IN VITRO ANTILEISHMANIAL ACTIVITY AND CYTOTOXICITY OF SOME INDIAN TRADITIONAL MEDICINAL PLANTS AGAINST SENSITIVE AND RESISTANT STRAINS OF LEISHMANIA DONOVANI

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ABSTRACT
Visceral leishmaniasis is a renowned neglected tropical disease responsible for affecting poor people in developing countries like India. However, the treatment options are very few due to resistance to first line drugs like sodium stibogluconate, toxicity and high cost of other available drugs. Hence, this is of utmost importance to develop safe, effective and inexpensive drugs especially from plant resources. Therefore, the present study is an effort to discover plants whose extracts can be used as antileishmanial agents in future. Three plants have been examined for their phytochemical constituents, antileishmanial activity and cytotoxicity. These plants significantly inhibited promastigote growth at concentrations ranging from 10 to 100 µg/ml against sensitive and resistant strains of Leishmania donovani and showed negligible cytotoxicity against HeLa cells. Therefore, it can be suggested from present study that these plants extracts and their active components can be isolated and further exploited to check their efficacy against murine visceral leishmaniasis.

KEYWORDS: Visceral leishmaniasis, plant extracts, antileishmanial activity, cytotoxicity.

INTRODUCTION
The usage of medicinal plants is well acclaimed in traditional health care systems like Ayurveda, Siddha, Unani and Rasashastra as early as 7000 B.C. and they are still used in various tribes of India and Africa for the treatment of a number of ailments. Moreover, 60% of people around the world depends upon traditional herbal remedies for their primary health care requirements and 25 % of modern medicines are made from traditionally used plants.
Plants are important drug candidates, particularly against parasites because of their long association with them.\(^3\) One of major milestone in this field was the revelation of artemisinin, a sesquiterpene lactone obtained from *Artemisia annua* as a drug for curing malaria. This discovery encouraged the scientific community to identify new lead compounds from plant resources with antiparasitic activity, especially those to treat leishmaniasis.\(^4\)

Leishmaniasis is a neglected tropical disease which affects about 350 million people worldwide and 2 million new cases are reported annually. It is widespread in 22 New World countries and 66 Old World countries.\(^5\) The two most common clinical forms, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are mainly seen with approximated incidence of 1.5 and 0.5 million cases respectively. India, Bangladesh and Nepal harbour an estimated 67% of the global VL disease burden.\(^6\) *Leishmania donovani* is the causative agent of VL, which is fatal in the absence of treatment.\(^7\) Since the available drugs for the treatment of VL are associated with severe side effects. The pentavalent antimonial drugs, a first line therapeutics is conjoined with emergence of acquired resistance which is a major obstacle in treating VL in Bihar, India now a days.\(^8\) The other second line drugs like amphotericin B, its formulations and miltefosine are being used for the treatment with more efficacies and dramatic potential to cure leishmaniasis. But they are comparatively costlier and require long treatment regimes than the generic antimony. Moreover, miltefosine is associated with very severe side effects like teratogenicity.\(^9\) Other drugs like paromomycin and pentamidine have shown some usefulness and could be a potential supplement in the drug regimen but their use and availability in disease endemic regions is limited.\(^10\) Therefore, in the scarcity of an effective antileishmanial drug, there is urgent need to explore new antileishmanial compounds especially from traditional medicinal plant resources which can cure leishmaniasis completely. It has also encouraged the researchers to exploit the traditional herbal remedies as sources for the development of new chemotherapeutic compounds. India is well known for its rich flora with a high percentage of endemic species which can be used for the treatment of parasitic diseases. However, much efforts have not been done till date to identify plants or their phytoconstituents as sources of drugs. The World Health Organization has also advocated the use of traditional medicine for the treatment of these tropical diseases.\(^11,12\) Therefore, the objective of this study is to evaluate the antileishmanial efficacy of some Indian traditional medicinal plant extracts against sensitive and resistant strains of *L. donovani* promastigotes in comparison to sodium antimony gluconate (SAG) and their cytotoxic effect on cells.
MATERIALS AND METHODS

