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**Original Article**

# Safety and Efficacy of *Prosopis juliflora* Leaf Extract as a Potential Treatment against Visceral Leishmaniasis in Balb/c Mice

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***Abstract***

***Background:*** Visceral Leishmaniasis caused by *Leishmania donovani* is a major health problem in the tropics and sub-tropic regions where it is endemic. We aimed in testing the leishmanicidal activity and toxicity of *Prosopis juliflora* leaf extract in BALB/c mice and in vitro test systems respectively.

***Methods:*** In the year 2017 until 2019, BALB/c mice of mixed sexes aged between 6 and 8 weeks in groups of 8 were used. Group I treated with 100 mg/kg of *P. juliflora* extract, Group II -1 mg/kg of Sodium stibogluconate (SSG) and Group III treated with normal saline. All mice were anaesthized and sacrificed to obtain blood, spleen samples for antibody measurements, and determination of parasite loads.

***Results:*** There was significant inhibitory effect ($P<0.05$) exhibited by *P. juliflora* leaf extract on promastigote growth during the in vitro test whereby up to 98% parasites were killed at the highest concentrations of 100 µg/Ml of the extract as compared to SSG, which showed less inhibitory effect on promastigotes. *P. juliflora* exhibited a higher splenic antiamastigote effect after 21 days of administration as compared to SSG. *P. juliflora* methanolic leaf extract induced a higher total IgG level as compared to the reference drug which could be attributed to higher titer in IgG2a subtype in mice treated with the extract, which was not induced in mice, treated with SSG.

***Conclusion:*** *P. juliflora* exhibited higher inhibitory effects against *L. donovani* promastigotes as well as amastigotes and induced significantly higher IgG antibody levels as compared to SSG ($P<0.05$). Furthermore, it was safer than SSG on Vero E6 cells.

## Introduction

Leishmaniasis is a parasitic disease caused by an intracellular protozoan parasite of the genus *Leishmania* that invades the macrophages. It is a vector borne parasitic disease transmitted through the bite of an infected female sand fly belonging to the genera *Phlebotomus*, *Lutzomia* or *Psychodopygus* during a blood meal. Sand flies are tiny, silent, short distance fliers and usually bite at night. The sand flies live in forests, cracks of mud walls, animal burrows and anthills (1).

Visceral leishmaniasis, if left untreated, is a fatal disease with fatality rates as high as 100% within two years of infection (2). This is a major health challenge in the endemic areas, which have high poverty levels hence; access to antileishmanial drugs is a challenge due to their high cost.

The current antileishmanial drugs are costly, cause life threatening side effects including arthralgia, myalgia, leucopenia, pancreatitis, liver problems, cardio-toxicity and cardiac arrhythmia (3). Due to these devastating side effects, the drugs have to be administered at low dosages, which increases the risk of drug resistance by the parasite and lowers the efficacy of the drugs (4). This presents a major drawback to the treatment of visceral leishmaniasis since currently there is no licensed vaccine against *Leishmania* for prevention.

There is an urgent need for safe, affordable and effective leishmanicidals in the endemic areas (5). This can be developed from locally growing medicinal plants moreover herbal medicines are now in great demand in developing countries since they are readily available, affordable, locally acceptable and have minimal side effects (6).

We therefore aimed at determining the antileishmanial effect of *Prosopis juliflora* leaf extract on *L. donovani* infections in BALB/c mice for possible use in the treatment of Leishmania disease.

## Methods

### *Study area*

The present study was carried out in the year 2017 until 2019 at the Department of Zoological Sciences laboratories Kenyatta University, Nairobi, Kanya.

### *Sample collection*

Young leaves of *P. juliflora* were collected from several trees in the same location from Marigat, Baringo County in Kenya. Leaves of *P. juliflora* were washed with water and shade dried at room temperature for three weeks. Crushing and grinding into fine powder was done at the Jomo Kenyatta University of Science and Technology (JKUAT) chemistry laboratories using an electric miller. Cold sequential extraction was carried out using methanol as extracting solvent. One hundred grams of the fine powders of every plant sample were macerated in 1000 ml methanol at room temperature. The extracts were filtered using Whatman Filter Paper No.1 and concentrated using a rotary evaporator (BUCHI R 200, Labortechnik Switzerland) at (40 $^{0}$C) (7).

