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Assessment of Antileishmanial Potential of *Solanum nigrum* and *Alstonia scholaris*: New Hope for Leishmaniasis Treatment

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ABSTRACT: In the present investigation, the leishmanicidal efficacy of ethanolic extracts of leaves of *Alstonia scholaris* and bark of *Solanum nigrum* were investigated against murine *Leishmania donovani* infection. The *in vitro* investigations showed effective suppression of parasites. The results showed that the IC_{50} values obtained for *S. nigrum* were comparatively lower than *A. scholaris*, leading to the selection of this particular plant for subsequent *in vivo* investigations. The leishmanicidal efficacy of the *S. nigrum* leaf extract was evaluated by analyzing the parasite count and humoral immune responses. After a week of administration of plant extracts to all the infected and treated BALB/c mice, the parasite load decreased significantly compared to the only infected animals. The administration of plant extracts at concentrations 100 mg/kg and 200 mg/kg b.wt resulted in higher IgG2a and lower IgG1 levels in treated animals compared to infected controls. The SSG-treated animals demonstrated a decrease in IgG2a and an increase in IgG1 levels and higher DTH Responses. This finding indicates that the examined plant extracts possess the potential to counteract the immunosuppressive effects of the parasitic infection. The administration of a larger dose of the plant extract derived from *S. nigrum* has been observed to provide protection against experimental murine visceral leishmaniasis.

Keywords: *Solanum nigrum, Alstonias cholaris,* leishmanicidal activity, *Leishmania donovani, in-vivo* studies, *in-vitro* studies, immune response, and plant extracts.

INTRODUCTION

An obligatory intracellular parasite from the genus Leishmania is the culprit behind the parasitic disease leishmaniasis. This disease is transferred to humans through the bite of a female sandfly. It causes high morbidity and mortality, making it a severe public health issue (Gharirvand Eskandari *et al.,* 2020). The disease is characterised by fever, weight loss, fatigue, enlarged liver and spleen, and anaemia. Every year, 2 million new cases are reported, and 350 million people are at risk. The parasite kills thousands of individuals and disables millions (Parham *et al.*, 2020).

The present therapeutic approach for leishmaniasis relies on the utilization of pentavalent antimonials, Amphotericin B, Ambisome, paromomycin, miltefosineetc (Et-Touys *et al.*, 2017). The efficacy of some of drugs is progressively decreasing as a result of the development of resistance (Soosaraei *et al.*, 2017). Therefore, it is widely believed that the discovery of a new drug with the same or higher efficacy than existing agents and lower toxicity can be a research priority.

Leishmaniasis can be treated in a variety of ways, but most of them are inefficient, expensive, out-of-date, and linked to side effects and the emergence of resistance. Numerous research teams have looked at natural ingredients in pursuit of innovative and successful leishmaniasis treatments. Natural products, which mediate interactions between plants and their environment, are structurally varied secondary metabolites found in the roots, stems, leaves, fruits, seeds, and other plant components (Heidari-Kharaji *et al.*, 2016). According to Bouyahya *et al.* (2018), medicinal plants containing secondary metabolites exhibit a variety of pharmacological capabilities, including antibacterial, antioxidant, anticancer, antifungal, anti-litholitic, and antileishmanial actions. The effectiveness of these medicines in preventing the growth of multiple *Leishmania* species, including *L. major* (which causes cutaneous leishmaniasis) and *L. infantum* (which causes visceral leishmaniasis), has been shown in numerous investigations (Gharirvand Eskandari *et al.*, 2020; Soosaraei *et al.*, 2017). The synthesis of metallic nanoparticles using phytocompounds has drawn a lot of interest from scientists and the pharmaceutical sector (Rathika *et al.*, 2023).

Alstonia scholaris, also known as sapthparna and a member of the Apocynaceae family, is a potent medicinal plant. It contains diverse alkaloids, flavonoids, and phenolic acids, according to reports. "Antimicrobial, antiamoebic, antidiarrheal, bronchodilatory,

antiplasmodial, hepatoprotective immunomodulatory, anti-cancer, free radical scavenging, antioxidant, analgesic, and anti-inflammatory properties" have been reported (Patil, 2019). On the other hand, *Solanum nigrum* Linn. (Solanaceae), also known as "Black nightshade", is frequently used to treat liver conditions in traditional medicine in India and other countries across the world, chronic skin conditions (psoriasis and ringworm), etc. It contains total alkaloids, steroid alkaloids, steroidal saponins, and glycoproteins, which account for its medicinal properties. Numerous scientific studies have demonstrated its anticancer, immunomodulatory, antimicrobial, nematicidal, antioxidant, hepatoprotective, antiulcerogenic, and anti-inflammatory properties (Xufei Chen *et al.*, 2022).