Parasite Culture
For the current study, the Indian strain of *Leishmania donovani*, MHOM/IN/80/Dd8, originally obtained from the London School of Hygiene and Tropical Medicine, U.K. and SSG resistant strain P.B.-0014 (a kind donation by Prof. Pradeep Das, Director, Rajendra Memorial Research Institute of Medical Sciences, Bihar, India) were used. The promastigotes of both the strains were maintained in modified Novy, McNeal and Nicolle’s (NNN) medium and RPMI-1640+10% FCS medium and subcultured after every 48-72 hours.\[^{13}\]

Plant Material
The leaves of *Delonix regia*, and *Sida acuta* were collected from the Botanical Garden of Panjab University, Chandigarh. The leaves of *Euphorbia hirta* were collected from village Kasba Bhraal near Raikot, Punjab, India. The specimens were deposited at the Department of Botany, Panjab University, Chandigarh, India and voucher numbers were obtained. Plants were washed thoroughly with water, dried at room temperature and then powdered.

Preparation of Extracts
The air-dried and milled plant parts were successively extracted with alcohol by Soxhlet extraction method. Approximately 250 ml of alcohol was added to 70g dried and powdered plant specimen in a glass flask. After filtration, the extracts were concentrated under vacuum in a rotary evaporator. The residues obtained were then lyophilized and stored at -4°C till further use.

Phytochemical analysis
The plant extracts were screened for their phytochemical constituents by applying standard protocols.\[^{14-16}\] Qualitative tests were performed to detect alkaloids, saponins, phenols, anthraquinones, steroids, diterpenes, triterpenes, flavonoids, cardiac glycosides, tannins and phytosterols.

Antileishmanial activity of plant extracts
The promastigotes of sensitive and resistant strains of *L. donovani* promastigotes were cultured in RPMI-1640 complete medium supplemented with 10% heat inactivated fetal bovine serum. The culture was checked for any contamination and centrifuged at 3000 rpm for 10 minutes. The pellet obtained was washed thrice with phosphate buffer saline and promastigotes were counted with Neubauer chamber under light microscope. The final
concentration of $2 \times 10^6$ promastigotes/ml was made by diluting with fresh culture media. For *in vitro* analysis, $2 \times 10^6$/ml *L. donovani* promastigotes were dispensed in each well of 24 well culture plates. The stock solutions were prepared by dissolving known quantity (10 mg/ml) of extract in 0.3% DMSO. The stock solutions were further diluted to make various concentrations (1, 10, 20, 40, 60, 80 and 100 µg/ml) of extracts. The wells were then supplemented with plant extracts in triplicate series. Each test was repeated three times. Negative control cultures were supplemented with equal volume of working DMSO solution. Positive control cultures were incubated with similar concentrations of SSG. Cultures were further incubated at 22±1°C and analysed under microscope after 24, 48 and 72 hours using a 0.1-mm Neubauer chamber with trypan blue dye exclusion test and 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

**Cytotoxic Activity of plant extracts on HeLa Cells**

**MTT Assay:** Growth of HeLa cells was quantitated by studying the ability of living cells to reduce the yellow dye 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. For this purpose, HeLa cells were harvested from tissue culture flask by trypsinization with 0.25% trypsin. Then the cells were centrifuged at 2500 rpm for 5 minutes. Cell suspension was made in 1ml of complete media and counted by trypan blue dye exclusion method in Neubauer’s chamber. The required concentration of $6 \times 10^3$ cells of HeLa Cell line were cultured in 100 µl of DMEM Media for 24 hrs in incubator at 37°C with 5% CO$_2$ in 96 well culture plate. The cells were subsequently supplemented with different concentrations (10, 50, 100, 200, 400, 600, 800 and 1000 µg/ml) of plant extracts and further incubated for another 48 hrs. Dilutions of stock solution were made in culture medium yielding final extract concentrations with a final DMSO concentration of 0.3%. This concentration of DMSO doesn’t affect cell viability. Negative control cells were incubated in culture medium and 0.3% DMSO only. Each concentration was tested in triplicate. Then the medium in each well was replaced by MTT solution (1 mg/ml). The plates were incubated for 3-4 hrs in an incubator at 37°C with 5% CO$_2$. MTT reagent was then removed and the formazan crystals produced by viable cells were dissolved in 100 µl of DMSO and gently
shaken. The absorbance was then measured in ELISA plate reader at 560nm. The percentage cell viability was calculated using the following formula

\[
\text{Percentage cell viability} = \left(\frac{A_t - A_b}{A_c - A_b}\right) \times 100
\]

Where, \( A_t \) = absorbance value of test compound,
\( A_b \) = absorbance value of blank and
\( A_c \) = absorbance value of control.