### *Culture of Leishmania donovani promastigotes*

*L. donovani* strain NLB 065 were obtained from the spleen of an infected patient in Baringo County, Kenya and maintained by intra-cardiac hamster-to-hamster passage at the Institute of Primate Research (IPR) was used. Cellular material was isolated from the spleen of the infected Hamster, cultured in Schneider's Drosophila Complete Media (Sigma Aldrich USA), Incubated at 25$^{o}$C and observed for growth. Stationary phase promastigotes were then harvested by centrifugation at 2500 rounds per minute (rpm) for 15 min at 4 $^{0}$C. Promastigotes were counted using a haemocytometer (8).

### Experimental design

BALB/c mice of mixed sexes aged between 8 and 10 weeks obtained from the animal house, Department of Zoological Sciences, Kenyatta University were used. The use of mixed sexes was guided by the need to avoid sex bias during experimentation (9). The mice were maintained in the animal house throughout the experiment, and food and water was provided *ad libitum*. The mice were marked for individual identification purposes. The study-involved tests for plant extract efficacy and toxicity. For determination of efficacy of test compounds in a murine model of visceral leishmaniasis, mice were infected intraperitoneally with $2x10^6$ virulent *L. donovani* parasites. Infected mice were then kept for five weeks for disease establishment after which three mice were selected randomly and sacrificed then splenic impression smears prepared to confirm presence of parasites.

Infected mice were then put into three groups of 8 mice each and treated as follows: Group I was treated with *P. juliflora* leaf extract at a dose of 100mg/kg of body weight; Group II was treated with a combination 1 mg/kg of Sodium stibogluconate, while Group III was injected with normal saline and served as an infected untreated control. The drug compounds were constituted in normal saline to make 100 µL per dose. The *P. juliflora* leaf extract was given orally while SSG and normal saline injections were done intraperitoneally once daily for 21 consecutive days. Experimental and control groups of mice were weighed once weekly for 3 weeks during the treatment experiment to determine the effect of test compounds on body weight. All mice were anaesthized and sacrificed to obtain blood and spleen samples. Blood samples were used to prepare serum for antibody measurements while the spleen was used to determine the parasite load. Pre-parasite injection mice sera were also included as a naïve control for the IgG antibody level analysis (8).

The experimental design was approved in the year 2017 by the Kenyan National Commission for science, Technology and innovation through a proposal submitted and vetted through Kenyatta University. The permit number is NACOSTI/P/17/20489.

### Determination of parasite load

Parasite load was determined by obtaining a thin section from mice spleen. Impression smears were then prepared on microscopic slides and stained using Giemsa stain. Counting of the number of amastigotes was done as described (10).

### In vitro evaluation against L. donovani promastigotes

Plant extracts of serial concentrations ranging from 100 to 0.78125 µg/ml were prepared and the extracts, negative control (normal saline), and positive control (with sodium stibogluconate) were added to the wells on a 96 well plate (11). The tests were performed as previously described earlier (12). Late Stationary-phase promastigotes harvested (8) were counted and suspended in a concentration of $2.0 \times 10^6$ parasites/ml in culture medium. Parasite observations and counting was done using a haemocytometer and a microscope and results expressed as the mean (± SD) drug concentration inhibiting parasite growth by 50% (IC50) after 72 h incubation period.

### Cytotoxicity Assay

Vero cells in their log phase of growth were harvested and counted. Twenty µL of MTT (0.5 mg/ml to each well) reagent were aseptically added to each well and plates incubated for 3 hours before addition of MTT solvent (Isopropanol-HCL). Death of cells per well was calculated using the surviving cells and results expressed as percentage cell death as well as lethal dose (ld 50). A dose responsive curve was plotted to enable calculation of the concentrations that killed 50% of the vero cells.

The experiment was performed as described earlier (12).

