

Original Research Article **IN VITRO AND IN VIVO EVALUATION OF ANTILEISHMANIAL ACTIVITY OF PHYLLANTHUS NIRURI**

ABSTRACT

Aim: The current study was aimed at evaluating the *in vitro* and *in vivo* leishmanicidal activity of *Phyllanthus niruri* against *Leishmania donovani* and compare the same with sodium stibogluconate.

Methodology: The present study was designed to assess *vitro* antileishmanial activity was by trypan blue dye exclusion test. Whereas, the parasite load in liver smears was calculated in Leishman Donovan units. In addition, various immunological and biochemical changes induced by it in uninfected and *Leishmania donovani* infected BALB/c mice were investigated.

Results: The ethanolic extract of this plant showed *in vitro* inhibitory activity with IC₅₀-59 µg/ml against the promastigotes of *L. donovani* stain MHOM/IN/80/Dd8. For *in vivo* studies, BALB/c mice were infected with the promastigotes of *L. donovani* and after 30 days they were treated with two oral doses of the ethanolic extract of *P. niruri* (100 and 200 mg/kg body weight) for seven days. Animals were sacrificed on 1 and 15 post treatment days. The ethanolic extract of *P. niruri* reduced the parasite load to a significant level and generated a protective immune response which was evident from the enhanced DTH responses, elevated levels of IgG2a and lower levels of IgG1. Moreover, the results of liver and kidney function tests indicated that the extract of *P. niruri* has no toxic effect on liver and kidneys.

Conclusion: Therefore, antileishmanial efficacy of this herbal drug was established and further it could be tested alone or in combination with other herbal formulations in higher models for control of leishmaniasis.

Keywords: *Phyllanthus niruri*, Visceral leishmaniasis, Ethanolic extract, Treatment

1. INTRODUCTION

Visceral leishmaniasis (VL) is the second largest parasitic disease caused by the flagellated protozoan parasite *Leishmania donovani* after malaria that affects about 350 million people. In 95 percent of VL cases, death can be avoided by proper and timely drug treatment [1]; however, current treatment options are not satisfactory. Intense efforts are being made to exterminate visceral leishmaniasis by 2015 in India, Nepal and Bangladesh where 60 percent of the total cases occur [2]. The best available drugs for visceral leishmaniasis treatment are liposomal amphotericin B and miltefosine. Obviously, both drugs are far superior to the older drugs, but with some constraints. The major flaws of miltefosine are its teratogenicity, treatment for a longer time and strong potential for developing unresponsiveness [3]. The major drawbacks related with amphotericin B are its high cost, parenteral administration and resistance in some patients in Sudan [4].

Considering the current situation, it is important to develop and introduce new drugs for treatment of leishmaniasis patients. New drugs that are less toxic, easily available, cost effective and within the reach of poor people which are most afflicted by the disease should be discovered. Plant derived compounds can be considered as paramount source for exploring better leishmanicidal compounds, as these are easily available and inexpensive [5]. Only a few species out of total 250,000 plant species of world show antiparasitic properties [6]. However, a lot still remains to be discovered to identify plants with potent phytoconstituents which can be used as sources of antileishmanial agents. A programme has been initiated by Drugs for Neglected Diseases Initiative (DNDi) for evaluating natural products. A diverse range of natural compounds are examined which show antileishmanial activities which include phenolics, alkaloids, luteolin, quassin, artemisinin and berberine chloride [7-11]. The plants having immunomodulatory, antioxidant and free radical scavenging properties have been found to have promising leishmanicidal potential. According to WHO estimates, more than 80% of the world's population uses plant products [12] for their health care purposes. Some plants having antileishmanial properties include *Adenia lobata*, *Citrus macroptera*, *Curcuma longa*, *Piper auritum* [13].

Phyllanthus niruri (family: Euphorbiaceae) is a native plant of India and occurs as winter weed throughout the warmer parts. It has been used in traditional medicine for treatment of jaundice, asthma, hepatitis and malaria [14,15]. This herb has been reported to possess antioxidant,

hepatoprotective, antibacterial and anti-inflammatory activity [16-19]. Its extract can heal liver injuries caused by oxidative stress [20]. The flavonoid contents of the plant are suggested to be responsible for its antioxidant properties [21] and are also capable of scavenging free radicals [22,23]. In red blood cells, polyphenols enhance cell resistance to oxidative insult [24]. A significant difference of antioxidant potential among the three *Phyllanthus* species ($p < 0.05$) has also been observed for both water and methanol crude extract. Among the three tested species, *P. niruri* scored the highest antioxidant potential activity followed by *P. urinaria* and *P. amarus* [25]. Many active bioconstituents of *P. niruri* constitute potential qualities in its curative action. Hence this plant may be exploited for developing various medicines and drugs [26]. In view of the foresaid background, the present study was planned to evaluate the leishmanicidal potential of *P. niruri* in the *in vitro* and *in vivo* system.

2. MATERIALS AND METHODS

2.1 Parasite

The strain of parasite used for the present study was MHOM/IN/80/Dd8 originally obtained from London School of Tropical Hygiene and Medicine, U.K. The promastigotes of these strains were maintained in modified NNN medium with MEM as overlay and RPMI-1640 medium with 10% FCS [27].