The effect of extracts was expressed by \( \text{CC}_{50} \) values i.e. the drug concentration which reduces the absorbance of treated cells by 50% with respect to untreated cells.

**STATISTICAL ANALYSIS**

For the inhibitory and cytotoxic assays of plant extracts, the \( \text{IC}_{50} \) and \( \text{CC}_{50} \) values were calculated by probit analysis using biostat software 2009.

**RESULTS AND DISCUSSION**

Throughout the history of medicinal chemistry and pharmaceutical drug development, natural products have played an invaluable role in drug discovery.\(^{[17]}\) The compelling demand for alternate therapeutic agents for treatments has initiated a program to investigate natural plant products for their possible use in the therapy of leishmaniasis. In fact, the WHO advocated the use of traditional medicine where appropriate health services are not available.\(^{[18,19]}\) Plant extracts and their phytochemical constituents can be a potential source of lead compounds plant-derived compounds are likely to provide a valuable source of new medicinal agents.\(^{[20-24]}\) Natural products having secondary metabolites like quinones, alkaloids (quinolines and isoquinoline analogues, indole analogues and steroidal alkaloids), terpenes (iridoids, monoterpenes, sesquiterpenes, diterpenes, triterpenes), saponins, phenol derivatives (chalcones and flavonoids) and other metabolites such as acetogenins showed antileishmanial activity.\(^{[25,26]}\)

Three plant extracts were selected based upon their medicinal properties and tested for their antileishmanial activity against \( L. \ donovani \) promastigotes. The medicinal properties of plants are listed in Table 1. Phytochemical analysis of plant extracts in the present study revealed the presence of various active secondary metabolites such as terpenes, alkaloids, flavonoids, polyesterols, phenolic compounds, tannins and saponins (Table 2). Presence of these metabolites might provide leishmanicidal property to the plant extracts.\(^{[26]}\)
Table 1. Description of plants

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plants</th>
<th>Family</th>
<th>Plant part used</th>
<th>Solvant used</th>
<th>Medicinal importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Delonix regia</td>
<td>Fabaceae</td>
<td>Leaves</td>
<td>Methanol</td>
<td>Anti-diabetic [50], immunostimulant and hepatoprotective [51], antimicrobial [52]</td>
</tr>
<tr>
<td>2.</td>
<td>Euphorbia hirta</td>
<td>Euphorbia ceae</td>
<td>Leaves</td>
<td>Methanol</td>
<td>Antimutagenic [53], antimicrobial [54], diuretic [55]</td>
</tr>
<tr>
<td>3.</td>
<td>Sida acuta</td>
<td>Malvaceae</td>
<td>Leaves</td>
<td>Ethanol</td>
<td>Antimalarial [56], hepatoprotective [57]</td>
</tr>
</tbody>
</table>

Table 2. Phytochemical constituents of plant extracts

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plant extract</th>
<th>Alkaloids</th>
<th>Triterpenes</th>
<th>Diterpenes</th>
<th>Saponins</th>
<th>Favonoids</th>
<th>Tannins</th>
<th>Phenolic compounds</th>
<th>Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Delonix regia</td>
<td>++</td>
<td>++</td>
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<tr>
<td>2.</td>
<td>Euphorbia hirta</td>
<td></td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>3.</td>
<td>Sida acuta</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
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</tr>
</tbody>
</table>

