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Leishmania donovani: Assessment of leishmanicidal effects of herbal extracts obtained from plants in the visceral leishmaniasis endemic area of Bihar, India

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ABSTRACT

One obstacle faced in the effective control of visceral leishmaniasis (VL) is the limited number of available treatment options. Furthermore, control efforts have been hindered further by the emergence of *Leishmania* resistance to many of the available drugs. In this study, we investigated the anti-leishmanial properties of 30 medicinally important plants from the VL endemic area of Bihar, India and compared them to two available anti-leishmanial drugs (sodium antimony gluconate and amphotericin B) and two plant lectins (phytohemagglutinin and concanavalin A) on *Leishmania donovani* promastigotes *in vitro* at 24 and 48 h after initiation of culture. We identified eight plant extracts in addition to phytohemagglutinin and amphotericin B that significantly inhibited the growth of promastigotes ($p < 0.03$). We further studied the minimum effective concentrations as well as the effect on axenic amastigotes viability and the cell cytotoxicity on human peripheral blood of four (*Agave americana, Azadirachta indica, Eclipta alba* and *Piper longum*) of the eight plant extracts that induced significant promastigotes killing ($p = 0.00098$). Effect-based dose finding analysis revealed that the threshold concentration of *A. americana* required to eliminate *L. donovani* after 24 h was 0.05 mg/ml. *A. indica* and *P. longum* plant extracts eliminated *L. donovani* promastigotes after 48 h at concentrations of 0.1 and 0.5 mg/ml, respectively. *E. alba* eliminated the promastigotes at a concentration of 0.5 mg/ml within 24 h. The axenic amastigote killing response was 1.90-, 2.52- and 1.3-fold higher than the promastigote killing response with *A. indica, A. americana* and *E. alba* plant extracts, respectively. *A. americana* and *A. indica*, respectively, led to approximate 2.5- and 1.3-fold declines in mitochondrial dehydrogenase activity compared with control. *E. alba* stimulation resulted in an up-regulation of dehydrogenase activity ($p = 0.00329$). The CSA from *P. longum* was found to be least cytotoxic; the observed difference in mitochondrial activity was insignificant ($p = 0.16314$). Further studies may reveal the pharmacological significance of many of the plants with anti-leishmanial properties identified in the present study.
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1. Introduction

Visceral leishmaniasis (VL), also known as Kala azar, is a major public health problem in India and many parts of the world (WHO Expert Committee, 1984); more than 20,000 cases of VL are reported annually in India alone (Ashford et al., 1992). The disease is fatal if left untreated. One of the available anti-leishmanial drugs, sodium antimony gluconate (SAG), is clinically unsatisfactory because many VL cases are not responsive to it; furthermore, the cases that do respond tend to relapse at a later stage (Thakur et al., 1998; Sundar et al., 2000). Pentamidine, another anti-leishmanial drug, is unsuitable as a first line treatment due to its toxicity. Amphotericin B and its liposomal formulation are effective, though such drugs are expensive and their use requires hospitalization. Reported clinical results using oral miltefosine treatment are encouraging; however, this drug is linked to potential teratogenicity and should not be given to pregnant women and those of childbearing age (Bhattacharya et al., 2004). The number of reported VL cases is increasing, and VL has also emerged as an opportunistic infection in patients infected with the human immunodeficiency virus (HIV) (Tremblay et al., 1996). Therefore, the development of cost-effective alternative therapeutic strategies has become a high-priority. Efforts aimed at screening medicinally important plants to identify novel therapeutic agents have attracted great attention in recent years (Wright and Phillipson, 1990; Iwu et al., 1994; Akendengue et al., 1999). Many plants possessing activity against *Leishmania* have been identified (Fournet et al., 1992; Singha et al., 1992; Chenari et al., 1999; Torres-Santos et al., 1999; Delorenzi et al., 2001; Plock et al., 2001; Ferreira et al., 2002; Salvador et al., 2002; Khalid et al., 2004; Luize et al., 2005; Mishra et al., 2005; Singh et al., 2004; Singh et al., 2005; Sharif

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et al., 2006; Lakshmi et al., 2007; Bafghi et al., 2008; El-On et al., 2009; Getti et al., 2009; Kivcak et al., 2009; Yousefi et al., 2009; Biswas et al., 2010). In this study, we assessed the leishmanicidal activities of crude soluble antigens (CSAs) derived from different parts of 30 plants from the VL endemic area of Bihar, India. We then tested their leishmanicidal activities against *Leishmania donovani* promastigotes *in vitro* in comparison to sodium antimony gluconate (SAG), amphotericin B, phytohemagglutinin (PHA) and concanavalin A (Con A). Studies on selected CSAs were extended to establish their minimum effective concentrations (MECs), their leishmanicidal effects on axenic amastigotes and their cytotoxic effects against human peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Preparation of crude soluble antigens (CSAs) from parts of medicinally important plants