2.2 Plant material

Phyllanthus niruri (voucher number: 8343) as whole plant was collected from botanical garden of Panjab University, Chandigarh, India. The identification was confirmed from the Department of Botany, Panjab University, Chandigarh, India. Whole plants of *P. niruri* were washed thoroughly with water and dried at room temperature and then powdered.

2.3 Preparation of the extracts

Dried and powdered whole plant of *P. niruri* was found to be soluble in ethanol. Thus ethanolic extract was prepared by Soxhlet extraction method. Approximately 250 ml of ethanol was added to 100g dried and powdered plant specimens in a glass flask. The solvent extracts were filtered and then concentrated under vacuum in a rotary evaporator. The residues obtained were then lyophilized and stored at -4°C till further use.

2.4 Preparation of the standard drug

The Sodium stibogluconate (SSG) was purchased from Sigma Aldrich Co., USA and required concentration of 40 mg/kg b.wt was prepared by dissolving it in distilled water at 72°C in water bath.

2.5 Phytochemical screening

The phytochemical screening was carried out using crude ethanolic extract to identify various constituents using standard protocols [28,29]. Qualitative tests were performed to detect alkaloids, saponins, phenols, anthraquinones, steroids, diterpenes, triterpenes, flavonoids, cardiac glycosides, tannins and phytosterols.

2.6 Acute Toxicity Test

The LD_{50} of the extract was determined by using the limit test of Lorke [30]. A concentration of 5g/kg body weight of extract was administered by oral route to five animals after a fasting of four hours. This dose is considered as the highest concentration to be given in rodents for evaluating acute toxicity of any drug. These animals were monitored for fourteen days for mortality and general behaviour such as feeding, hair erection and other symptoms of toxicity manifestations.

2.7 In vitro antileishmanial activity

For *in vitro* testing, *L. donovani* promastigotes at a concentration of $2 \times 10^6/\text{ml}$ in RPMI-1640 complete media with 10% FCS were dispensed into 24 well tissue culture plates. The stock solutions of extracts were prepared by dissolving known quantity (10 mg/ml) of extract in 3% DMSO. The stock solution was further diluted to make various concentrations (10, 20, 40, 60, 80 and 100 $\mu\text{g}/\text{ml}$) of extracts. Then the cultures were supplemented with plant extracts. The negative control cultures were supplemented with 3% DMSO. In addition, positive control cultures were incubated with different concentrations i.e. 10, 20, 40, 60, 80 and 100 $\mu\text{g}/\text{ml}$ of SSG. Cultures were further incubated at $22 \pm 1^{\circ}\text{C}$ and cell number was counted after 24 hours by trypan blue dye exclusion test using Neubauer Chamber after trypan blue staining.

The percentage growth inhibition was calculated as follows:

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

$$\text{Percentage growth inhibition} = 100 - \text{percentage viability}$$

IC_{50} was obtained by plotting linear dose response curve of extracts (10-100 $\mu\text{g}/\text{ml}$) used [31].

2.8 In vivo antileishmanial activity of plant extract

2.8.1 Inbred BALB/c mice

Mice in age group of 5-6 weeks (20-25 gm) were purchased from the Institute of Microbial Technology and maintained at Central Animal House of Panjab University, Chandigarh.

2.8.2 Ethical clearance

The permission for conducting experiments was obtained from Institutional Animal Ethics Committee (Approval no. IAEC/284-295/3/9/2015) and experiments were conducted in experimental wing of Central Animal House, Panjab University, Chandigarh.

2.8.3 Infection and treatment of animals

The animals were infected with 1×10^7 promastigotes of *L. donovani* intracardially [32]. Mice were treated with two different doses 100 mg/kg and 200 mg/kg b.wt. of *P. niruri* prepared by dissolving in standard suspension vehicle (SSV) which was prepared by mixing carboxy methyl cellulose (5g), benzyl alcohol (5ml), Tween-80 (4ml) in 0.9% aqueous sodium chloride (1000ml) after 30 post infection days (p.i.d.). One group of mice was injected intraperitoneally with sodium stibogluconate (SSG 40 mg/kg b.wt.) for 5 days [33]. Mice receiving PBS only served as normal controls. Infected untreated mice were considered as infected controls, and the treated controls consisted of uninfected mice receiving either of the two doses (100 or 200 mg/kg body weight) of the drug.

All animals were divided into seven groups. Each group contained 12 mice and six mice in each group were sacrificed on different p.i.d/p.t.d.

2.8.4 Assessment of Infection: Six mice from each group were sacrificed after a week of treatment. The drug treated animals were sacrificed on 1 and 15 p.t.d. Impression smears of liver were made on clean, grease free slides, fixed in methanol and stained with giemsa. The parasite load was calculated in the form of Leishman Donovan Units [34].