The promastigote stage which was used in this study has been extensively used in various other studies since it is easier to maintain in laboratory conditions [27-30]. The antileishmanial activities and cytotoxicity of extracts from six plants studied are listed in Table 3 and 4. All plants tested showed significant antileishmanial activity with IC₅₀ values in the range of 5 to 15 μg/ml after 48 hrs against sensitive strain and 6 to 16 μg/ml against resistant strain. The maximum activity was seen with S. acuta with IC₅₀ of 5.45±1.98 μg/ml after 48 hrs against sensitive strain and 6.38±0.62 μg/ml against resistant strain. It also exhibited least cytotoxicity against HeLa cells amongst other two plants and highest selectivity index of more than 1000 (Table 3,4). Phytochemicals such as tannins, steroids, alkaloids, glycosides, flavonoids, saponins and terpenes which were found to be present immensely in S. acuta are also reported [31] and might be responsible for its antileishmanial activity. Among the phytochemicals analysed, alkaloids that were present enormously in this plant extract have been found to kill parasite by causing morphological changes like swelling and rounding of parasite, vacuolated cytoplasm, swollen mitochondria, disrupted parasite membrane hence interrupting its integrity [32]. Phenolic compounds such as tannins also show biochemical and pharmacological activities including antioxidant, antitumor and free radical scavenging properties. They disrupt cell membrane of the parasite [33]. Saponins have been found to exhibit their leishmanicidal effect through apoptosis by disturbing the integrity of cell
membrane as detected by annexin V and propidium iodide. It also disturbs the mitochondrial membrane potential which results in cell-cycle arrest at the G_{0}/G_{1} phase and DNA nicking shown by deoxynucleotidyl transferase mediated dUTP end labeling (TUNEL)\cite{34}. Similarly, flavonoids were also present extensively in this plant extract which also disrupts parasite structure and mitochondria function as it causes swelling of parasite and formation of vacuoles in cytoplasm\cite{35}. Arginase is an enzyme helpful in the synthesis of polyamine in Leishmania and flavonoids are the compounds that target this enzyme to treat Leishmania infection. Disruption of the arginase gene in Leishmania block the parasite development. Natural compounds such as flavonoids when used against Leishmania promastigotes infection have shown the reduction of promastigote load by 70%. The plants like Allium sativum containing quercitrin and other flavonoids have also shown activity against CL and VL\cite{36,37}. Querlin (flavonoid) inhibits enzymes arginase and ribonucleotide reductase\cite{38} and induce topoisomerase II for causing DNA cleavage which results in kDNA linearization\cite{39}. Chalcones (flavonoid) inhibit activity of fumarate reductase, succinate dehydrogenase, NADH dehydrogenase and NADH-cytochrome reductase in parasite mitochondria\cite{40}. The antileishmanial effect of this plant could be attributed to one of these groups of compounds. The crude extracts of S. acuta also exhibited intense activity against malaria parasites in vitro\cite{41}, significant analgesic and anti-inflammatory activities in experimentally infected mice\cite{42}. In addition, aqueous acetone extracts of S. acuta have shown negligible level of toxicity when administered orally\cite{43}.

Similarly, D. regia had shown comparable activities against sensitive and resistant strains after 48 hrs. The predominant phytochemicals found in D. regia were sterols, terpenes, flavonoids and phenolic compounds. The ergosterol and its derivatives constitute cell membranes, which play an important role in normal functioning of various biological processes\cite{44}. Therefore, the sterol biosynthesis pathway can be a auspicious target which can be explored in the development of new therapeutic agents for the treatment of parasitic diseases\cite{45}. Moreover, it has been reported in recent studies that a number of sterol derivatives have shown antileishmanial activities, and some of them such as azasterols are found to inhibit the activity of certain enzymes involved in the sterol biosynthesis pathway of these parasites\cite{46-48}. Based on these facts, the active sterols of D. regia in the present study might exhibit their effect by acting as competitive molecules of endogenous sterols and thus interfering or inhibiting sterol biosynthesis and cause organism’s death. The activity of this extract may also be due to the presence of flavonoids which provide protective role as chemo
preventive agent in cancer by interfering with signal transduction in cell proliferation and angiogenesis\textsuperscript{[49]}.

