Original Article

In vitro and *In vivo* Efficacy of a New Herbaceous Indian Plant- *Abutilon indicum* Against *Leishmania donovani* Infection

Prashant Khare[1], Preeti Rastogi[2], Swati Gupta[1], Rakesh Maurya[2] and Anuradha Dube*[1]

[1]Division of Parasitology, Central Drug Research Institute, Lucknow- 226 031 (India)
[2]Division of Medicinal & Process Chemistry, Central Drug Research Institute, Lucknow- 226 031 (India).

Address for Correspondence

Division of Parasitology, Central Drug Research Institute
New Campus, BS 10/1, Sector 10, Jankipuram Vistar, Lucknow-226031
E-mail:
anuradha_dube@rediffmail.com

ABSTRACT

The control of visceral leishmaniasis caused by *Leishmania donovani* still remains a challenging problem. Due to limitations of available therapies, search for new antileishmanial agents becomes necessary. India, being rich in traditional medicinal plant species, provides good opportunity for exploration of new, effective and safe antileishmanials. The seeds of *Abutilon indicum,* a herbaceous plant commonly known as Thuthi and used as a remedy for jaundice, piles, ulcer and leprosy, were evaluated for the first time for its antileishmanial activity. The crude methanolic extract and two of its four fractions - n-hexane and n-butanol were found to be highly active against *Leishmania* promastigotes and intracellular amastigotes. When administered orally to *L. donovani* infected hamsters the extract and its two fractions at a dose of 500 mg/kg x5 days exhibited ~75% efficacy. The results suggested towards the potentiality of this plant's seeds as a source of new antileishmanial leads for further investigations.

Keywords: *Abutilon indicum*, Antileishmanial efficacy, *Leishmania donovani*; Amastigote, Macrophage, Hamster.

INTRODUCTION

Visceral leishmaniasis (VL) caused by protozoan parasite *Leishmania donovani* is regarded as a major public health problem causing significant morbidity and mortality in tropical and sub-tropical countries. First-line treatment for VL is dependent on an age old pentavalent antimonials against which Indian sub-continent has witnesses a marked unresponsiveness as upto 65% treatment failures were observed in Bihar state of India. Other drugs, amphotericin B desoxycholate and liposomal amphotericin B in use were however, expensive and cause toxicity complications. Recently two new drugs, Miltefosine and Paromomycin were registered for VL treatment in India and in spite of their excellent efficacy the possibility of emergence of resistance against these drugs has remained[1].

In recent years, there has been growing interest in the use of plants derived natural products and India being rich in traditional medicinal plant species, provides good opportunity to explore them in a search of new, effective and safe antileishmanial compounds. *Abutilon indicum* belonging to the family Malvaceae is commonly known as Thuthi and has been used in the Siddha system of medicine as a remedy for jaundice, piles, ulcer and leprosy[2]. The plant grows as a weed and is found abundantly in wasteland from the seashore to 2000 meters high in India, and in sub Himalayan tracks[3]. It is a herbaceous or shrubby, softly tomentose plant and its stems are round often tinged with purple colour. It is not a poisonous plant and there is no report of any adverse effects. Its roots are used as diuretic and for leprosy; leaves for ulcers, headaches, gonorrhea & bladder infection. *A. indicum* having β-sitosterol isolated from the petroleum ether extract of leaf of showed mosquito larvicidal activity[4]. The plant possesses analgesic activity[5] and in some places, juice from leaves of this plant is used in combination with the liquid extract of *Allium cepa* to treat jaundice and liver disorders. The plant has also been reported to possess antibacterial activity against gram positive bacteria *Bacillus cereus*, *Staphylococcus aureus* and gram negative bacteria *Klebsiella pneumoniae, Escherichia coli*[6]. In this communication we have for the first time explored seed extracts of this plant for its antileishmanial potential.

MATERIALS AND METHODS

Plant Material

A. indicum seeds were collected from Regional Research Laboratory campus, Bhuvaneshwar, Orissa, India in February, 2006, and identified by Division of Botany, Central Drug Research Institute. Voucher specimen (No.8507) is kept in the herbarium of the institute.