2.8.5 Delayed Type Hypersensitivity (DTH) Responses: The mice were given a subcutaneous injection of leishmanin in the right foot pad and PBS (control) in the left foot pad. The leishmanin was prepared by harvesting promastigotes from NNN medium. The parasites were washed three times with PBS (phosphate buffered saline) and pellet was dissolved in 0.5% phenol. This was left at room temperature for 10 mins. The pellet was washed with sterile PBS and the number of parasites was adjusted to a concentration of 2×10^8 per ml. The left control footpad was injected with PBS. After 48 hr, the thickness of both foot pads was measured using vernier callipers and the percentage increase in the thickness of leishmanin injected and control foot pad was calculated [35].

2.8.6 Humoral Immune Response: The levels of IgG1 and IgG2a antibodies were measured by indirect ELISA in serum samples of different groups of animals [33].

2.8.7 Evaluation of biochemical parameters

2.8.7.1 Liver Function Tests

Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT) and bilirubin were tested by using commercially available kits (Reckon Diagnostics Pvt. Ltd. Vadodra, India).

2.8.7.2 Kidney Function Tests

Urea and creatinine was estimated by using commercially available kits (Reckon Diagnostics Pvt. Ltd. Vadodra, India).

2.9 Statistical analysis

The student's t-test was performed to calculate p-value by employing Graph pad software.

3. RESULTS

3.1 Phytochemical Screening of plant extracts

The phytochemical constituents present in the whole plant extract of *P. niruri* were alkaloids, flavonoids, saponins, terpenoids and steroids.

3.2 Acute Toxicity

The LD₅₀ of *P. niruri* was found to be more than 5g/kg body weight in normal mice, which proves the clinical safety of extract. Histopathological examination of liver, spleen and kidney did not reveal significant pathological changes. No hepatomegaly/ splenomegaly was observed. Size and colour of organs was also comparable to normal tissues. The concentration of 5g/kg in mouse is prescribed upper limit for administration of test material in one dose in rodents according to limit test of Lorke.

3.3 In vitro efficacy of herbal extracts against *L. donovani* promastigotes

In vitro antileishmanial efficacy of the ethanolic extract showed significant inhibitory activity against the promastigotes of *L. donovani* and IC_{50} was found to be 59 μ g/ml (Fig. 1).

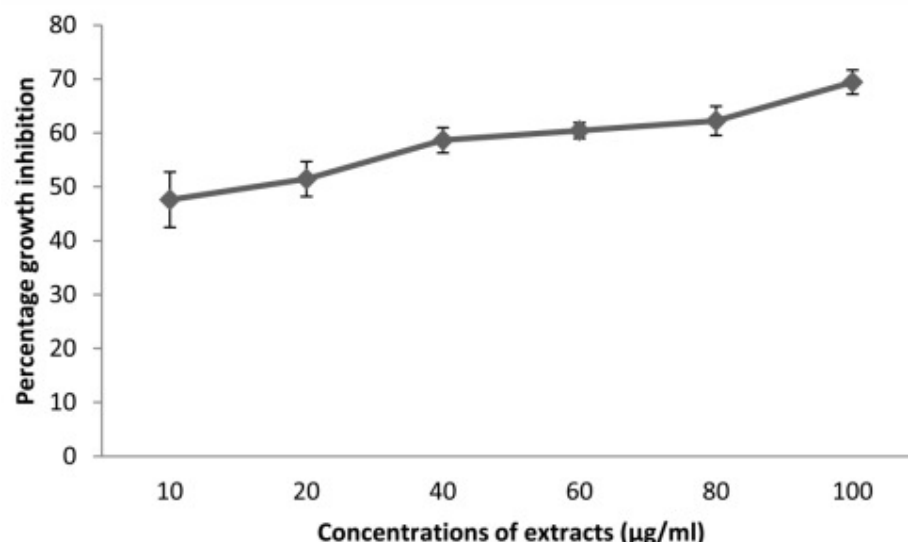


Fig 1: Percentage growth inhibition of plant extract.

Results are expressed as Mean $\pm$ SD.

3.4.1 Hepatic parasite load

The parasite load was significantly reduced ($p<0.05$) in animals of *P. niruri* treated groups when compared with infected animals. The oral treatment of *P. niruri* at a dosage of 200 mg/kg b.wt. for seven days exhibited 93% reduction in parasite burden as compared to those treated with 100 mg/kg b.wt. in which 84% decline in parasite load was observed. The percent decrease in parasite load was found to be in the range of 64–93% in animals treated with *P. niruri*. Similarly, the subcutaneous treatment of SSG at a dose of 40 mg/kg b.wt. significantly declined ($p<0.05$) the parasite load of liver in the range of 77-89% on 1 and 15 p.t.d. (Fig. 2).