This study was done to examine the antileishmanial efficacy of *Alstonia scholaris and Solanum nigrum* against *Leishmania donovani*. The main aim of this study was to evaluate the "*in-vitro* and *in-vivo"* leishmanicidal capabilities of both plants. The advantages of the proposed study are exploration of novel treatment options, assessment of *Solanum nigrum* and *Alstonia scholaris* potential, addressing Leishmaniasis, a neglected tropical disease, diversifying antileishmanial strategies, and promising alternatives for current therapies.

MATERIAL AND METHODS

A. Parasites and culture conditions

The present investigation utilized two strains of *Leishmania donovani* from India: "MHOM/IN/80/Dd8", which is responsive to SSG treatment and was initially received from the "London School of Tropical Hygiene & Medicine" in the United Kingdom, and P.B.0014, a strain that is resistive to SSG treatment and was obtained from RMRI in Patna, Bihar. The promastigotes of these strains were cultured and sustained on a modified "Novy, McMeal and Nicolle's medium supplemented with Eagle's Minimum Essential Medium **(**MEM) as an overlay" (Keshav *et al.*, 2021).

B. Maintenance of the parasite culture

The log phase promastigotes of both the strains were utilized to sustain the strain in a modified NNN and RPMI-1640 + 10% FCS medium at a temperature of 22±1ºC. The promastigote culture underwent regular monitoring and subculturing at intervals of 48-72 hours. This was achieved by transferring a volume of 0.5-1.0 mL of the culture solution. The culture was subjected to analysis via wet mount preparation, revealing the presence of motile promastigotes (Aydogdu *et al.*, 2019).

C. Plant material

The leaves specimens of *Solanum nigrum* (voucher number. 8056) and the bark of *Alstonia scholaris* (voucher no. 5718) were obtained from the "Botanical Garden of Panjab University, Chandigarh". The plants were procured and certified by the Department of Botany. The plant samples were thoroughly rinsed with water, at room temperature then air dried, and ground into powder.

D. Preparation of extracts

For extraction, dried and powdered *S. nigrum* leaves were used. The extraction was conducted using the Soxhlet procedure. In a glass flask, approximately 250 mL of methanol was added to 100g of desiccated and powdered plant specimens. The obtained extracts were concentrated in a rotary evaporator under vacuum.

E. In vitro antileishmanial activity of plant extracts

For *in-vitro* studies, *L. donovani* promastigotes of SSG responsive and unresponsive strains were dispersed into 24 well culture plates. The method was followed as per the previous literature of Nigatu *et al.* (2021).

F. In vivo antileishmanial activity of plant extracts

(i) Animals used. The BALB/c mice used in the current study were obtained from "IMTECH and the Central Animal House of Punjab University, Chandigarh". The mice were 5-6 weeks old and weighed between 20-25g. The subjects were provided with unrestricted access to water and a diet consisting of mouse feed. The "Institutional Animal Ethics Committee of Panjab University in Chandigarh", India, gave its approval for the experimentation (IAEC approval number-IAEC/284-295/3/9/2012).

(ii) Groups of animals used for the study

Group	Treatments
Group I	"Normal inbred BALB/c mice."
Group II	"Inbred BALB/c mice infected with 1×10^8 promastigotes of *L. donovani."*
Group III	"Infected mice treated intraperitoneally with SSG at the dose of 40 mg/kg daily for 5 days"
Group IV	"Normal mice treated orally with *S. nigrum* at the dose of 100 mg/kg body wt. for 7 days daily"
Group V	"Infected mice treated orally with *S. nigrum* at the dose of 100 mg/kg body wt. for 7 days daily"
Group VI	"Normal mice treated orally with *S. nigrum* at the dose of 200 mg/kg body wt. for 7 days daily"
Group VII	"Infected mice treated orally with *S. nigrum* at the dose of 200 mg/kg body wt. for 7 days daily"

(iii) Infection of BALB/c Mice. "Two groups of BALB/c mice", one infected and one normal, were administered with infection taking the method of Nelson *et al.* (2023).