### Data analysis

Parasite burden data was expressed as the mean per 1000 per cell nuclei of spleen cells ± standard deviation. The in vitro data on promastigote count and data was subjected to one-way analysis of variance (ANOVA) to compare means of groups. The data on weight was analyzed by Paired T test. A *P*-value less than or equal to 0.05 was considered statistically significant. Results were presented in graphs and tables following presentation of descriptive text and statistics.

## Results

### Anti-promastigote effect of Prosopis juliflora methanolic leaf extract

The inhibitory effect of *P. juliflora* on promastigote growth in vitro was dose dependent; thus increase in *P. juliflora* concentration showed more inhibitory effect with 98% lethal effect being observed at the highest concentration. As compared to Sodium stibogluconate (SSG), *P. juliflora* leaf extract was the more efficacious as exhibited by reduction of the number of promastigotes from the initial 18.4x $10^5$ at the lowest concentration to 0.2x$10^5$ at the highest *P. juliflora* concentration. Sodium stibogluconate showed a promastigote count of 4.5x$10^5$ at the highest concentration. *P. juliflora* killed significantly more promastigotes at each test compound concentration ($P<0.05$ compared to the group treated with SSG. Treatment with *P. juliflora* or SSG significantly reduced promastigotes numbers as compared to the untreated control group ($P<0.05$) (Fig. 1).

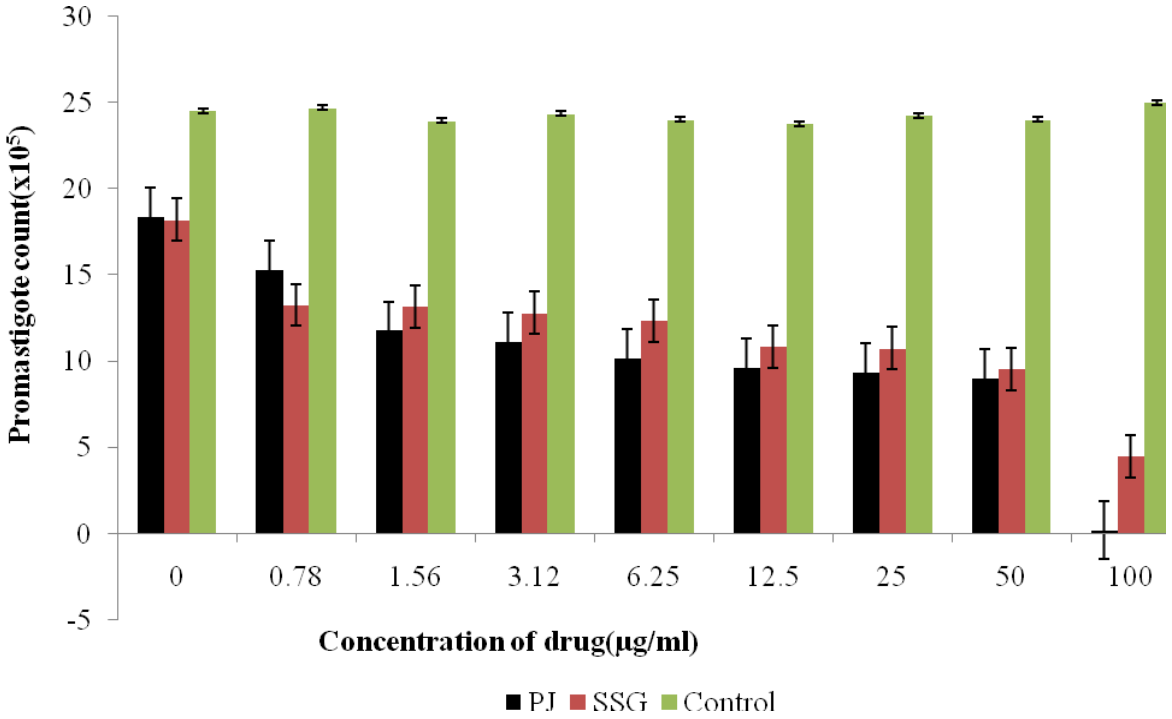


**Fig. 1:** Leishmania donovani promastigotes counts

Promastigotes were incubated *in vitro* with various concentrations (100 µg/mL to 0.78µg/mL) of PJ or SSG for 72 hours before counting surviving parasites using a haemocytometer. Data represent promastigotes. PJ-*Prosopis Juliflora*, SSG - Sodium stibogluconate. C – Control (Normal saline)