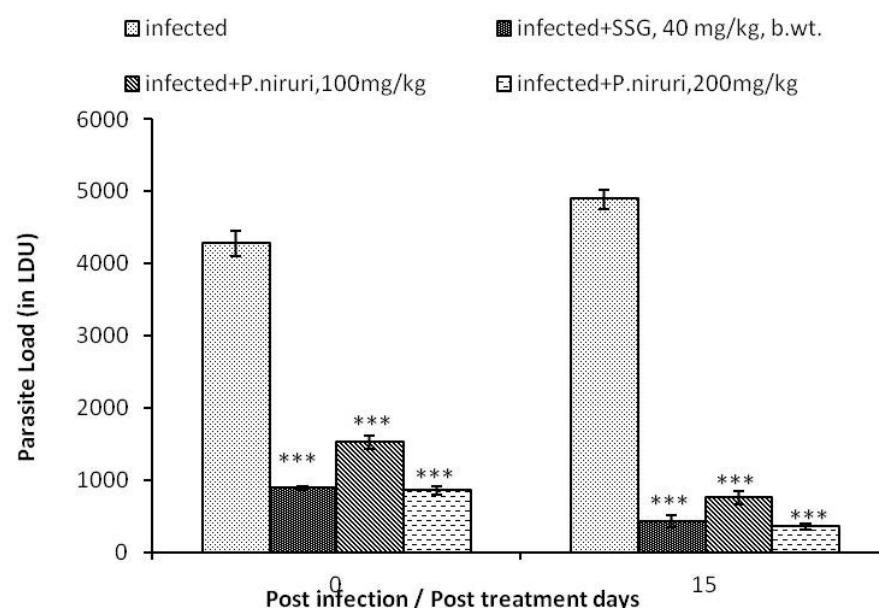


Fig 2: Parasite Load in various groups of animals.

Results are expressed as Mean $\pm$ SD of 6 animals.

Test drugs significant from infected control *** $p<0.001$.

3.4.2 Delayed type hypersensitivity response

Delayed type hypersensitivity response is directly correlated with the generation of cell-mediated immune responses. The treatment of infected animals with 200 mg/kg b.wt. of *P. niruri* showed significant increase ($p<0.05$) in footpad thickness as compared to those treated with 100 mg/kg b.wt. of plant extract. In addition, the DTH responses were found to be enhanced with increase in post treatment days in comparison to infected controls. The increase in footpad thickness in treated animals was comparable to SSG treated animals (Fig. 3).

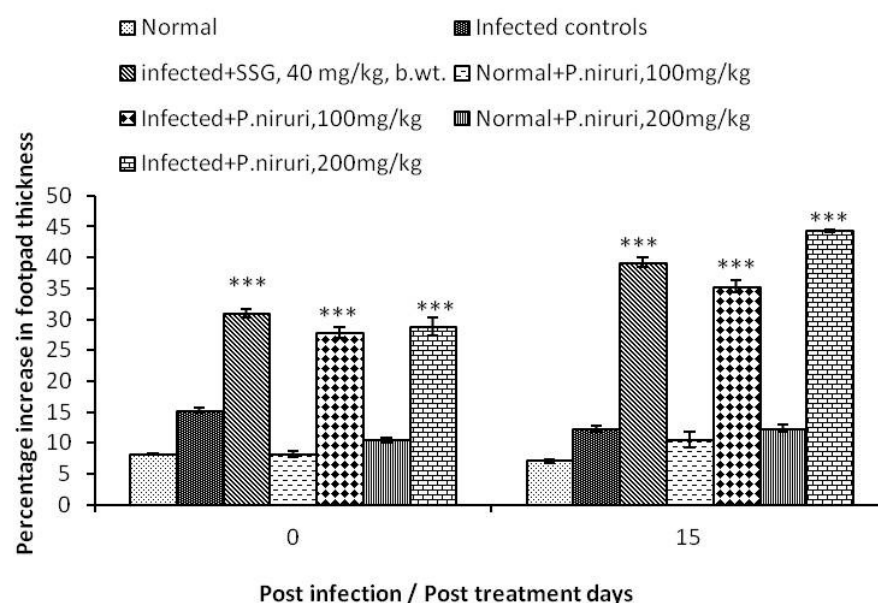


Fig 3: DTH responses in various groups of animals.

Results are expressed as Mean $\pm$ SD of 6 animals.
Test drugs significant from infected control *** $p<0.001$.

3.4.3 Humoral immune Responses

IgG1 and IgG2a antibodies play a crucial role in the generation of Th2 and Th1 type immune responses. The infected animals exhibited higher levels of IgG1 as compared to the treated animals. When compared with infected animals, a significantly reduced ($p<0.05$) levels of IgG1 were observed in infected mice treated with *P. niruri* (200 mg/kg b.wt. and 100 mg/kg b.wt.). Moreover, the IgG1 levels were found to be higher in mice treated with 200 mg/kg b.wt. of *P. niruri* as compared to those treated with 100 mg/kg b.wt. of the plant extract. The decline in IgG1 titre in treated groups was comparable to SSG treated animals (Fig. 4A).

Both the doses of *P. niruri* (200 mg/kg b.wt. and 100 mg/kg b.wt.) significantly enhanced ($p<0.05$) the IgG2a antibody levels on 1 and 15 p.t.d. as compared to infected animals. However, the higher dose has more pronounced effect on infected animals than the lower dose. The levels of IgG2a antibody in SSG treated animals were comparable to *P. niruri* treated animals (Fig. 4B).

