 µL of ethanol followed by centrifugation at 13,000 rpm for 10 min at 4°C.

11. Ethanol was discarded and the pellet was air dried.

12. At the end pellet was dissolved in 30 µL of DPEC water.

### 3.24.2 Quantification and Quality check

Quantity and quality was analysed using the NanoDrop spectrophotometer (Thermo Scientific). Samples with 260/280 ratio of 2 or close to 2 were selected for further cDNA conversion step.

### 3.24.3 Reverse Transcription (cDNA Synthesis)

First strand complementary DNA (cDNA) was formed from the RNA via reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) and was stored at -20°C until use. Gene expression of iNOS and NF-κβ was measured in different groups of animals using β-actin housekeeping gene as a reference gene. Real-time PCR System® was run at the following thermal profile: denaturation at 95°C for 5 min, next amplification and quantification program (94°C for 10 s, amplification temp. for 20 s, 72°C for 30 s and 72°C for 1 min) with 35 cycles, melting curve program (60-95°C and a continuous fluorescence measurement) and finally a cooling step at 40°C.
The quantification of the target genes expression was calculated using $2^{-\Delta\Delta CT}$ method. The final result was presented as the fold change of target gene expression relative to uninfected sample, normalized to a reference gene. The relative gene expression was set to 1 for the uninfected samples because $CT$ is equal to 0 and $2^0$ is equal to 1 (Livak and Schmittgen, 2001).

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence and PCR programme</th>
<th>Cycles</th>
</tr>
</thead>
</table>
| β-actin | Forward primer: 5'-GCATTGCTGACAGGATGCAG-3'  
Reverse primer: 5'-CCTGCTTGCTGATCCACATC-3' | Amplification  
95°C 5 minutes (initial denaturation)  
94°C 10 seconds  
57°C 20 seconds  
72°C 30 seconds  
63°C 1 minute (melting temperature)  
40°C 10 seconds (Cooling) | 35 |
| NF-κB | Forward primer: 5'-CTGGTGAGCACATACAGGAAGAC-3'  
Reverse primer: 5'-ATAGGCACTGTCTTTCACCTC-3' | Amplification  
95°C 5 minutes (initial denaturation)  
94°C 10 seconds  
57°C 20 seconds  
72°C 30 seconds  
63°C 1 minute (melting temperature)  
40°C 10 seconds (Cooling) | 35 |
| iNOS | Forward primer: 5'-GATCCGATTTAGTAGCTGCTTGGTG-3'  
Reverse primer: 5'-TCCCTTGCTAGTGCTTCAG-3' | Amplification  
95°C 5 minutes (initial denaturation)  
94°C 10 seconds  
60°C 20 seconds  
72°C 30 seconds  
75°C 1 minute (melting temperature)  
40°C 10 seconds (Cooling) | 35 |

3.25 QUANTIFICATION OF NITRIC OXIDE (NO)

To detect intracellular NO production in spleen, Griess assay was used.

1. The spleen was homogenized in 0.1 M phosphate buffer and then cool centrifuged at 10,000 rpm for 15 minutes.
2. A total of 50 μL of the supernatant was collected and incubated with the same volume of Griess reagents (sulfanilamide (1%) and naphthylethylenediamine dihydrochloride (0.1%) in 5% orthophosphoric acid).

3. Following 10 min of incubation at room temperature total nitrite was measured at 550 nm on microplate reader.

4. The standard curve was prepared with different dilutions (1.56-100 μM) of sodium nitrite (Bhardwaj and Kumar, 2016; Paik et al., 2016).

3.26 MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species were quantified in the spleen by 2'7'-dichlorodihydrofluorescein diacetate (H2DCFDA). This dye gets oxidized in the presence of ROS and becomes fluorescent green.

1. Fresh stock solution of carboxy-H2DCFDA (Sigma.) was prepared in the sterile DMSO.

2. The splenocytes were washed with phosphate-buffered saline (PBS) and 1X10⁶ cells were incubated with the dye (10 μM) for 30 min in the dark in incubator at 37°C and 5% CO₂.

3. The extra dye was removed by washing the cells twice with PBS.

4. Cells were added to the 96 well plate and ROS was assessed in the cells by fluorescence plate reader at the excitation of 485 nm and emission wavelength of 535 nm.

5. The fold change of ROS was calculated as compared to normal control (Anand et al., 2015).

3.27 BIOCHEMICAL INVESTIGATIONS

The liver and kidney function tests were carried out to survey the toxicological retort of HERERI, SAL, RTN and reference drugs in the unhaemolysed serum samples of mice by using commercially available biochemical reagent kits. The kits were based on kinetic method endorsed by International Federation of Clinical Chemistry (IFCC) (Howell et al., 2014; Sharma et al., 2012).

3.27.1 Liver Function Tests

The levels of enzymes like SGOT (Serum Glutamate Oxaloacetate Transaminase), SGPT (Serum Glutamate Pyruvate Transaminase), LDH (Lactate Dehydrogenase), ACP (Acid Phosphatase) and ALP (Alkaline Phosphatase) were used as a suggestive measure to inspect any kind of liver related disorders.
3.2.7.1 Estimation of Serum Glutamate Pyruvate Transaminase (SGPT)

SGPT is found in high concentration in the liver so the estimation of its levels in the serum is an indication of liver related disease. The levels of enzyme, SGPT were studied by modified IFCC method as per manufacturer’s (Fischer Scientific) guidelines and the results were expressed in U/L (Units/Litre).