Table 3: Inhibitory and cytotoxic concentrations of plant extracts and SSG drug on SSG sensitive strain with respect to time

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plants</th>
<th>Inhibitory Concentration (IC$_{50}$±S.D) µg/ml</th>
<th>Cytotoxic concentration (CC$_{50}$±S.D) µg/ml</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>After 24 hours</td>
<td>After 48 hours</td>
<td>After 72 hours</td>
</tr>
<tr>
<td>1</td>
<td>D. regia</td>
<td>58.42±1.20</td>
<td>14.71±1.34</td>
<td>4.93±1.25</td>
</tr>
<tr>
<td>2</td>
<td>E. hirta</td>
<td>101.78±2.05</td>
<td>43.22±3.08</td>
<td>15.81±3.14</td>
</tr>
<tr>
<td>3</td>
<td>S. acuta</td>
<td>10.06±1.01</td>
<td>5.45±1.98</td>
<td>0.56±0.08</td>
</tr>
<tr>
<td>4</td>
<td>Standard drug</td>
<td>70.21±2.52</td>
<td>53.20±1.56</td>
<td>39.78±1.89</td>
</tr>
</tbody>
</table>

Table 4: Inhibitory and cytotoxic concentrations of plant extracts and SSG drug on SSG resistant strain with respect to time

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Plants</th>
<th>Inhibitory concentration (IC$_{50}$±S.D) in µg/ml</th>
<th>Cytotoxic concentration (CC$_{50}$±S.D) in µg/ml</th>
<th>Selectivity Index (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>After 24 hrs</td>
<td>After 48 hrs</td>
<td>After 72 hrs</td>
</tr>
<tr>
<td>1</td>
<td>D. regia</td>
<td>55.08±2.14</td>
<td>15.08±0.14</td>
<td>4.92±1.98</td>
</tr>
<tr>
<td>2</td>
<td>E. hirta</td>
<td>142.32±2.75</td>
<td>46.57±0.38</td>
<td>20.48±2.24</td>
</tr>
<tr>
<td>3</td>
<td>S. acuta</td>
<td>8.85±0.15</td>
<td>6.38±0.62</td>
<td>0.76±0.34</td>
</tr>
<tr>
<td>4</td>
<td>Standard drug (SSG)</td>
<td>142.15±2.61</td>
<td>115.49±2.1</td>
<td>87.16±2.5</td>
</tr>
</tbody>
</table>

Hence, a number of plant extracts have been exploited in the present study which exhibited interesting antileishmanial properties \textit{in vitro}, seeming to authenticate their use in traditional healthcare systems. Therefore, the vital components can be isolated from these natural sources which will represent a preliminary advancement in the search for novel antileishmanial agents at a time since there is an urgent need for new innovative drug leads. Moreover, they can be further explored for \textit{in vivo} studies so as to check their efficacy in experimental models.
Figure 1. Inhibitory concentrations of plant extracts and standard drug (SSG) on SSG sensitive and SSG resistant strain after 24 hrs
Results are expressed as Mean±S.D. for triplicate cultures
p value: SSG drug Vs D.regia/E.hirta/ S.acuta against SSG sensitive strain *p<0.05
p value: SSG drug Vs D.regia/E.hirta/ S.acuta against SSG resistant strain )#p<0.05

Figure 2. Inhibitory concentrations of plant and standard drug (SSG) on SSG sensitive and SSG resistant strain after 48 hrs
Results are expressed as Mean±S.D. for triplicate cultures
p value: SSG drug Vs D.regia/E.hirta/ S.acuta against SSG sensitive strain *p<0.05
p value: SSG drug Vs D.regia/E.hirta/ S.acuta against SSG resistant strain )#p<0.05
Figure 3. Inhibitory concentrations of plants and standard drug (SSG) on SSG sensitive and SSG resistant strain after 72 hrs

Results are expressed as Mean±S.D. for triplicate cultures

p value: SSG drug Vs D.regia/E.hirta/S.acuta against SSG sensitive strain *p<0.05

p value: SSG drug Vs D.regia/E.hirta/S.acuta against SSG resistant strain #p<0.05

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