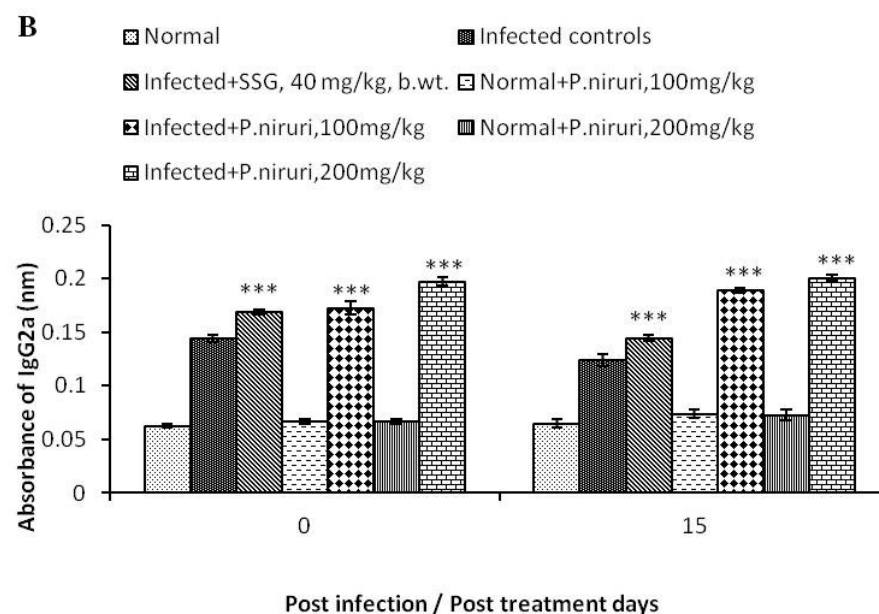
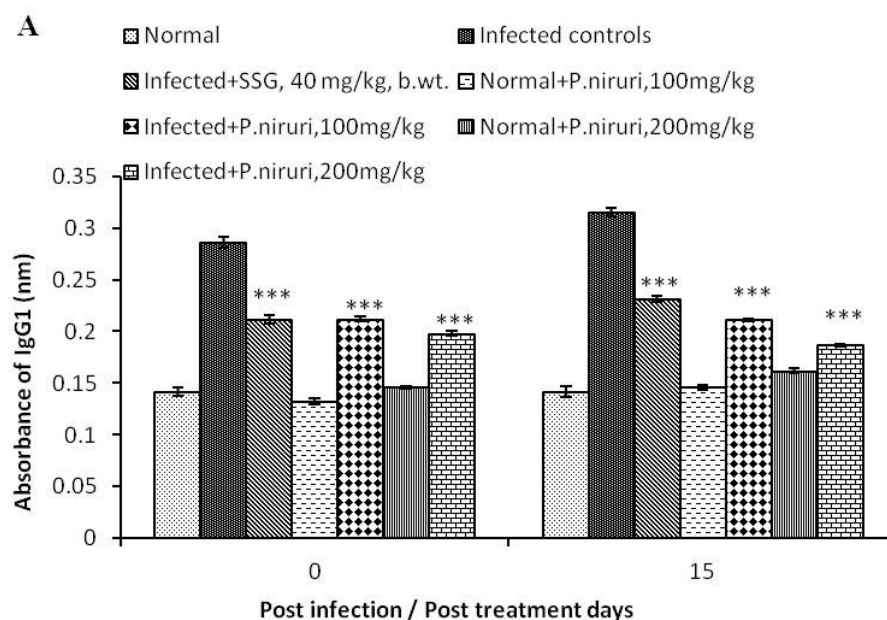


Fig 4A,B: IgG1 and IgG2a antibodies production in various groups of animals.

Results are expressed as Mean±SD of 6 animals.

Test drugs significant from infected control ***p<0.001

3.4.4 Liver function tests

SGOT and SGPT enzymes levels were significantly increased ($p<0.05$) and above the normal range in infected controls as compared to normal controls. However, after treatment of animals with both doses of plant extract, the enzyme activity was restored to their normal range on both post treatment days. The concentrations of these enzymes in infected animals treated with SSG were slightly higher (above the normal range) than normal controls but they were significantly lower ($p<0.05$) than infected controls (Fig.5A,B).

Serum bilirubin levels were found to be within the normal range (0-1 mg/dl) in all the groups of animals (Fig.5C).