(iv) Preparation of Leishmanin. The preparation of leishmanin was done following the previous literature of Tadele *et al.* (2020). The findings were presented as the mean ± standard deviation of the %growth in the width

of the right foot-pad in comparison to the left footpad in mice.

Each group of mice received a subcutaneous injection of 40µl of leishmanin in the right foot pad and PBS in the left foot pad. The thickness of the right and left foot pad was measured after a 48-hour period using a set of vernier calipers. The results were reported as the mean ± standard deviation of the percentage increase in the thickness of the right footpad compared to the left footpad of mice (Kaur *et al.*, 2008).

Percentage increase in thickness of foot pad was calculated as:

$$\frac{(\text{Thickness of right foot pad} - \text{thickness of left foot pad})/(\text{Thickness of left foot pad})}{\text{Thickness of left foot pad}} \times 100$$

(v) Enzyme Linked Immunosorbent Assay (ELISA). Six animals from each group were slaughtered, and blood was collected on various days following infection or therapy. Separated serum was used for ELISA testing. "IgG1 and IgG2a levels" in serum samples were evaluated by ELISA according to Xinhai Chen *et al.* (2022) to assess antibody immune responses.

G. Statistical analysis

Data is represented as mean ±S.D. (mean=6). Statistical tests were performed to determine significance between different groups by applying student's t-test.

RESULT AND DISCUSSION

In this study, an examination was conducted on the "*in-vitro* and *in-vivo"* antileishmanial effectiveness of the ethanolic extracts derived from the leaves of *S. nigrum* and bark of *A. scholaris*.

A. In vitro Anti-leishmanial activity

The *in-vitro* antiparasitic activities of *A. scholaris* and *S. nigrum* were evaluated along with controls against two strains (MHOM/IN/80/Dd8 and SSG-unresponsive, P.B. 0014) of promastigote forms of *L. donovani*. The results showed that the concentration of DMSO tested had no effect on the growth of parasites. *A. scholaris* and *S. nigrum* plant extracts were found to be active, with IC_{50} values of 72.26g/ml mL and 55.16g/mL, respectively, against the promastigote form of the susceptible strain of *L. donovani*. With an IC_{50} of 62.26g/mL and 43.16g/mL, respectively, *A. scholaris* and *S. nigrum* were found to be efficacious against the sensitive strain of parasite. *S. nigrum* demonstrated greater potency than *A. scholaris*. Higher concentrations of both extracts elicited discernible cytopathological alterations in the promastigote form of *L. donovani*, encompassing ovoid cells, flagellum loss, granulation, and cellular rounding, alongside additional morphological modifications in the parasites, such as diminished promastigote mobility, transformation into round to oval shapes, reduced size accompanied by condensed cytoplasm, and enlarged nuclei. Since the IC50 values for Solanum nigrum were lower, only this botanical specimen was taken into consideration for subsequent *in vivo* investigations. The *in vitro* anti-leishmanial activity comparison between *A. scholaris* and *S. nigrum* revealed both as active against *L. donovani*, but *S. nigrum* exhibited greater potency with lower IC50 values. Consequently, only *S. nigrum* was chosen for subsequent in vivo investigations, aligning with recent studies (Nelson *et al.*, 2023) emphasizing its efficacy.

B. In vivo Anti-leishmanial activity

(i) Hepatic Parasite Load. "The quantification of the parasite load was conducted using Leishman Donovan Units as a means of evaluating the level of infection. On day 30 post-infection days (p.i.d.), the most parasites were detected in the infected controls. After treatment, the parasite load decreased significantly in all treated groups compared to the infected controls. Following treatment with *S. nigrum* at a dose of 100 mg/kg b.wt., the parasite load was reduced by 78.81% and 87.53 % on the 1st and 15th post-treatment day. *S. nigrum* at a dose of 200 mg/kg b.wt. increased the level of protection to 82.57 and 90.62% on 1 and 15, respectively (Fig. 1). The decrease in parasite load was shown to be statistically significant ($p < 0.01$) in animals administered with a dosage of 200 mg/kg body weight of *S. nigrum* compared to animals treated with a dosage of 100 mg/kg body weight of *S. nigrum* on days 1 and 15 post-treatment. There was a statistically significant difference ($p < 0.01$) observed between the two doses on the 15th post-treatment day. The parasite burden was shown to be higher in animals treated with the extract at both doses, in comparison to the standard drug SSG. Similar results were obtained in the study on "*in vivo* and *in vitro* response of *L. major* infection to *Tephrosia vogelii* extracts in BALB/c mice" (Marango *et al.*, 2017).