### *Safety of P. juliflora leaf extract on vero cells*

Results indicated that after 72 hours of incubation of vero cells with *P. juliflora* or SSG, *P. juliflora* leaf extract had lower toxicity to vero cells as compared to SSG, which induced a higher vero cell death. The toxicological effect of PJ and SSG was significantly different ($P<0.05$). The $LD_{50}$ of *P. juliflora* was recorded at 0.85µg\ml while that of SSG was of 0.57µg/ml (Fig. 2).

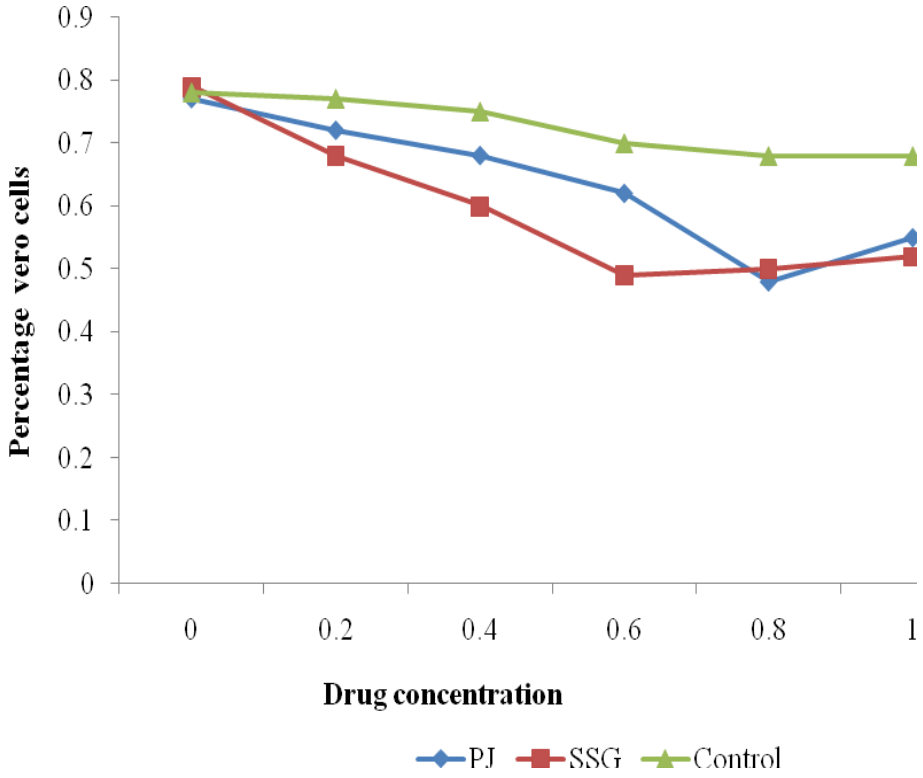


**Fig. 2:** Percentage vero cell death after treatment with drug compound and leaf extract in vero cells

Vero cells surviving were calculated after 72 hours of incubation with different concentrations of test compounds. The data represented above shows the number of surviving cells vero cells at the end of incubation period. P.J-P. *Juliflora*, SSG - Sodium stibogluconate, C – control (Normal saline)

### *Effect of treatment compounds on mice weight during treatment*

Through the treatment period, there was a gradual increase in mice body weights. Differences in body weights in the treated mice groups did not differ significantly. One-week post treatment, the weights in mice treated with PJ remained unchanged while the mean weight in the SSG group appeared to drop slightly as compared to the corresponding weights obtained at week 3. The body weights in the untreated control mice seemed to increase slightly at a higher rate than the rate of increase observed between week 1 and week 3 (Fig. 3).