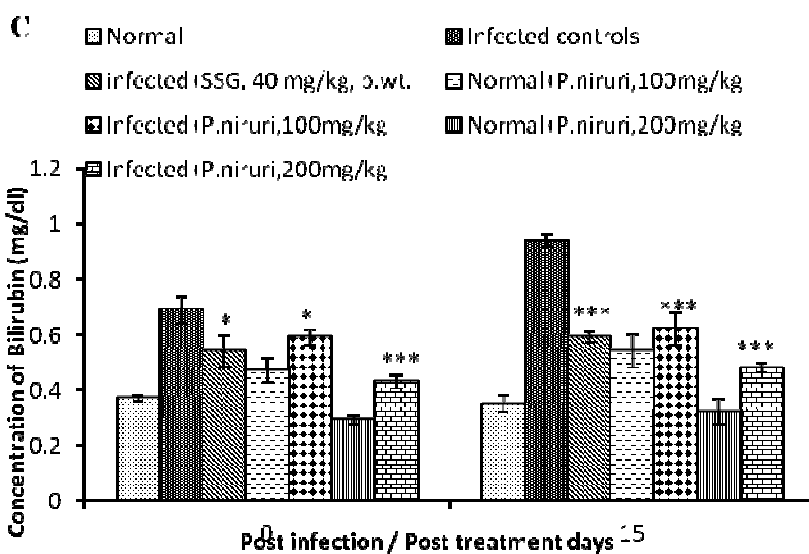
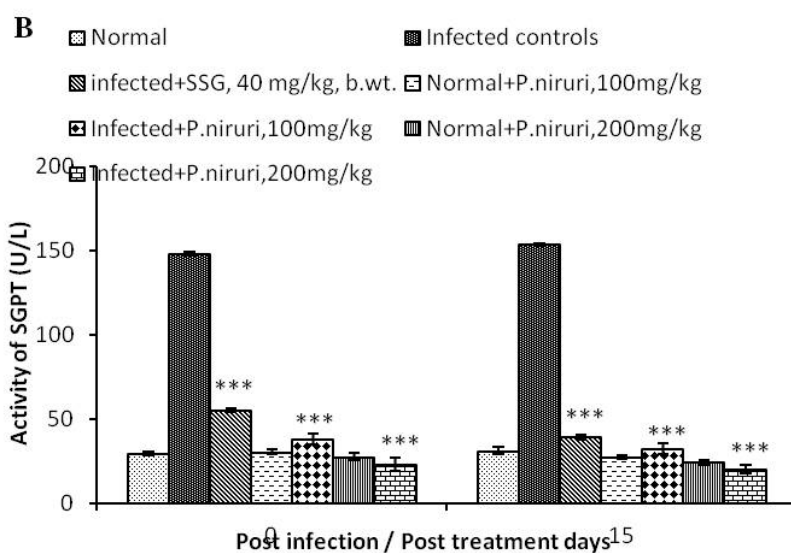
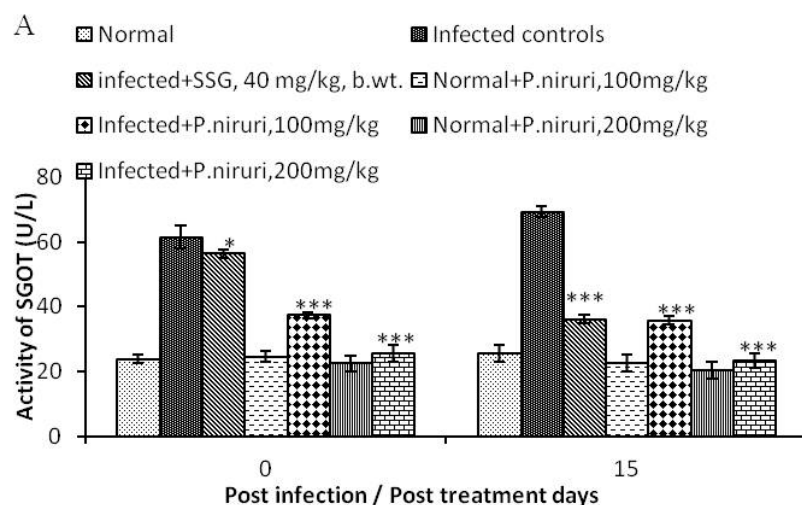


Fig 5A,B,C: SGOT, SGPT, bilirubin activities in various groups of animals.

Results are expressed as Mean±SD of 6 animals.

Test drugs significant from infected control ***p<0.001, *p<0.05

3.4.5 Kidney function tests

The functionality of kidneys was checked by estimating the concentrations of creatinine and serum urea. They increased significantly ($p<0.05$) after infection and were above the normal range as compared to normal controls. However both the levels were found to be within normal range after treatment with both the doses of *P. niruri*. This reveals that the plant extract of *P. niruri* does not cause any nephrotoxicity and is safe (Fig. 6A,B).