Extraction and Fractionation

A powdered *A. indicum* seed (1.7Kg) with 95% ethanol (5 L) in a glass percolator was allowed to stand at room temperature for about 24 h. The percolate was collected. This process of extraction was repeated four times. The combined extract was filtered, concentrated under vacuum using rotavapor at 40^{o}C and weighed (EE 65 g, 3.82%). This ethanolic extract (35g) was partitioned successively with n-hexane (200mLx5), chloroform (200mLx5) and n-butanol saturated with water (200mLx6) affording a hexane (F002, 13g) $CHCl_3$ (F003, 7g), n-butanol (F004, 8g) and aqueous (F005, 6g) soluble fractions.

Parasite and Hosts

L.donovani (Dd8 strain) promastigotes were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (Sigma, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 26^{0}C. Transgenic parasites expressing green fluorescent protein (GFP) were maintained in presence of 100 µg/ml of antibiotic G418[7]. Mouse macrophage adherent cell line J774A.1 was maintained in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% heat inactivated FBS at 37ºC in humidified atmosphere with 5% CO_2.

Male golden hamsters (*Mesocricetus auratus*) weighing approximately 50g were used as the experimental host in this study. Animals were kept in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton India, Mumbai) and water *ad libitum*. Infection to naive hamsters was established by spleen-derived amastigotes as per method described elsewhere[8]. The use of animals for all the experiments was in compliance with the relevant guidelines of the institutional animal ethics committee

Experimental Protocols

In vitro testing

For testing against transgenic GFP-expressing log phase promastigotes, 1×10^6 cells/ml were put into 48-well tissue culture plates (CellStar) and different concentrations of test samples were administered. At 72 h after treatment the effect of extract and fractions was assessed by flow cytometry as described by Dube et al[8]. Untreated cells served as control. The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated parasites with control parasites.

The efficacy was also checked against intracellular amastigotes and for this J774A.1 macrophages (10^5 cells/well) growing in 24 well plates were infected with metacyclic GFP-promastigotes at 10:1 (parasite /macrophage) ratio and incubated at 37°C in 5% CO_2 for 12-24 h. Non-phagocytosed parasites were removed by washing and finally the wells were supplemented with complete medium. At 72 h after treatment, cells were washed with PBS, transferred to tubes and analyzed by FACS as described above. The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated infected MQs with that of untreated infected MQs. Miltefosine was used as a reference antileishmanial drug against both the stages. Each assay was performed in triplicate.

In vivo testing

Antileishmanial activity of seeds of *A. indicum* against *L.donovani* was assessed *in vivo* at different doses by oral route (p.o.). Twenty five day old *L.donovani* infected hamsters were subjected to splenic biopsy to estimate the initial parasite load. Thereafter the animals were divided into groups of 3 animals each and the treatment was initiated in one group at a dose level of 500mg/kg and continued for 5 consecutive days. The second group of animals was treated orally with the reference drug - Miltefosine at a dose of 40mg/kg×5. Third group served as untreated control. Splenic biopsies were performed on days 7 and 28 p.t. and the percent inhibition was assessed using the formula as described elsewhere[8]. The other doses of test samples were also tested to obtain optimum efficacy.

Assessment of NO activity

The presence of NO was assessed using Griess reagent in the culture supernatants of macrophage cell lines (J774.1). LPS (10ug/ml Sigma, USA) was used as mitogen. Cultured macrophages were suspended in culture medium and plated at 10^6cells/well and exposed to the methanolic extract and fractions of above described from all the study groups. The supernatants (100 ul) collected from macrophage cultures 24 h after incubation was mixed with an equal volume of Griess reagent (Sigma, USA) and left for 10 min at room temperature. The absorbance of the reaction was measured at 540nm in an ELISA reader[9].

Statistical Analysis

Two to three replicates were done in case where activity was observed for confirmation and results were expressed as mean ± SD. Statistical analyses were performed using Graphpad prism software.