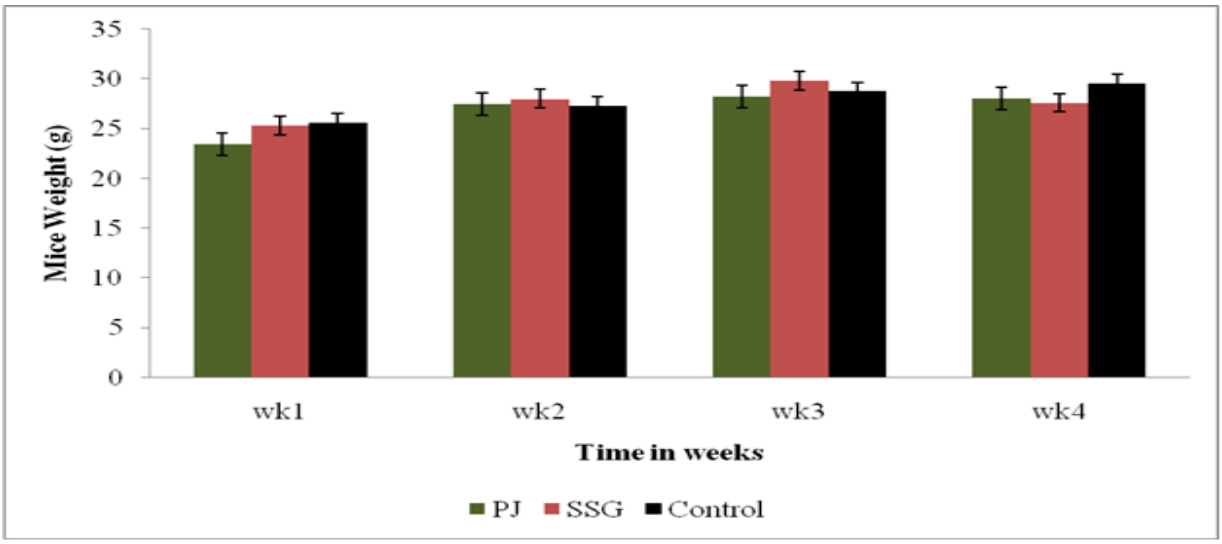


**Fig. 3:** Effect of treatment compounds on mice weight during treatment

Weight changes before, during and after treatment were represented as mean. The mice were weighed weekly during the treatment period. The initial weights (week 1) were taken before treatment started and weights were taken after treatment stopped (week 4) (*Prosopis Juliflora)*, SSG (Sodium stibogluconate)**,** C – control (Normal saline)

### *Splenic impression smears prepared from infected mice*

Following 21 days of treatment of *L. donovani* infected mice with *P. juliflora* or SSG, splenic impression smears showed fewer amastigotes parasites in the PJ group than the SSG treated group (Fig. 4).

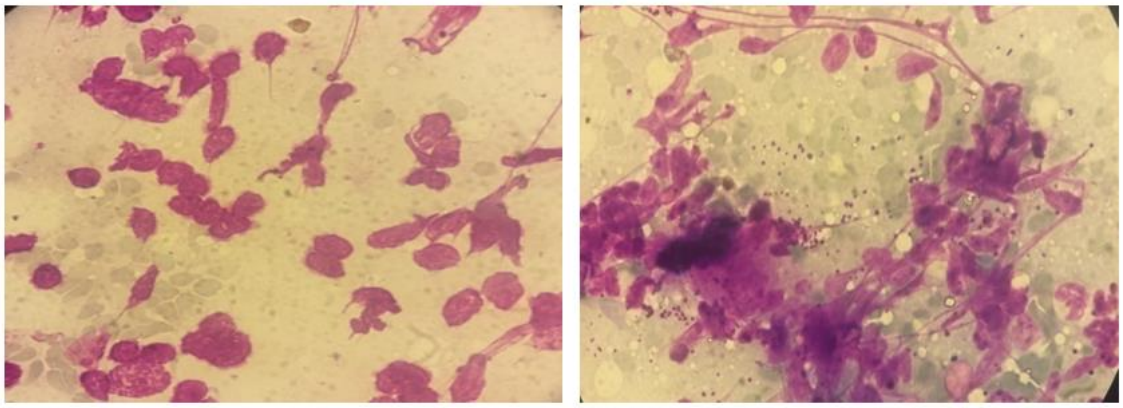


**Fig. 4:** Splenic impression smears from treated mice

Mice were infected with *L. donovani* and treated with PJ or SSG for 21 days before they were sacrificed after a further 7 days for assessment of amastigotes in splenic impression smears. PJ treated group showed less amastigotes (A) than SSG treated group (B).