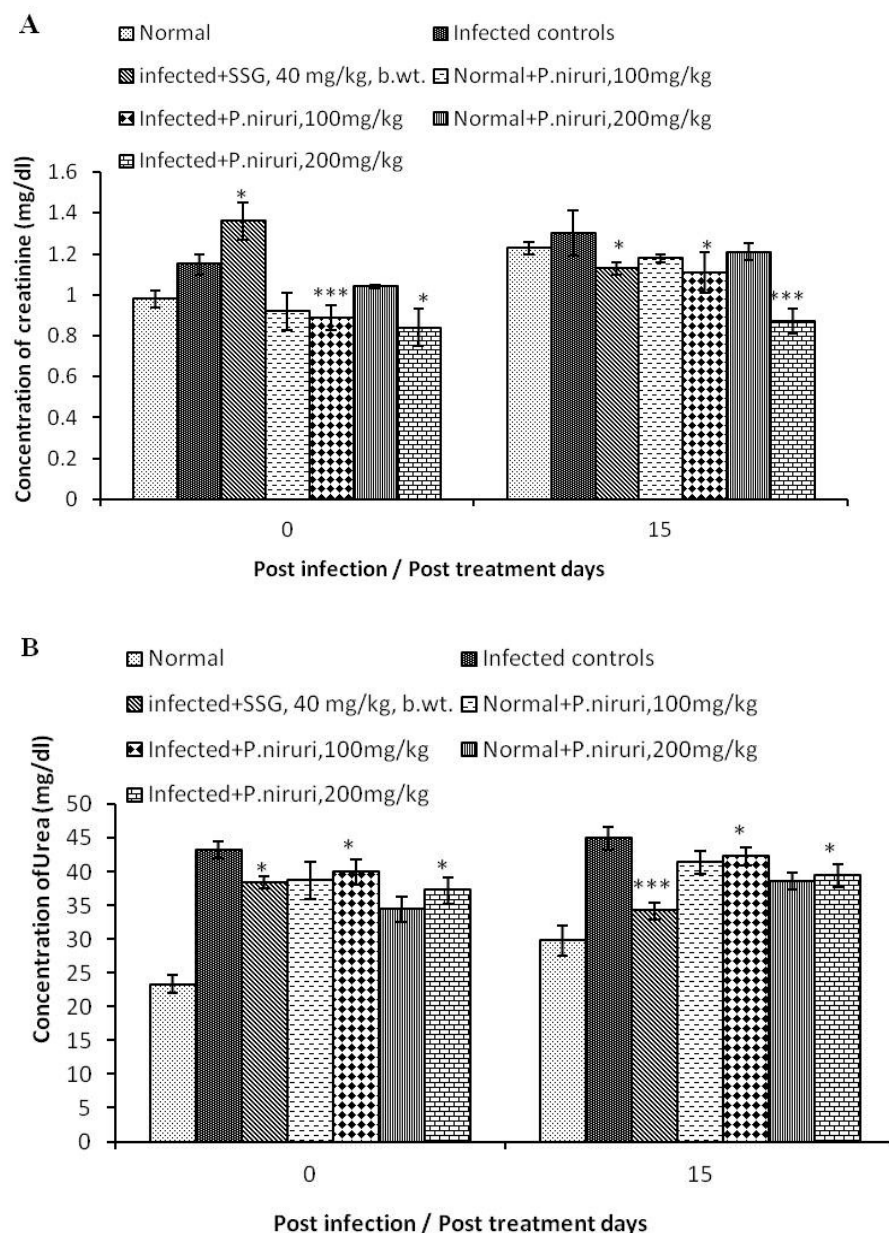


Fig 6A,B: Concentration of creatinine and blood ures in various groups of animals.

Results are expressed as Mean $\pm$ SD of 6 animals.

Test drugs significant from infected control *** $p<0.001$, * $p<0.05$

4. DISCUSSION

For more than half a century, the standard treatment of kala-azar has relied on the use of heavy metal-based pentavalent antimonials which are highly toxic and are known to be associated with resistance in some parts of India [36]. The other second line drugs like miltefosine and paromomycin also show serious side effects [37,38]. Therefore, an impelling need is felt for the

development of new, effective, safe and nontoxic drugs from plant sources for treatment of visceral leishmaniasis. The interest in medicinal plants or their extracts has risen up all over the world due to the belief that many herbal extracts have been extensively used by endemic natives for the treatment of leishmaniasis. Scientific reports have also demonstrated their potential lately [39].

Cure of leishmaniasis, even during chemotherapy, depends primarily upon the activation of an effective immune response that stimulates macrophages to kill the intracellular amastigotes. This activity of macrophages is downregulated by the infection itself, which suppresses the requisite signalling between macrophages and T cells. Therefore, the use of therapy involving immunomodulators which enhance host immune responses may have many uses as a means for reforming current therapeutic system [40]. In clinical research, *P. niruri* has been demonstrated to be hepatoprotective, antiviral, antibacterial, antispasmodic and antimutagenic. Hence, we tried to explore the antileishmanial efficacy of crude extract of *P. niruri* *in vitro* and *in vivo*.

In the present study different concentrations of the crude ethanolic extract of *P. niruri* were tested for their *in vitro* antileishmanial activity. The crude extract of the plant showed inhibitory activity against promastigotes of *L. donovani* in a concentration dependent manner. Notably there was a concordance in antileishmanial effect of the ethanolic extract with an increase in concentration of the extract. The leishmanicidal effect of *P. niruri* extract can be attributed to the cumulative effect of various biologically active components like alkaloids, flavonoids, saponins, terpenoids and saponins present in it.

The oral treatment with *P. niruri* significantly reduced the parasite load in *L. donovani* infected mice as compared to infected controls. There was a significant reduction in parasite burden in animals treated with a higher dose of 200 mg/kg b.wt. as compared to lower dose. These results correlate with another study in which Niranthin, a lignin isolated from the aerial parts of the plant *Phyllanthus amarus*, has showed potent antileishmanial activity [41].