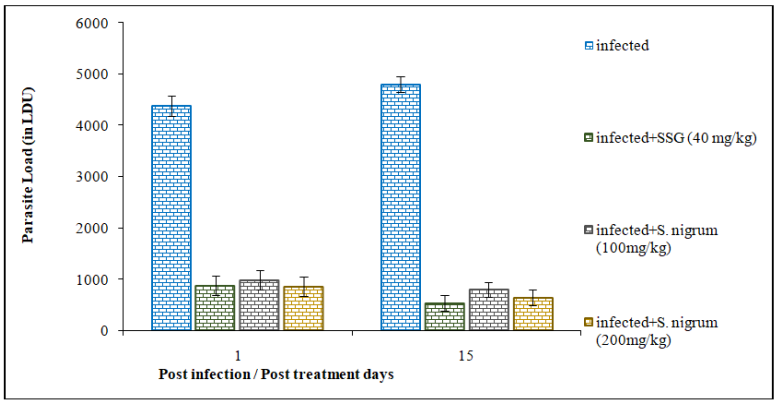

Fig. 1. Parasite load in various groups of animalsexpressed as Mean ± S.D. of 6 animals.

(ii) Generation of Immune response. ELISA was employed to evaluate serum samples obtained from various animal groups in order to determine the presence of IgG1 and IgG2a antibodies. The analysis was conducted at a wavelength of 450 nm.

(iii) Humoral Immune Responses

IgG1 antibody responses- The investigation assessed "serum levels of parasite-specific IgG1 and IgG2a isotypes" to validate cytokine responses & understand the potential mechanism of protection. The levels of IgG1 antibody in the normal animals were measured to be 0.126 ± 0.003 and 0.146 ± 0.003, respectively. These levels increased to 0.281 ± 0.005 and 0.311 ± 0.004 in infected animals on 1 and 15 post-treatment days (p.t.d.), respectively. In the group of animals subjected to standard treatment, the observed values were found to be comparable to those of the control group. In animals that were infected and then treated with a low dose of 100 mg/kg body weight of *S. nigrum*, the measured values were 0.202 ± 0.002 and 0.209 ± 0.001 on days 1 and 15 post-treatment, respectively. Similarly, in infected animals treated with a higher dose of 200 mg/kg body weight of *S. nigrum*, the measured values were 0.141 ± 0.001 and 0.161 ± 0.002 on days 1 and 15 post-treatment, respectively (Fig. 2). There was a statistically significant drop in antibody levels observed in animals at a higher dose ($p < 0.0001$). In the present study, mice were infected and then treated with SSG at a dosage of 40 mg/kg body weight. The absorbance values determined on day 1 and day 15 post-treatment were 0.212 ± 0.004 and 0.231 ± 0.003, respectively. There was a statistically substantial difference in antibody levels between animals treated with SSG and animals treated with plant extract ($p < 0.0001$).

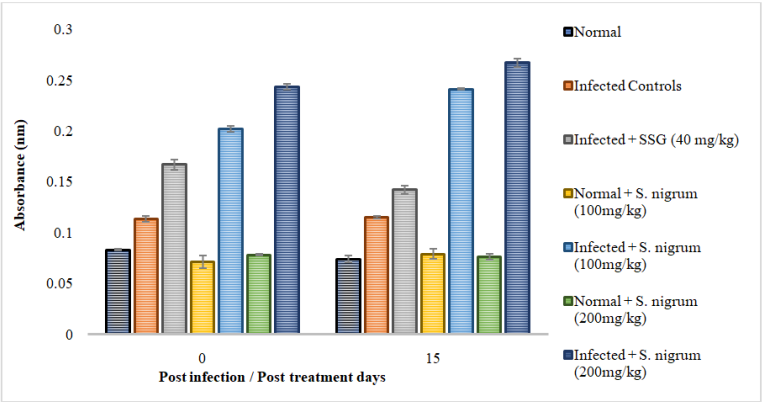

Fig. 2. IgG1 antibody production in various groups of animals expressed as Mean ± S.D. of 6 animals.

IgG2a antibody responses-When comparing the two doses, it was observed that the absorbance values in animals treated with the greater dose were considerably higher ($p < 0.001$) compared to those treated with the lower dose. The levels of IgG2a were shown to be significantly elevated in animals administered with a dosage of 200 mg/kg body weight of *S. nigrum*, in comparison to those treated with the same herbal extract at a dosage of 100 mg/kg body weight, on both days following the treatment (Fig. 3).