### *Effect of P. juliflora leaf extract on splenic amastigote count*

Splenic tissues from treated mice showed average parasite burden of 123 amastigotes per 1000 splenic cell nuclei in the *P. Juliflora* treated group and 230 amastigotes per 1000 splenic cell nuclei in the group treated with SSG while the negative control mice group recorded 830 amastigotes/1000 splemic cell nuclei. Therefore, *P. juliflora* leave extract had a higher antileishmanial effect on *L. donovani* amastigotes as compared to SSG. However, amastigote numbers in the *P. juliflora* and SSG treated mice groups did not differ significantly. One week after post 21 days of treatment, the number of amastigotes in the spleen were significantly ($P < 0.05$) reduced for both treatment mice groups as compared to the negative control group (Fig. 5).

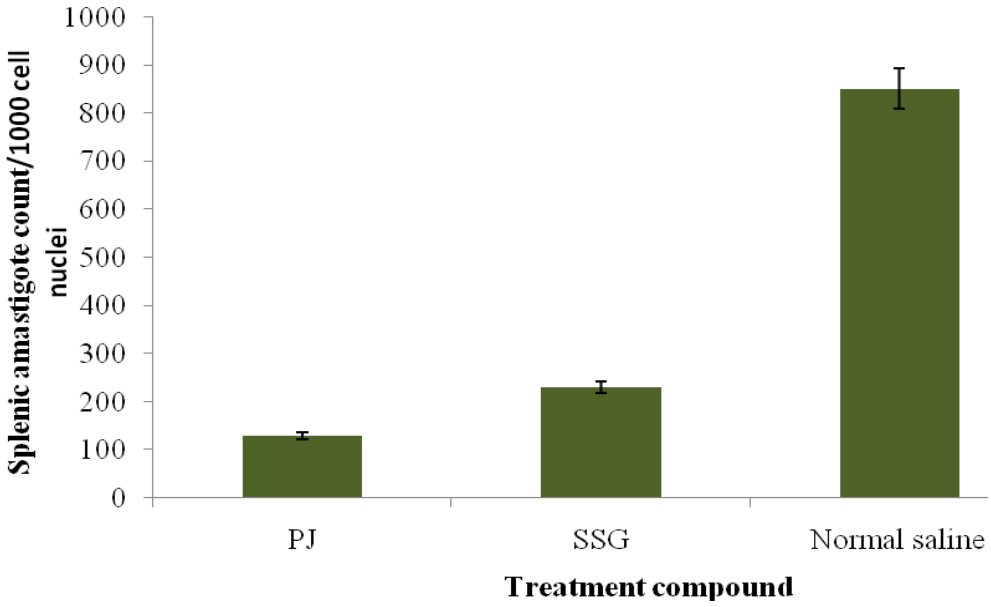


**Fig. 5:** Splenic amastigote numbers in *L. donovani* infected and treated BALB/c mice

Data represented in the graph above shows the antiamastigote effect of *Prosopis juliflora* leaf extract on the spleen amastigote count following treatment with the drug compounds one for 21 consecutive days and splenic impression smears made. The number of parasites was expressed as the number of parasites observed in 1000 cell nuclei of spleen cells, mean. P.J - *Prosopis Juliflora,* SSG-Sodium stibogluconate.

### *Effect of the P. juliflora leaf extract on total IgG levels in mice infected with L. donovani*

Treatment of mice with PJ leaf extract induced higher levels of anti-leishmanial IgG antibody than in mice treated with SSG. Mice in the control groups had lower IgG levels compared to mice treated with *P. juliflora.* The *P. juliflora* treated mice showed significantly higher total IgG antibody levels as compared to the SSG treated mice group ($P<0.05$). Mice in the naïve group had the lowest levels of IgG. The IgG levels were significantly higher in *P. juliflora* as compared to the control mice groups ($P < 0.05$) (Fig. 6).