Delayed type hypersensitivity (DTH) responses are antigen-specific reactions which are used to assess cell-mediated immune (CMI) responses *in vivo*. The CMI functions to enhance antimicrobial actions of macrophages to eliminate microbes. The macrophages are activated by various inflammatory cytokines produced by Th-1 cells. The DTH responses and parasite load are inversely associated in *Leishmania* infection. The higher DTH response is linked with lower parasite load and enhanced immune response. Our results demonstrated that the DTH responses were significantly reduced in *L. donovani* infected BALB/c mice as compared to all the animals treated with herbal drugs. The DTH responses in infected animals treated with *P. niruri* at a dose of 200 mg/kg b.wt. were higher as compared to those treated with 100 mg/kg b.wt. The SSG treated group of animals also showed positive DTH responses as compared to infected animals. This is in agreement with a previous study by [42] where successful drug (SSG) therapy in patients with leishmaniasis resulted in higher DTH responses. In a similar study, the flavonoids isolated from leaf extract of *Kalanchoe pinnata* (Crassulaceae) showed antileishmanial activity by the development of delayed type hypersensitivity (DTH) responses in *Leishmania* infected mice. Quercetrin is an active flavonoid isolated from *K. pinnata* which may played a major role in leishmanicidal activity [43-46].

It has been demonstrated that the Th1 cytokines instigate immunoglobulins to swop from IgM to the IgG2a isotype. At the same time, Th2 cytokines promote switch from IgM to IgG1 and IgE. Indeed, IgG2a and IgG1 kinetics indirectly reflect the Th1/Th2 responses in mice. Therefore, the relative production of these antibodies can thus represent the activation of Th1-like and Th2-like immune responses, respectively. In particular, there are many studies in murine model of leishmaniasis which show the correlation of Th response and IgG isotypes [47-49]. Therefore, to assess the type of immune response generated we measured serum levels of parasite-specific IgG1 and IgG2a isotypes. In this study, all animals treated with *P. niruri* revealed higher levels of IgG2a and lower levels of IgG1 as compared to infected controls. The greater levels of IgG2a and lower levels of IgG1 were observed in higher dose of *P. niruri* (200 mg/kg b.wt.) treated animals as compared to the animals treated with 100 mg/kg b.wt. on both 1 and 15 p.t.d. Our results are in agreement with another study in which it has been shown that mice deficient in IgG1 develop resistance to the disease which confirms that the levels of IgG1 increase during progression of disease [50]. Our results also support another study, wherein niranthin a component from *P. amarus* supported 2.1-fold higher SLA-specific IgG2a production [42] which confirms upregulation of Th1 type of immune response.

Drug-induced liver injury remains the most common type of detrimental drug reactions and is the most universal reason cited for removal of a drug from the market. The liver is cardinal to the metabolic discarding of most drugs and xenobiotics, so it is more vulnerable to metabolism-based injury. The indications of drug-induced hepatotoxicity are highly flexible, ranging from asymptomatic uplift of liver enzymes to perilous hepatic failure. Although, there have been wonderful advancement

in modern pharmacology, there are hardly any convincing drugs that protect the liver from injury and help in modification of hepatocytes. Many active plant extracts have been consistently exploited to manage a broad variety of liver disorders. Analysis of serum enzymes has been reported to be of value and is early warning sign of liver injury. Infected controls and infected animals treated with SSG showed increase in the activity of SGOT and SGPT which may be due to hepatopathy. This is in accordance with our previous study in which both the enzyme levels were higher than the upper limit of normal range [51]. However, in the infected and normal groups treated with herbal extract of *P. niruri* at a dose of 100 mg/kg b.wt. and 200 mg/kg b.wt. the levels of SGOT, SGPT and bilirubin were found to be within normal range. This indicates hepatoprotective efficacy of the herbal extract. The groups of animals treated with 40 mg/kg b.wt. SSG also exhibited an increase in enzyme levels as compared to normal controls. It correlates with a previous study which demonstrated that the hepatotoxicity caused by SSG could be compensated by picroliv which is also hepatoprotective compound [52]. Another study also demonstrated that the elevated levels of serum SGPT and SGOT in nimesulide-induced rats were brought to normal levels after intraperitoneal and oral administration of *P. niruri* as compared to control group. In a similar study, it has been demonstrated that *P. niruri* extract has potential to control the abnormal hepatic biochemical activities [53].

Renal function tests are used to determine the extent of renal dysfunction. We tested the levels of creatinine and blood urea for evaluating abnormal renal functions. Creatinine, a low molecular weight cation is a waste product produced at a constant rate and is not bound to plasma proteins and freely filtered by the renal glomeruli with little or no reabsorption. Therefore, its level rise in blood when there is reduced glomerular filtration [54]. The concentration of urea concentration is the amount of nitrogen derived from urea. The enzyme urease catalyses the hydrolysis of urea to form ammonia and carbon dioxide [55]. In current study the kidney parameters were found to be in normal range in all the infected animals treated with *P. niruri* as compared to the infected controls. In SSG treated animals the blood urea levels were raised in comparison to *P. niruri* treated animals but were found to be within normal range. Creatinine levels were also above the normal range suggesting that SSG is nephrotoxic. In a similar study, the ethanolic extracts of leaves and unripe pods of *Bauhinia purpurea* normalized the increased levels of serum creatinine, serum uric acid and blood urea nitrogen levels produced due to gentamicin toxicity [56].

From the above study it can be concluded that *P. niruri* has a potent antileishmanial as well as convincing immunostimulatory activity. A significant reduction in parasite burden and absence of any side effects proves the efficacy of this drug as a treatment option for visceral leishmaniasis. The drug can be further tested in higher animal models alone or in combination with other herbal formulations for proving its potential against disease.

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