In the current study, all infected and treated animals had "higher IgG2a levels and lower IgG1 levels compared to infected controls". The animals treated with SSG at a dose of 40 mg/kg b.wt had lower IgG2a levels and higher IgG1 levels than those treated with *S. nigrum* at both doses. This finding is consistent with a previous study that had shown a correlation between SSG administration and the occurrence of acute immunosuppression in treated individuals (Rostamian *et al*., 2017). The low levels of IgG1 antibody and higher levels of IgG2a detected in the sera of animals treated with herbal extract suggest that the disease is being controlled in the treated animals, whereas them infection is progressing in the control animals. In fact, the kinetics of "IgG2a and IgG1" indirectly reflect the "Th1/Th2 responses". Indicators of the "induction of Th1- and Th2-" like immune responses can therefore be found in the comparative production of these isotypes.

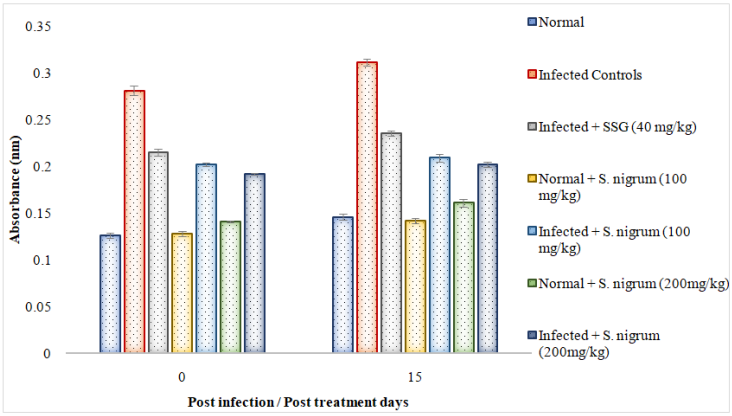

Fig. 3. IgG2a antibody production in various groups of animals expressed as Mean ± SD of 6 animals.

The study delves into the in vivo anti-leishmanial activity of *S. nigrum*, focusing on hepatic parasite load and immune response generation. On day 30 post-infection, significant parasite reduction occurred with *S. nigrum* treatment, particularly at 200 mg/kg. ELISA analysis revealed a distinctive IgG2a-dominant response in treated animals, indicating effective control of infection. These findings align with previous studies (Nigatu *et al*., 2021), underscoring the potential of *S. nigrum* as an anti-leishmanial agent.

C. Delayed type Hypersensitivity (DTH) responses

Delayed type Hypersensitivity is manifested as an index of cell mediated immunity. In normal animals the percentage increase in footpad thickness was 5.78±0.19 and 6.67±0.33 on 1 and 15 p.t.d. respectively which increased in infected animals to 8.21±0.34 and 9.86±0.45 on 1 and 15 p.t.d. respectively. The DTH responses in infected animals treated with *S. nigrum* at a dose of 100 mg/kg b.wt. were 30.92±0.89 and 38.31±0.92 and in infected animals treated with *S. nigrum* at a dose of 200 mg/kg b.wt., they were 32.82±1.53 and 41.24±0.12 on 1 and 15p.t.d. respectively. In both the treated groups of animals, DTH responses increased significantly ($p<0.0001$) as compared to the infected controls. The increase in footpad thickness was found to be significantly ($p<0.0001$) more in animals treated with higher dose as compared to lower dose on 15 p.t.d. In infected animals treated with SSG at a dosage of 40 mg/kg b.wt, the DTH responses were 45.06±0.73 and 50.12±0.67 on 1 and 15 p.t.d. respectively. The DTH responses were significantly ($p<0.0001$) higher in animals treated with plant extract as compared to SSG treated animals on 15 p.t.d. (Table 1, Fig. 4).

Table 1: DTH responses in various groups of animals.

