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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 ethnobotanical plant extract are in use for treatment of the protozoan diseases. *Sterculia villosa* is an ethnomedicinal 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 IC_{50} 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 IC_{50} 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-medicinal 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 45ºC. 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 22ºC 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 37ºC in presence of 5% CO_2. 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* paraite (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 (Biotek Synergy H1 Hybrid Reader). The standard curve for nitrite was prepared by using 10-100 µM sodium nitrites 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 un-treated 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:

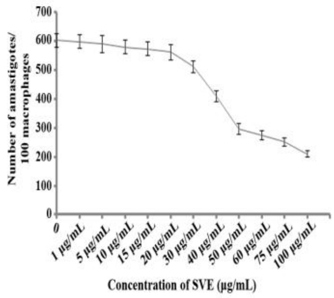

Figure 1: Determination of antileishmanial activity of SVE on the parasite burden in *L. donovani*-infected murine peritoneal macrophages. Macrophages were cultured in complete RPMI 1640 medium overnight and infected with *L. donovani* promastigotes for 4 h at a macrophage-to-parasite ratio of 1:10. 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.

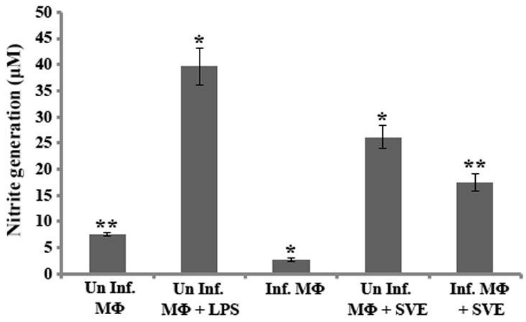

Figure 2: Determination of antileishmanial activity of SVE through nitric oxide generation in *L. donovani* infected murine peritoneal macrophages. Macrophages isolated from BALB/c mice were cultured and incubated with *L. donovani* promastigotes (macrophage-to-parasite ratio, 1: 10), LPS (100 ng/mL) 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.

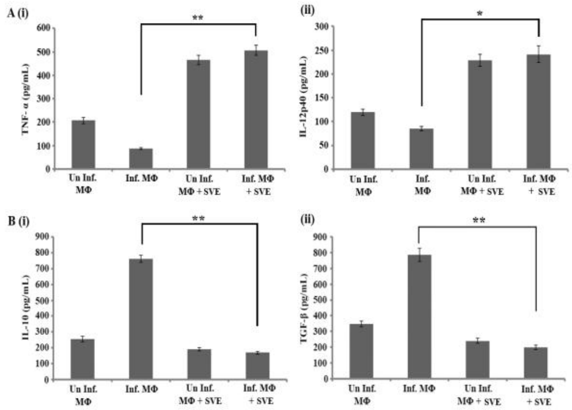

Figure 3: Effect of SVE on pro-inflammatory and anti-inflammatory cytokine production in *L. donovani* infected peritoneal macrophages. Peritoneal macrophages were cultured and then infected with *L. donovani* promastigotes (macrophage-to-parasite ratio, 1: 10), washed after 4 h and treated with SVE (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), (ii) 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.[18, 19] *Sterculia villosa* is known to have ethnomedicinal importance against skin disease, inflammation, anthelmintic, microbial infection and rheumatism.[20, 21, 22, 23] 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 amastogote 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 IC_{50} 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 (O_2^-) 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 participates in parasite killing process. It is well established that during leishmanial infection there is an alteration in cytokine release for the intracellular survivability 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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REFERENCES

1. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J, Boelaert M. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol, 2007; 5: 873–882.
2. Wortmann G, Zapor M, Ressner R, Fraser S, Hartzell J, Pierson J, Weintrob A, Magill A. Lipsosomal amphotericin B for treatment of cutaneous leishmaniasis. Am J Trop Med Hyg, 2010; 83:1028–1033.
3. Rahman M, Ahmed BN, Faiz MA, Chowdhury MZ, Islam QT, Sayeedur R, Rahman MR, Hossain M, Bangali AM, Ahmad Z, Islam MN, Mascie-Taylor CG, Berman J, Arana B. Phase IV trial of Miltefosine in adults and children for treatment of visceral leishmaniasis (kala-azar) in Bangladesh. Am J Trop Med Hyg, 2011; 85:66–69.
4. Ashutosh Sundar S, Goyal N. Molecular mechanisms of antimony resistance in *Leishmania*. J Med Microbiol, 2007; 56: 143–153.
5. Haque A, Alam MR, Raton M, Hassan MM, Kadir MF, Islam SMA. Anthelmintic and diuretic activity of bark extracts of *Sterculia villosa*. J Appl Pharm Sci, 2012; 2: 86-89.
6. Tania KN, Islam MT, Mahmood A, Ibrahim M, Chowdhury MMU, Kuddus MR. Pharmacological and phytochemical screenings of ethanol extract of *Sterculia villosa* Roxb. J Biomed Pharmacol, 2013; 2: 09-14.
7. Nwodo NJ, Ibezim A, Ntie-Kang F, Adikwu MU, Mbah CJ. Anti-Trypanosomal Activity of Nigerian Plants and Their Constituents. Molecules, 2015; 20: 7750-71.
8. Kumar R, Suman NR, Dash SS. Traditional uses of plants by tribals of Amara kantak region, Madhya Pradesh. Indian J Trad Knowledge, 2004; 3: 383-90.
9. Namsa ND, Tag H, Mandal M, Kalita P, Das AK. An ethnobotanical study of traditional anti-inflammatory plants used by the Lohit community of Arunachal Pradesh, India. J Ethnopharmacol, 2009; 125: 234-45.
10. Courret N, Fréhel C, Gouhier N, Pouchelet M, Prina E, Roux P, Antoine JC. Biogenesis of *Leishmania*-harbouring parasitophorous vacuoles following phagocytosis of the metacyclic promastigote or amastigote stages of the parasites. J Cell Sci, 2002; 115: 2303-16.
11. Olivier M, Gregory DJ, Forget G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. Clin Microbiol rev, 2005; 8: 293‑305.
12. Palit P, Ali N. Oral therapy with sertraline, a selective serotonin reuptake inhibitor, shows activity against *Leishmania donovani*. J Antimicrob Chemother, 2008; 61:1120-4.
13. Fahey TD, Tracey KJ, Tekamp-Olson PA, Cousens LS, Jones WG, Shires GT, Cerami A, Sherry B. Macrophage inflammatory protein 1 modulates macrophage function. J Immunol, 1992; 148: 2764-9.
14. Rudrapaul P, Sarma IS, Das N, De UC, Bhattacharjee S, Dinda B. New flavonol methyl

ether from the leaves of *Vitex peduncularis* exhibits potential inhibitory activity against *Leishmania donovani* through activation of iNOS expression. Eur J Med Chem, 2014; 87: 328-35.

15. Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages: comparison of activating cytokines and evidence for independent production. J Immunol, 1988; 141: 2407e2412.
16. Carvalho EM, Teixeira RS, Jr Johnson WD. Cell-mediated immunity in American visceral leishmaniasis: reversible immunosuppression during acute infection. Infect Immun, 1981; 33: 498-502.
17. Carvalho EM, Badaro R, Reed SG, Jones TC, Jr Johnson WD. Absence of interferon and interleukin 2 production during active visceral leishmaniasis. J Clin Invest, 1985; 76: 2066-2069.
18. de Carvalho PB, Ferreira EI. Leishmaniasis phytotherapy. Nature's leadership against an ancient disease. Fitoter, 2007; 2: 599-618.
19. Kayser O, Kiderlen AF. *In vitro* leishmanicidal activity of naturally occurring chalcones. Phytother Res, 2001; 15: 148-152.
20. Kunwar RM, Shestha KP, Bussmann RW. Traditional herbal medicine in Far-west Nepal: a pharmacological appraisal. J Ethnobiol Ethnomed, 2010; 6: 1.
21. Haque A, Alam MR, Raton M, Hassan MM, Kadir MF, Islam SMA. Anthelmintic and diuretic activity of bark extracts of *Sterculia villosa*. J Appl Pharm Sci, 2012; 2: 86-89.
22. Hossain MK, Prodhan MA, Hasan IMSA, Morshed H, Hossain MM. Anti-inflammatory and antidiabetic activity of ethanolic extracts of *Sterculia villosa* barks on albino Wistar rats. J Appl Pharm Sci, 2012; 2: 96-100.
23. Tania KN, Islam MT, Mahmood A, Ibrahim M, Chowdhury MMU, Kuddus MR. Pharmacological and phytochemical screenings of ethanol extract of *Sterculia villosa* Roxb. J Biomed Pharmacol, 2013; 2: 09-14.