METHANOLIC BARK EXTRACT OF Sterculia villosa ATTENUATE THE INTRACELLULAR LEISHMANIAL LOAD THROUGH NITRIC OXIDE GENERATION AND IMMUNOMODULATION

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ABSTRACT
Visceral leishmaniasis is an immunosuppressive disease caused by the protozoan parasite Leishmania donovani. Visceral leishmaniasis affects millions of people all over the world, especially in subtropical countries. Presently available drugs against visceral leishmaniasis are restricted due to their higher toxicity. This condition further aggravated due to the development of resistance of the parasites against the available drugs. Nowadays several ethnomedical plant extract are in use for treatment of the protozoan diseases. Sterculia villosa is an ethnomedical plant and possess antimicrobial, antiprotozoal property. In the present study methanolic bark extract of Sterculia villosa (SVE) was prepared to evaluate its antileishmanial and immunomodulatory activity. To evaluate its antileishmanial study, intracellular parasitic burden, nitric oxide (NO) generation and cytokine production was studied in L. donovani infected murine peritoneal macrophages. It was observed that SVE inhibited the intracellular parasitic load in the murine peritoneal macrophages and IC₅₀ dose was found to be 50 μg/mL. It was also observed that SVE induces killing of intracellular parasite by up regulating pro-inflammatory and down regulating anti-inflammatory cytokine release. The present study explores that SVE at IC₅₀ dose can induce parasite killing through modulation of cytokine expression and NO generation in comparison with un-treated infected macrophages.

KEYWORDS: Leishmania donovani, SVE, amastigotes, nitric oxide, cytokines.

INTRODUCTION
Leishmania donovani, a protozoan parasite transmitted by female sandflies, causes visceral leishmaniasis or kala-azar. The parasites exist in two different morphotypes in their life cycle: the promastigote in sand fly vector and the amastigote in mammalian host. Millions of people are being affected by this fatal disease in tropical and subtropical countries such as India, Bangladesh, Nepal, Sudan, Brazil, and Ethiopia.[1] The available drugs [sodium antimony gluconate (SAG), amphotericin B, miltefosine] for the treatment of leishmaniasis have been shown to have toxicity, side effects, high cost and emergence of drug-resistant strains.[2, 3, 4] Therefore, there is a need to search for cheaper, more effective, easily available and less toxic chemotherapeutic agents for combating leishmaniasis. In this concept, the ethno-medical plants are gaining specific importance nowadays. Sterculia villosa Roxb (Malvaceae) possess anthelmintic, antimicrobial, antithrombotic and antiprotozoal activity.[5, 6, 7] Traditionally, the different parts of this plant is known to have diuretic, cooling, aphrodisiac and anti-inflammatory properties.[8, 9]

Infection by promastigote form of leishmania parasite initiated through phagocytosis by the host cells, including neutrophils, dendritic cells and macrophages that are involved in the clearance of invading microbes. Internalized promastigotes in the host cells can replicate after differentiating into non-motile aflagellar amastigotes within lysosome like compartments or parasitophorous vacuoles (pVs) and induces their pathogenicity in that amastigote form.[10] Since macrophages are specialized for the destruction of invading pathogens by the generation of oxidative stress (nitric oxide and reactive oxygen species) and immune modulation.[11] The present study was carried out with an aim to evaluate the antileishmanial and immunomodulatory efficacy of methanol bark extract of Sterculia villosa against amastigote form of L. donovani.
MATERIALS AND METHODS

Plant material
The stem bark of Sterculia villosa was collected from the region of Suryamaninagar, Agartala, Tripura. The plant was identified by Taxonomist Prof. B. K. Datta, Department of Botany, Tripura University. A voucher specimen (Code No. TR0115) was deposited in the herbarium of the Department of Botany, Tripura University and an accession No. 0495 was assigned to the specimen. Bark of Sterculia villosa were washed properly, cut into small pieces and then shade dried for 30 days.

Preparation of plant extract
Air dried small pieces of S. villosa (3.3 kg) bark was extracted with MeOH (6.0 L each) (Himedia, India) at room temperature (three times) for 7 days. The whole mixtures were then filtered through a fresh cotton plug and finally with a Whatman No.1 filter paper. Following filtration the volume of filtrate was reduced using rotary evaporator (Superfit Rotavat, model no. PBV-7D, India) and dried in vacuum oven at 45°C. Finally, 113 gm of crude methanol bark extract of Sterculia villosa (SVE) was prepared.