RESULTS AND DISCUSSION

In a bid to explore those plant species which are commonly used in traditional herbal medicine for treating various parasitic diseases for their antileishmanial potential, *A.indicum* was taken as it was reported to possess various pharmacological activities. In this study we have assessed the antileishmanial activity of the seeds of this plant. The ethanol extract of the seeds of *A. indicum* as well as two of its fractions, n-hexane fraction and n-butanol fraction have exhibited dose-dependent antileishmanial

activity against promastigote and intracellular amastigote forms of *L.donovani in vitro*. The crude extract exhibited 94.3±6.9% and 78.2±5.5% efficacy against *Leishmania* promastigote and intracellular amastigotes, respectively, at 100 µg/ml concentration (Table 1). At a lower dose of 50 µg/ml, it demonstrated 57.6%±5.1% and 49.7±6.2% efficacy against promastigotes and intracellular amastigotes respectively. Among the fractions, n-Hexane fraction was the most potent resulting in 90.3±7.4% and 72.2±6.4% efficacy against promastigotes and 76.7±3.7% and 54.4±4.2% efficacy against amastigotes at 100- and 50µg/ml concentrations followed by n-butanol fraction (89.1±7.7% and 67.8±8.4% efficacy against promastigotes and 69.6±6.4%and 46.23±5.2% efficacy against amastigotes) at respective concentrations of 100 and 50µg/ml. Chloroform and aqueous fractions were inactive against both the stages of the parasite (Table-1). Good antileishmanial activity of this plant against clinical relevant (amastigote) stage is encouraging.

NO are firmly established to play a role as antimicrobial effector molecules produced by activated Mϕs[10,11], hence its production in J774.1 macrophage was studied after 24 h of incubation in the presence of extract and fractions. For comparison, NO production in mitogenic (LPS) stimulated and untreated cells served as positive and negative controls respectively (Fig.1). NO production was recorded to be higher in n-hexane fraction (22.9µM±0.13) at 100µg/ml which was very close to mitogen (LPS) stimulated NO (23.9 µM±0.71) production and exhibited significantly more than 2-fold increase when compared with untreated cells. Treatment with methanol extract and other fractions viz n-butanol, chloroform and aqueous fraction at 100 µg/ml were lesser effective in stimulating NO production (Fig.1). These findings substantially increased the NO production in Mϕs confirms their NO enhancing ability.

The activity of the potential extracts/fractions was further tested in *L.donovani* infected hamsters. Ethanol extract exhibited dose-dependent efficacy with highest activity being observed at an initial dose of 500 mg/kg x5 days to the tune of 74.4±7.4% inhibition of parasite multiplication. Among the fractions tested at 250-mg/kg dose the n-hexane fraction showed superior antileishmanial efficacy 76.4±7.1 followed by n-butanol fraction 71.3±8.1%. This indicates that the active principles are concentrated in these fractions. The efficacy of n-butanol was similar to methanol extract. The chloroform and aqueous fractions were totally inactive. Miltefosine, the reference drug, exhibited 94.0±2.3% inhibition at a 40-mg kg−1 day−1×5 dose (Fig. 1).These findings further substantiate the *in vitro* observations.

On the basis of these results, it is very clear that apart from the aerial part of *A. indicum* its seeds also possess potential which may provide promising leads for the development of new drugs against leishmaniasis. This is a preliminary report and further studies are needed to elucidate the pure chemical entities of n-hexane fraction of the seeds for its antileishmanial efficacy.

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Table 1. Antileishmanial effect of extract and fractions of *Abutilon indicum* against *Leishmania donovani* promastigotes and intracellular amastigotes. Data (mean ± standard deviation) represents results of three independent experiments

Plant extract and fractions	Activity against promastigotes		Activity in MQ-amastigote system	
	Dose (μg/ml)	% Inhibition (Mean ± S.D.)	Dose (μg/ml)	% Inhibition (Mean ± S.D.)
Ethanol extract (EE)	100 50	94.3 ± 6.9 57.6 ± 5.1	100 50	78.2±5.5 49.7±6.2
n-Hexane fraction (F002)	100 50	90.3 ± 7.4 72.2 ± 6.4	100 50	76.7±3.7 54.4±4.2
Chloroform fraction (F003)	100 50	NI* NI	100 50	NI NI
n-Butanol fraction (F004)	100 50	89.1 ± 7.7 67.8 ± 8.4	100 50	69.6±6.4 46.23±5.2
Aqueous fraction (F005)	100 50	NI NI	100 50	NI NI
Miltefosine	10 5	100 100	10 5	100 64.3 ± 6.8