Animal Groups	Percentage Increase in Footpad Thickness	
	1 p.t.d. (30p.i.d)	15p.t.d. (45p.i.d)
Normal	5.78±0.19	6.67±0.33
Infected	8.21±0.34	9.86±0.45
Infected + SSG, 40 mg/kg b. wt	45.06±0.73	50.12±0.67
Normal + *S. nigrum*,100mg/kg	7.71±0.45	8.14±1.34
Infected + *S. nigrum* 100 mg/kg b.wt	30.92±0.89	38.31±0.92
Normal + *S. nigrum*,200mg/kg	9.45±0.41	11.33±0.54
Infected + *S. nigrum* 200mg/kg b.wt	32.82±1.53	41.24±0.12

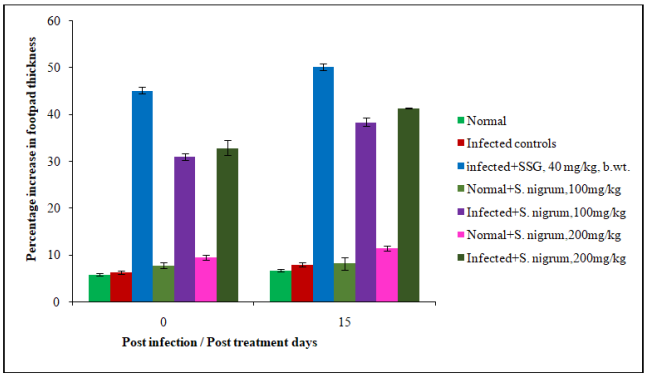

Fig. 4. DTH responses in various groups of animals.

Results are expressed as Mean±SD of 6 animals
p.t.d: post treatment days.
p.i.d: post infection days.

p values: Infected vs Infected+*S. nigrum* 100 mg/kg b.wt; Infected vs Infected+ *S. nigrum* 200mg/kg b.wt

p values: Infected+*S. nigrum* 100 mg/kg b.wt vs Infected+ *S. nigrum* 200mg/kg b.wt.

p values: Infected+SSG vs Infected+ *S. nigrum*100 mg/kg b.wt; Infected vs Infected+*S. nigrum* 200mg/kg b.wt

The observed variations in delayed type hypersensitivity (DTH) responses among animal groups reflect the immunomodulatory effects of *Solanum nigrum* and *Alstonia scholaris*. The substantial increase in footpad thickness, especially in infected groups treated with SSG (Goyal *et al.,* 2021) and *S. nigrum*, underscores their potential in enhancing cellular immune responses against leishmaniasis.

The delayed type hypersensitivity (DTH) responses, indicative of cell-mediated immunity, were assessed in animals treated with *S. nigrum* against leishmanial infection. The increase in footpad thickness was significantly higher in treated groups, especially at 200 mg/kg compared to infected controls. Notably, *S. nigrum* outperformed SSG, consistent with prior studies (Parham *et al.,* 2020).

The present study's findings align with recent research endeavors (Sajwan *et al.,* 2021) focusing on the exploration of novel treatment options for Leishmaniasis. In concordance with these studies, our assessment of *Solanum nigrum* and *Alstonia scholaris* highlights their potential as promising antileishmanial agents. This correlation with recent works strengthens the validity of our results, reinforcing the growing consensus on the significance of these botanical extracts in combating Leishmaniasis. Leishmaniasis, classified as a neglected tropical disease, has garnered increased attention in recent years. Our study contributes to addressing this global health challenge by providing insights into alternative treatment strategies. By diversifying antileishmanial approaches, we aim to offer a more comprehensive toolkit for combating the disease. This diversification is crucial, given the complex nature of Leishmaniasis and the need for adaptable solutions to tackle its various manifestations.

In the context of promising alternatives for current therapies, our results exhibit a noteworthy potential shift in the treatment paradigm. Comparisons with recent studies (Kumar *et al.,* 2017) underscore the uniqueness of our findings, emphasizing their contribution to the evolving landscape of antileishmanial research. This discussion not only validates the relevance of our study but also positions it within the broader narrative of ongoing efforts to innovate and improve treatment options for Leishmaniasis.

CONCLUSIONS

The result of this investigation indicates that the plant extract is effective in vitro against both SAG-resistant and susceptible strains of *Leishmania donovani*. A higher dose of *S. nigrum* plant extract provides protection contrary to experimental murine visceral leishmaniasis, as evidenced by a reduction in parasite burden and the production of humoral immunity. Even though the lessening in parasite load following herbal treatment was less than that observed with the standard drug SSG, hepatotoxicity and nephrotoxicity were not observed. DTH assessments affirm the potential of *Solanum nigrum* and *Alstonia scholaris* in leishmaniasis treatment, advocating for their consideration in therapeutic strategies. More research is required on animal models such as hamsters to determine their antileishmanial efficacy.

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