3.2.7.1.2 Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT)

Similarly SGOT was estimated by modified IFCC method as per manufacturer’s (Fischer Scientific) guidelines and the results were expressed in U/L (Units/Litre).

3.2.7.1.3 Estimation of Lactate Dehydrogenase (LDH)

LDH estimation was performed by UV-Kinetic method as per manufacturer’s (Reckon Diagnostics) instructions. Results were expressed in IU/L.

3.2.7.1.4 Estimation of Acid Phosphatase (ACP)

ACP estimation was done by Kinetic method as per manufacturer’s (Aspen Company) instructions. Results were expressed in IU/L.

3.2.7.1.5 Estimation of Alkaline Phosphatase (ALP)

ALP estimation was done by Kinetic method as per manufacturer’s (ERBA Diagnostics) instructions. Results were expressed in IU/L.

3.2.7.2 Kidney Function Tests

To outline the toxicity status, the testing of renal functions alongwith liver is an essential endeavour. To retrieve the functional repute of kidney the quantitative estimation of urea, blood urea nitrogen (BUN), creatinine and uric acid in the serum was performed.

3.2.7.2.1 Estimation of Blood Urea and Blood Urea Nitrogen

Blood Urea and BUN Estimation was done by the Modified Berthelot method as per manufacturer's (Reckon diagnostics Pvt. Ltd., Baroda) instructions. Results were expressed in mg/dL (deciliter) of plasma.

3.2.7.2.2 Estimation of Blood Creatinine

Creatinine, a waste product formed in muscles is excreted out by kidneys so the levels of creatinine in the serum were studied as a test to assess the renal function. The alkaline picrate method was performed following the protocol mentioned in the
diagnostic kit (Reckon diagnostics Pvt. Ltd., Vadodara) manual and results were computed in mg/dL of plasma.

3.27.2.3 Estimation of Blood Uric acid

The levels of Uric acid have diagnostic value in terms of kidney function. It was estimated by Uricase method as per the instructions of manufacturer (Reckon diagnostics Pvt. Ltd., Vadodara) and the results were expressed in mg/dL.

3.28 HISTOLOGY OF KIDNEY, LIVER AND SPLEEN BY HEMATOXYLIN AND EOSIN (H&E) STAINING

3.28.1 Processing of tissues

To further analyse the toxic effects of HERERI, SAL, RTN and reference drugs on liver, kidney and spleen the anatomy of these visceral organs was also studied by microtomy.

1. Different organs from all groups of animals were cut and fixed in Bouin's fixative (Pearse, 1968).
2. After 24 hrs bouin’s solution was removed from the tissues by washing in running water overnight.
3. The water was discarded and tissues were dehydrated using different grades of ethanol in increasing order of concentration. The tissues were placed in order of 30% for 1 h, 50% for 1 h and then again in 70% for 1 h.
4. The 70% alcohol was replaced by fresh 70% alcohol and the tissues were left in it overnight.
5. A third change of 70% alcohol was given again and the tissues were left as such for overnight.
6. The dehydration was commenced further by keeping tissues in 90% alcohol.
7. After 1 h 90% alcohol was replaced by absolute alcohol. After every 30 min, three changes of 100% alcohol were given to completely assure the removal of water.
8. To clear out the alcohol from the tissues, a clearing agent i.e. benzene was used in different formulations in steps. First it was used in 1:3 ratio with absolute alcohol for 30 min, then 2:1 ratio for 30 min, 1:1 for 30 min, 1:2 for 30 min, 1:3 for 30 min and lastly in pure benzene again for 30 min.
9. Then the change was given with fresh benzene for 30 min.
10. The tissues were placed in the mixture of benzene: molten paraffin wax (1:1) for 1 h.
11. After 1 h tissues were saturated with wax and kept at 60°C overnight.
12. To provide support to the tissues, the tissues were embedded in the blocks.
13. Wax blocks were trimmed and shaped.
14. 5-7 µ thick sections were cut with microtome equipped with clean blade.

### 3.28.2 Staining Method

1. The slides were cleaned and albumin was applied on them. Section ribbons were stretched in hot water on these slides.
2. The sections were dewaxed by giving two changes of xylene.
3. The downgrading of the sections was performed using different grades of ethanol in decreasing order, each for 3 minutes as 100%, 90%, 70%, 50%, 30% and water.
4. Then the sections were stained in haematoxylin for 15-20 minutes.
5. It was kept under running tap water for 15 minutes till the sections turned pink.
6. Then the tissues were differentiated in acid water and ammonia water (one or two dips in each).
7. They were then upgraded up to 90% alcohol by dehydrating the slides - 30% (4 min), 50% (4 min), 70% (4 min), 90% (4 min).
8. Then the tissues were stained with eosin (1 min or 30 sec), 90% (10 min), and 100% (10 min), xylene (10 min).
9. The sections were mounted in DPX and observed under light microscope using different magnification.

### 3.29 STATISTICAL ANALYSIS

Experiments were performed in triplicate. Statistical analysis was performed using one-way ANOVA tukey test. The results were expressed as mean±SEM and the differences were considered statistically significant at \( p < 0.05 \).