* NI = No inhibition, $ MΦs = Macrophages

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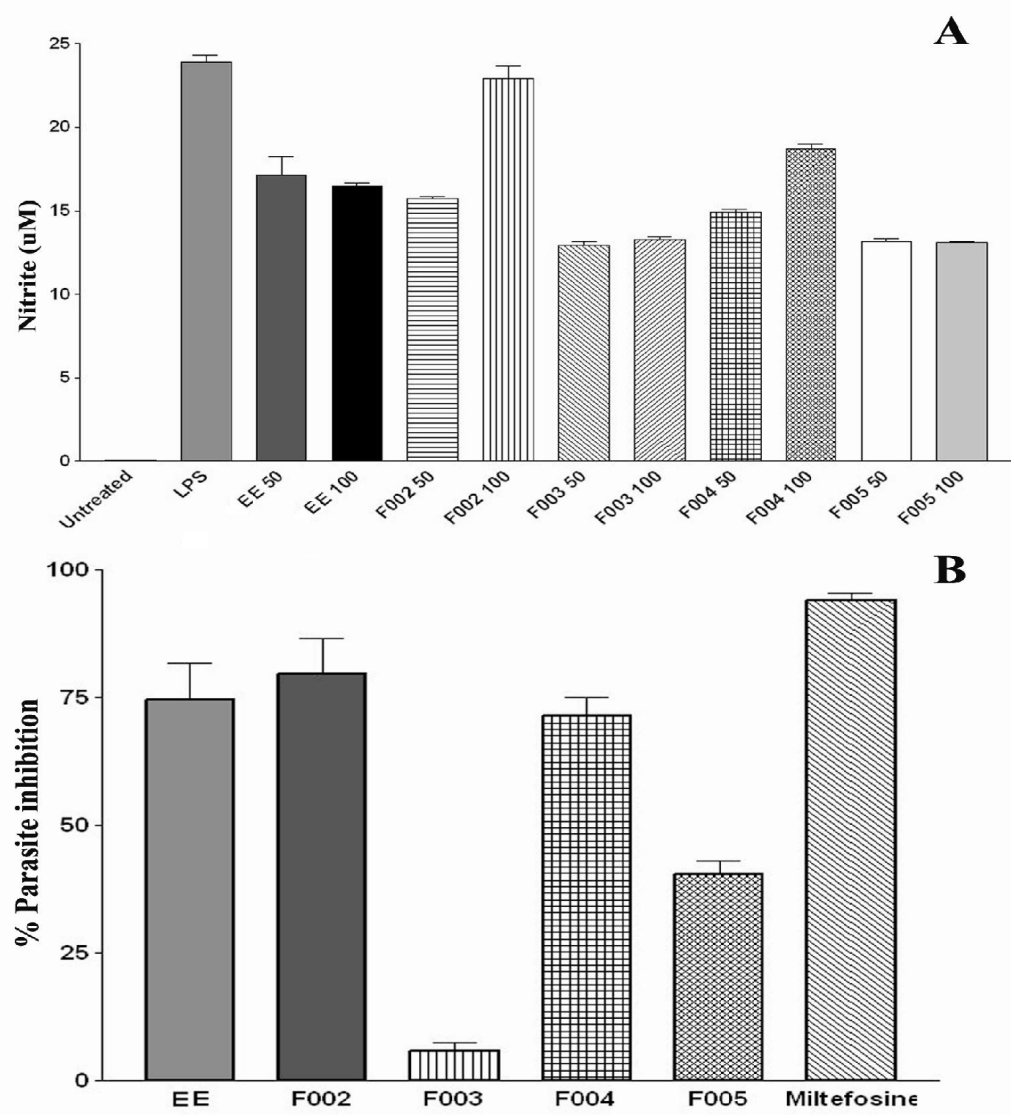

Figure 1. :(A) NO generation in J774 macrophages by extract and fractions of *Abutilon indicum*. Cells pretreated with test samples (50µg/ml and 100 µg/ml) were analysed by griess reagent Data represent the mean ± SD of three separate experiments; (**B)** *In vivo* efficacy of *Abutilon indicum* ethanol extract against established infection of *Leishmania donovani* in golden hamsters. Results are expressed as mean ± SD of three separate experiments