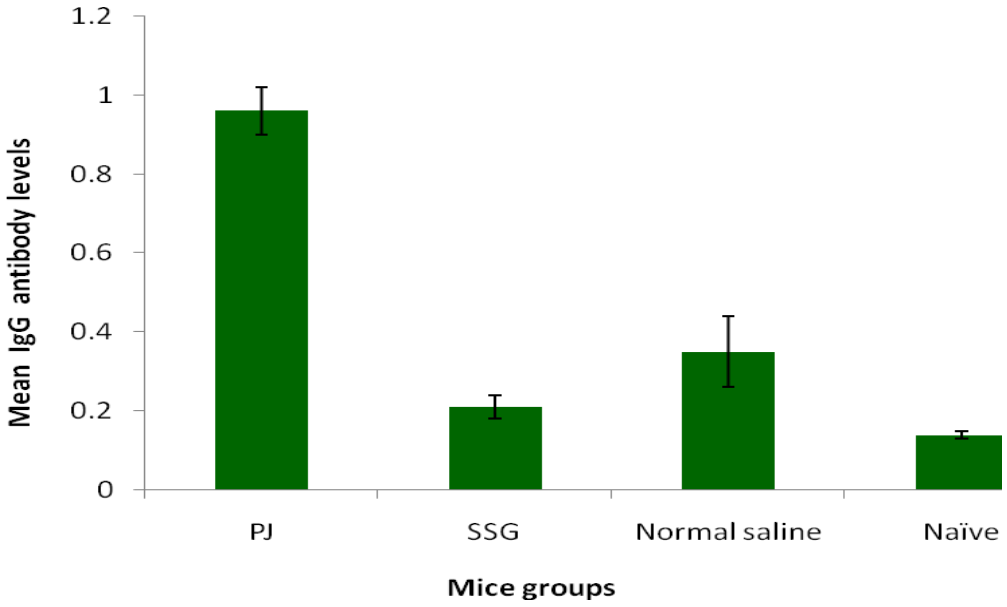


**Fig. 6:** IgG levels in *L. donovani* infected mice following treatment with PJ or SSG compounds

After infection, mice were observed for 5 weeks for disease establishment. Treatment started on the six week. It was done for 21 consecutive days after, which mice were sacrificed and blood samples obtained for serum quantification of IgG levels. Data represents mean±SD of total IgG levels in both experimental and control mice groups. PJ- *Prosopis juliflora*, SSG- Sodium stibogluconate and Naïve

## Discussion

The observation of significant reduction of promastigotes by the test compound, *P. juliflora* as compared to the reference drug SSG is a clear indication that *P. juliflora* is a potent natural product with potential for treatment of visceral leishmaniasis.

Many plant metabolites have been shown to be effective in treating a wide range of diseases ranging from bacterial, fungal and parasitic infections (13). It is likely that the potent leishmanicidal effect of *P. juliflora* leaf extract is attributed to the presence pharmacologically active metabolites like saponins, alkaloids, tannins, flavonoids, sterols and other secondary metabolites. Furthermore, *P. juliflora* has been reported to contain these metabolites (7, 14). Elsewhere, *P. juliflora* was effective in the treatment of helminthes following tests of the saponins from the bark, roots and leaves extract against *Haemonchus* (14). Thus, it may be an important natural medicinal product against a wide range of protozoan and helminthic diseases. The reference drug, which is a pentavalent antimonial, and the only approved drug in Kenya for treatment of leishmaniasis exhibited a slightly lower antileishmanial effect against *L. donovani* promastigotes at each concentration. Therefore, if the selection of one of the two drug compounds, *P. juliflora* and SSG were to be based on this single parameter, then PJ would be selected as a more effective antileishmanial compound as compared to SSG.