Parasites
Promastigote forms of Leishmania donovani (MHOM/IN/1983/AG83) parasites were cultured in RPMI 1640 liquid media (Himedia, India) (pH 7.4) at 22°C supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 IU/mL of penicillin and 100 mg/mL of streptomycin (Himedia, India). [12]

Animal
BALB/c mice (4–6 weeks old) weighing about 20 g were procured from the National Centre for Laboratory Animal Sciences, Hyderabad, India. Animals were maintained in an environmentally controlled room with 12-h light/dark cycle and fed standard diet and water ad libitum during experimental period. Experiments were carried out as per the guidelines of the Animal Ethics Committee of Tripura University (Registration Number: 1667/GO/a/12/CPSEA dated 12/11/2012), Suryamaninagar, Tripura, India.

Isolation of peritoneal macrophages
Mouse peritoneal macrophages were isolated by peritoneal lavage with ice-cold PBS after 48 h of intraperitoneal injection of sterile 4% thioglycolate broth (Sigma, India). Cells were cultured as described previously. [13]

Effect of SVE on intracellular parasitic load
To investigate the effect of SVE on intracellular amastigotes, peritoneal macrophages (10^6 cells) isolated from BALB/c mice were infected with L. donovani promastigotes (macrophage to parasite ratio at 1:10) and incubated at 37°C in presence of 5% CO2. Following infection for 4 h, the macrophages were treated for 48 h with graded doses (0–100 µg/mL) of SVE. After treatment, the antileishmanial efficacy of SVE towards the intracellular amastigotes was evaluated through microscopic counting of the number of amastigotes per 100 macrophages by the Giemsa staining method. Intracellular parasitic load in treated infected macrophages were compared with untreated infected macrophages. [14]

Nitrite generation assay
Nitrite accumulation in culture was measured colorimetrically by the Griess reaction as described previously. [15] Briefly, peritoneal macrophages (1 x 10^6 cells/mL) were infected with Leishmania parasite (macrophages to parasite ratio at 1:10) and incubated with SVE at IC_{50} concentration for 48 h. Cell-free supernatants were collected from different experimental sets (un infected macrophages, un infected macrophages treated with LPS, infected macrophages, SVE treated infected and un infected macrophages) after 48 h of treatment. 100 µL of supernatant were incubated with an equal volume of Griess reagent (Sigma, India) for 10 min. The absorbance at 550 nm was then measured by a microtitre plate reader (Bioread H1 Hybrid Reader). The standard curve for nitrite was prepared by using 10–100 µM sodium nitrates in distilled water. Data were expressed as micromoles of nitrite.

Measurement of cytokine release by sandwich ELISA
The level of murine pro-inflammatory cytokines (TNF-α, IL12p40) and anti-inflammatory cytokines (IL-10, TGF-β) were measured following treatment with or without SVE against Leishmania infected or uninfected macrophages using a sandwich ELISA kit (Abcam, US). The assay was performed as per the detailed instructions of the manufacturer.

STATISTICAL ANALYSIS
All experiments were performed in triplicate. The values were mean of three assays ± SD. Significance level was determined by using one way ANOVA. Data were presented as P value < 0.01 (noted with *), P value <0.001 (noted with **) and P value <0.0001 (noted with ***). Statistical software Graph Pad Prism 6.0 (Graph Pad, CA, US) was used for all statistical analysis.

RESULTS
Methanol bark extract of Sterculia villosa reduces intracellular parasitic load
Effect of SVE on intracellular amastigote was evaluated through counting the number of amastigotes per 100 macrophages by the Giemsa staining method. Macrophages were counted under microscope (40X). At 48 hrs of infection the number of amastigotes in infected macrophages was found to be 612/100 macrophages. It was found that SVE markedly inhibited the intracellular parasitic load in peritoneal macrophages (209 no. of amastigotes per 100 macrophages at 100 µg/mL dose of SVE). The IC_{50} dose of SVE was found to be 50 µg/mL (Figure 1).
Effect of SVE on NO generation in *L. donovani*-infected murine peritoneal macrophages

To study the effect of SVE on nitrite production in macrophages, NO release was determined in treated infected peritoneal macrophages with respect to untreated infected macrophages. It was observed that in untreated infected macrophages NO generation is significantly reduced in comparison to uninfected macrophages. NO production in SVE treated *L. donovani*-infected macrophages was significantly increased (about 6.5 fold) in comparison with untreated infected macrophages (Figure 2).

