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In vitro and *in vivo* evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection

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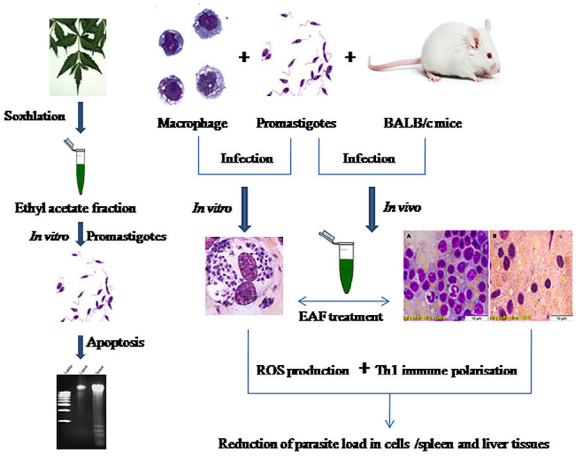
HIGHLIGHTS

- Anti-leishmanial activity of Neem leaf ethyl acetate fraction on promastigotes.
- Anti-proliferation activity and apoptosis like death in promastigotes.
- Anti-leishmanial activity on intracellular amastigotes both *in vitro* and *in vivo*.
- Th1 polarization and Th2 downregulation at m-RNA level by real-time PCR analysis.
- Increased production of reactive oxygen and nitrogen species from macrophages with extract treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity and emergence of resistance to available chemical drugs against visceral leishmaniasis is evoking to explore herbal treatment. One such attempt with the Neem is being reported here. The current study is primarily focused to evaluate the anti-leishmanial effects of Neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC_{50} to be 52.4 μg/ml on the promastigote stage. Propidium iodide (PI) staining of dead cells substantiated the aforementioned activity. Carboxy fluorescein–diaceate succinimidyl ester (CFSE) staining of promastigotes has affirmed its anti-proliferation activity. The characteristic features such as DNA fragmentation, reduced mitochondrial membrane potential, increased sub G_0/G_1 phase parasites and increased reactive oxygen species (ROS) production in EAF treated promastigotes indicate the apoptosis like death. In addition, the reduced parasite burden both *in vitro* (*viz.* ~45% in human monocytic leukemia cell line (THP-1) and ~50% in peripheral blood mononuclear cells) and *in vivo* (spleen and liver) provides the evidence for its anti-leishmanial activity on amastigote stage. The increase of ROS levels

Abbreviations: EAF, ethyl acetate fraction; VL, visceral leishmaniasis; THP-1, human monocytic leukemia cells; PBMCs, peripheral blood mononuclear cells; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ROS, reactive oxygen species; Ψ_m, mitochondria membrane potential; MFI, mean fluorescence intensity; LDU, Leishman–Donovan units; NO, nitric oxide.

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in THP-1 and nitric oxide (NO) production from J774.1 cell line (mouse macrophages) upon EAF treatment was evidenced for oxidative killing of intracellular amastigotes. Active immunomodulatory activity at m-RNA level (*viz.* upregulation of Th1 cytokines, and downregulation of Th2 cytokines) both *in vitro* and *in vivo* was also shown to be exhibited by EAF. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of EAF revealed the presence of 14 compounds.

1. Introduction

Visceral leishmaniasis (VL), commonly called as kala-azar, is caused by an intracellular protozoan parasite belonging to the genus *Leishmania*. This parasite commonly exists in two morphological forms: promastigotes and amastigotes, the former being the developmental and infective stage, occur only in the insect vector *i.e.*, female sandflies. During their blood meal, sandflies inject infective metacyclic promastigotes (highly motile form) into the vertebrate host. These are engulfed by host phagocytes and fused with lysosome to form phagolysosome wherein the parasites are transformed into amastigotes (non-motile form). This stage is responsible for the pathologies associated with leishmaniasis. It is a systemic infection, and affects vital organs such as spleen, liver, lymph nodes, etc. It is a serious public health problem in developing countries due to poor hygienic conditions and is fatal, if left untreated. It is estimated that 2–4 lakh new cases occur every year around the world with more than 10% deaths, among which 90% occur in the Indian subcontinent, Sudan, South Sudan, Ethiopia and Brazil (Alvar et al., 2012). In humans, protection against VL is mediated by Th1 response characterized by higher production of IFN-γ whereas pathogenesis is directly associated with IL-10 (Ghalib et al., 1993; Kharazmi et al., 1999). Peripheral blood mononuclear cells (PBMCs) from active VL patients fail to produce immune response when stimulated with leishmanial antigens *in vitro* suggesting immune dysfunction (Kumar and Nylén, 2012).