The lower toxicity exhibited by PJ than SSG against vero cells is a clear manifestation that *P. juliflora* is a safer compound than SSG. Thus, it can be used in the treatment of clinical leishmaniasis without the side effects associated with current antileishmanial drugs. Furthermore, current drugs used for treatment of leishmaniasis are associated with high toxicity

rates. Toxicity of drugs also makes patients less compliant with dosage leading to development of pathogen resistance and disease relapse (5). *P. juliflora* was safe against the peritoneal macrophages (15). *P. juliflora* did not exhibit cytotoxicity against glial cells of Winstar mice for up to 72 hours at concentrations of up to 3 µg/ml (16). Furthermore, plant extracts have proved more effective against *Leishmania* as compared to current chemotherapeutics in use due to less toxicity (17).

The difference in weights of mice due to treatments was not significant and could not have entirely been used as an efficacy parameter. However, increase in mice weight after the first week of treatment with *P. juliflora* leaf extract could be attributed to relieve of the symptoms due to efficacy of the extract leading to improved feeding and increase in weight. Weight loss is one of the symptoms associated with leishmaniasis (18). Increase in weight in the control group was because of natural growth in the mice. The slight decrease in weight in the SSG treated group which could be attributed to the side effects of the drug such as nausea, lack of appetite hence leading less feeding and weight loss (19).

The higher anti amastigote effect of *P. juliflora* leaf extract can also be attributed to the presence of biologically active metabolites in the plant. *P. juliflora* antiprotozoal effect has been demonstrated against *Plasmodium falciparum* and *T. cruzi* (20). *Prosopis* spp. have shown potential in pharmaceutical application such as acetycholine esterase inhibition, inhibition of hydrogen and potassium ions (21). This could be the same mechanism employed by the compound against amastigotes leading to the extract´s higher efficacy. The results of the present study were in agreement with those of a study conducted to test the effect of spleen amastigote count of *L. major* infected BALB\C mice using leaf, seed, stem and bark extracts of *C. tinctorius, P. anisum, C. cyminum, C. verum* and *A. persarum* which showed significant reduction of splenic amastigote count in comparison with the control groups though the difference in comparison with gluntamine treatment compound was not significant (22). The present study has revealed the chemotherapeutic activity of *P. juliflora* leaf extract against amastigotes of *L. donovani* was higher as compared to the reference drug making this plant a potential candidate for developing leishmanicidal compounds.

The presence of higher than normal levels of antibody in all infected groups was indicative of progressive active leishmaniasis infection. This supports a previous study (23) that presence of antileishmanial antibodies is indicative of disease. Although antibody responses are not generally associated with disease control, IgG2a in mice is associated with type 1 immunity, which is critical for solid control of leishmaniasis (24). It was interesting to observe that *P. juliflora* leaf extract induced higher IgG levels than SSG although disease control was more pronounced in the *P. juliflora* than SSG treated groups as shown by the significantly reduced amastigotes number in the former treated mice group. These findings may most probably point to one conclusion, that the highest fraction of IgG subclasses in the total IgG induced by *P. juliflora* was IgG2a, hence enhanced disease control in the *P. juliflora* treated mice may have been a result of the IgG2a subclass but further studies to fractionate the total IgG may be important to clarify this claim. If this explanation stands to be true, then the severity of leishmaniasis in the SSG treated mice than in the *P. juliflora* treated mice may be attributable to lack of IgG2a in the SSG mice group.

However, caution should be exercised in interpreting these data given that the lack of equivalent or greater disease control in the SSG treated mice may simply be due to less effectiveness of this drug compound as compared to *P. juliflora*.

## Conclusion

*P. juliflora* exhibited higher inhibitory effects against *L. donovani* promastigotes as well as amastigotes and induced significantly higher IgG antibody levels as compared to SSG. Furthermore, *P. juliflora* was safer than SSG on Vero E6 cells. This study recommends *P. juliflora* leaf as a high potential antileishmanial agent whose efficacy can be evaluated further in non-human primate models before application in the treatment of clinical visceral leishmaniasis.

## Acknowledgements

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## Conflict of interest

The authors declare no conflicting interests

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