Effect of SVE on cytokine production

Infection of peritoneal macrophages with *L. donovani* promastigotes resulted in the suppression of the release of pro-inflammatory cytokines (TNF-α and IL-12) and upregulation of anti-inflammatory cytokines (IL-10 and TGF-β). Following treatment it was observed that SVE (50 μg/mL) increases the release of TNF-α and IL-12p40 (5.8 and 2.8 fold respectively) in *L. donovani*-infected macrophages in comparison with untreated infected macrophages [Figure 3A(i)(ii)]. In addition to the generation of pro-inflammatory cytokine, effect of SVE (50 μg/mL) on the release of anti-inflammatory cytokines like IL-10 and TGF-β were also analyzed. It was observed that release of IL-10 and TGF-β were reduced to about 4.5 and 3.9 fold respectively with respect to untreated infected macrophages [Figure 3B (i) (ii)]. Thus, it can be infer that the treatment with SVE in *L. donovani* infected macrophages leads to inhibition of anti-inflammatory cytokines and upregulation of pro-inflammatory cytokines.

**Figure legends:**

**Figure 1:** Determination of antileishmanial activity of SVE on the parasite burden in *L. donovani*-infected murine peritoneal macrophages. Macrophages were cultured in complete RPMI 1640 medium overnight and infected with *L. donovani* promastigotes for 4 h at a macrophage-to-parasite ratio of 1:10. The cells were washed and treated with graded concentration of SVE (1 to 100 μg/mL). The cells were incubated for another 48 h, stained with Giemsa, and the numbers of intracellular amastigotes per 100 macrophages counted.

**Figure 2:** Determination of antileishmanial activity of SVE through nitric oxide generation in *L. donovani* infected murine peritoneal macrophages. Macrophages isolated from BALB/c mice were cultured and incubated with *L. donovani* promastigotes (macrophage-to-parasite ratio, 1: 10), LPS (100 ng/mL) SVE (50 μg/mL) or SVE plus *L. donovani*. The cells were kept for 48 h for maximum nitrite generation and the cell-free supernatants were collected and subjected to a nitrite generation assay, as described in the Materials and methods section.

**Figure 3:** Effect of SVE on pro-inflammatory and anti-inflammatory cytokine production in *L. donovani* infected peritoneal macrophages. Peritoneal macrophages were cultured and then infected with *L. donovani* promastigotes (macrophage-to-parasite ratio, 1: 10), washed after 4 h and treated with SVE (50 μg/mL). The cells were incubated for another 24 h, following which the cell-free supernatants were collected and subjected to sandwich ELISA to detect A(i) TNF-α (pg/mL), (i) IL-12 (pg/mL), B(i) IL-10 (pg/mL) and (ii) TGF-β (pg/mL) secretion.

**DISCUSSION**

Visceral leishmaniasis is the foremost pathogenic manifestation of *L. donovani* infection which is well associated with fever, cachexia, hepatosplenomegaly, blood cytopenia and immune suppression.[16,17] At present, there are many antileishmanial drugs such as sodium stibogluconate, meglumine antimoniate, amphotericin B and miltefosine, but due to their toxicity, high cost and gradual development of resistance to parasites emphasize the search for new antileishmanial agent. Plant extracts or plant-derived compounds are likely to be a valuable source of new therapeutic.
agents. S. villosa is known to have ethnomedicinal importance against skin disease, inflammation, anthelmintic, microbial infection and rheumatism. In the present work we have prepared methanolic bark extract of S. villosa (SVE) in the direction to evaluate its antileishmanial activity where we have focused mainly on L. donovani amastigotes. In general leishmania parasite has two phases such as intracellular amastigote and extracellular promastigote form. As amastigote is the infective state of the parasite where they can execute intracellular infection thus the present study was performed against amastigote form of the parasite. It was observed that SVE significantly reduces the intracellular parasitic burden in L. donovani infected peritoneal macrophages. The IC50 dose of SVE was determined as 50 μg/mL. Further it was observed that SVE induces intracellular parasitic killing by induction of the nitric oxide generation and pro-inflammatory cytokine response. SVE mediate significant induction of NO generation in SVE treated infected macrophages compared to untreated infected macrophages. Reactive nitrogen species (RNS) are antimicrobial molecules derived from nitric oxide (NO) and superoxide (O2-) mediated by the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase respectively. NOS2 is expressed primarily in macrophages after induction by cytokines and other molecules. Thus induction of NO generation by macrophages after SVE treatment may participate in parasite killing process. It is well established that during leishmanial infection there is an alteration in cytokine release for the intracellular survival of the amastigote form of the parasite. It was observed that SVE (50 μg/mL) treatment could induces the release of pro-inflammatory cytokine (TNF-α, IL-12p40) and inhibits the release of anti-inflammatory cytokine (IL-10, TGF-β) in L. donovani-infected murine peritoneal macrophages. Therefore, treatment with SVE resulted in generation of pro-inflammatory environment in infected macrophages which might be responsible for the suppression in intracellular parasitic burden.

CONCLUSION
The present study reveals that, SVE executed reduced intracellular parasitic load through NO generation and immunomodulation. On this observation we can infer that SVE is a potent antileishmanial agent against amastigote form of Leishmania donovani.

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