CSAs were isolated from various parts of 30 different plant species (Table 1). The plants were selected based on previous reports of therapeutic properties in the medical literature and were collected from the VL endemic area of Bihar, India. In brief, we isolated distilled water (DW)- or 90% ethanol- (Eth) soluble plant sap from the following sources: air-dried twigs of *Andrographis paniculata, Dipteracanthus prostrates, Eclipta alba, Euphorbia hirta, Phyllanthus fraternus, Swertia chirata* and *Tinospora cordifolia*; leaves of *Acacia nilotica, Azadirachta indica, Calotropis procerra, Calotropis gigantia, Canavalia gladiate, Catharanthes roseous, Murraya koenigii, Oscimum sanctum, Phaseolus vulgaris, Ricinus communis, Solanum nigrum, Vitex negundo* and *Vitex trifolia*; stem bark of *A. nilotica* and *A. indica*; stems of *Dracaena angustifolia*; fruit pulp of *A. nilotica* and *C. gladiate*; and the fruit of *S. nigrum* and the dried rhizome of *Curcuma longa*. CSAs from *Zingiber officinalis* rhizomes and other succulents, including *Agave americana, Allium cepa, Allium sativum* and *Aloe barbadensis*, were obtained directly from fresh material. Hot infusions of *Opuntia dillenii* were obtained by squeezing modified stems that had been treated with hot water for several minutes. Ethanol-soluble plant saps were obtained from the following sources: air-dried twigs of *D. prostrates, E. alba, E. hirta* and *P. fraternus*; leaves of *C. procerra, C. gigantia, R. communis, V. negundo* and *V. trifolia*; the seed oil of *A. indica* and *R. communis*; and fresh leaves from *A. americana* and *A. barbadensis*. Dried or fresh plant materials were soaked overnight in either distilled water or ethanol and ground using a mortar and pestle before being centrifuged at 30000×*g* for 30 min in a chilled centrifuge (4 °C; Sorval RC 5C plus, Germany). The supernatants (CSA) were placed in Petri dishes and evaporated under reduced pressure at 37 °C. The dry extracts obtained from these solutions were weighed and dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mg/ml and then stored at −70 °C until further use. Oils of some seeds were extracted directly using a mortar and pestle and were stored at room temperature.

2.2. Promastigote and amastigote culture condition

WHO reference strain (MHOM/IN/80/DD8) and five other isolates of *L. donovani* from the Rajendra Memorial Research Institute of Medical Sciences (RMRIMS), Patna, India were used in this study. The promastigote strains were maintained in Tobie's biphasic medium and mass-cultured in RPMI-1640 medium (GIBCO, Invitrogen, USA) containing 20% heat inactivated fetal bovine serum (FBS; GIBCO, Invitrogen, USA), pH 7.2–7.4, at 24 ± 1 °C. Axenic amastigotes of the *L. donovani* reference strain and the other isolates from the RMRIMS were generated from the promastigote forms at 37 °C in a CO_2 incubator using the media described above at pH 5.5 (Chattopadhyay et al., 1996; Saar et al., 1998). Cultures were maintained through serial sub-culturing for further studies.

2.3. Treatment of L. donovani promastigotes with plant CSAs

To identify plant CSAs with anti-leishmanial activity, the effects of plant CSAs on the growth on the promastigotes of six different strains of *L. donovani* were evaluated. For these tests, a total of 2×10^6/ml early stationary phase *L. donovani* promastigotes (72 h culture) in RPMI-1640 complete media with 20% FBS were dispensed into 24-well culture plates. The culture was supplemented with plant CSAs at a final concentration of 0.5 mg/ml in duplicate series. Commercially available PHA (DIFCO) and Con A (Bangalore Genei, India) were used at a final concentration of 0.02 mg/ml (w/v). Negative control cultures were supplemented with an equal volume of normal saline (0.85% w/v NaCl, MERCK, India). In addition, positive control cultures were incubated with the following concentrations of two well-known anti-leishmanial drugs: 20 μg/ml SAG (Albert Devid Ltd., Calcutta, India) or 1 μg/ml amphotericin B (E.R. Squibb & Sons, NJ). Cultures were further incubated at 24 ± 1 °C and subjected to microscopic analysis after 24 and 48 h (Table 1) using a 0.1-mm Naubauer Chamber (Fein Optic, JENA, Germany; Jaffe et al., 1984).

2.4. Evaluation of minimum effective concentrations of plant CSAs showing significant leishmanicidal properties

We evaluated the MECs of PHA and the plant CSAs (*A. americana, A. indica, E. alba* and *P. longum*) that showed significant levels of *L. donovani* killing according to the tests described above. The leishmanicidal activities of these compounds against six strains of *L. donovani* promastigotes (2×10^6/ml) were evaluated in duplicate series (Fig. 1a–e) at final concentrations of 0.001, 0.02, 0.05, 0.1, 0.5, 1.0, 1.5 and 2.0 mg/ml. The culture conditions and analyses used were the same as described above. The concentration of the CSAs at or above the optimal promastigote killing concentration was defined as the MEC for the selected plants (Fig. 1a–e).

2.5. Comparison of the leishmanicidal activities of the CSAs against axenic amastigotes and promastigotes

The plant CSAs that were found to eliminate *L. donovani* promastigotes in the initial microscope-based studies (*E. alba, A. americana, P. longum* and *A. indica*) were further tested to compare their leishmanicidal activities against axenic amastigotes and promastigotes. *L. donovani* promastigotes and axenic amastigotes (2×10^6/ml) were incubated either with or without the determined MECs of CSAs for 1 h. The cultured cells were washed with PBS and stained with 0.1 μg/ml propidium-iodide (PI, BD Pharmingen, USA) for 10 min in the dark (Martin et al., 1995). The stained cells (10,000) were subjected to FL2/SSC plot analysis using a FACS Calibur sorter and CellQuest software (Becton Dickinson, CA, USA). The cell population appearing on lower right quadrant was analyzed to compare the leishmanicidal effects exerted on promastigotes and axenic amastigotes (Fig. 2).

2.6. Evaluation of cell toxicity of the selected plant CSAs at the MEC against human peripheral blood mononuclear cells

The mitochondrial dehydrogenase-based 3-(4,5-diamethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicities of plant CSAs against human PBMCs (Mossman 1983; Vistica et al., 1991). The assay was performed using a MTT-based *in vitro* toxicology assay kit (Sigma–Aldrich, USA). Briefly, PBMCs were isolated by density gradient centrifugation (800×*g*, 15 min) over Histopaque-1077 (Sigma, USA) from peripheral blood samples taken from five healthy human volunteers.

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