There is no prophylactic vaccine for human VL, and the treatment has to rely on anti-leishmanial drugs. Oral miltefosine is currently recommended drug in the Indian subcontinent for VL elimination program. However, its high cost, teratogenic potential and rapid emergence of resistance are major drawbacks (Sundar et al., 2007). VL co-infected with human immunodeficiency virus (HIV) is another challenge (Alvar et al., 2008). Thus, there is an immediate necessity of effective, safe and low cost drugs to combat this disease. Plant based products may play a pivotal role in search of a better anti-leishmanial compound.

Azadirachta indica is one such plant with abundant medicinal values commonly called as Neem (Biswas et al., 2002). It is a traditional medicinal plant growing profusely in tropical countries where leishmaniasis is endemic. All kinds of Neem plant parts are sourced for many therapeutic agents. It has anti-inflammatory, immune modulatory and anti-carcinogenic properties (Dholi et al., 2011; Ray et al., 1996; Subapriya et al., 2006). It functions as an immune booster by inducing the humoral and cell mediated immunity, phagocytic activity of macrophages and NO production (Abhishek et al., 2009). Notably, Neem oil is able to kill multi-drug resistant bacteria species from human infections (Jain et al., 2013). Thus, there has been much interest in recent years regarding the use of various Neem products in ayurvedic and herbal medicines. This study was carried out with the objective of evaluating the anti-leishmanial and immunomodulatory activities of Neem leaf extract in *L. donovani* infection.

2. Materials and methods

2.1. Chemicals

M199 medium, fetal bovine serum (FBS), penicillin and streptomycin, propidium iodide (PI), phorbol 12-myristate 13-acetate (PMA) and Ficoll–Histopaque were purchased from Sigma Aldrich (India). JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide), carboxy fluorescein-diaceate succinimidyl ester (CFSE) were purchased from e-Bioscience Inc; H_2DCFDA (2′, 7′-dichloro dihydrofluorescein diacetate) was purchased from Molecular Probes. Miltefosine was purchased from Cayman Chemicals, India.

2.2. Parasite

L. donovani strain (Dd8) was obtained from ATCC (American Type Culture Collection, U.S.A.). Promastigote stage was cultured at 25 °C ±1 in M199 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES pH7.4, 4 mM $NaHCO_3$, 100 U/ml of penicillin and 100 mg/ml of streptomycin.

2.3. Preparation of Neem leaf extracts

Neem leaves were collected during November 2011 at University of Hyderabad campus, Hyderabad, Andhra Pradesh. Leaves were dried in shade and powdered for extract preparation. Extracts have been prepared using a Soxhlet apparatus with solvents like hexane, ethyl acetate, alcohol and water according to increasing order of the polarity (Chandrasekaran et al., 2013).

2.4. In vitro *studies on promastigotes*

2.4.1. Anti-leishmanial activity by MTT assay

Cytotoxicity was performed using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. Exponentially growing log phase promastigotes

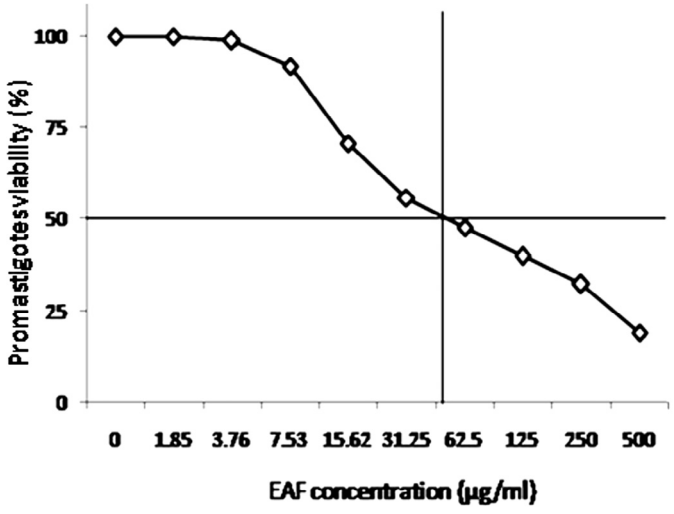

Fig. 1. MTT assay; X-axis represents serial dilution of EAF concentrations in µg/ml and Y-axis represents % of viable promastigotes. The graph used to determine the IC_{50} of EAF is 52.4 µg/ml. As the EAF concentration increases (0, 1.8, 3.9, 7.8, 15.6, 31.2, 62.2, 125, 250 and 500 µg/ml) the promastigotes viability proportionally inhibited.