The triterpenoid fraction from *Trichosanthes dioica* root exhibits *in vitro* antileishmanial effect against *Leishmania donovani* promastigotes

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ABSTRACT

Background: *Trichosanthes dioica* Roxb. (Cucurbitaceae), called pointed gourd in English is a dioecious climber found wild throughout the plains of the Indian subcontinent and traditionally used in India for several medicinal purposes. **Objective:** The present study was aimed at the evaluation of *in vitro* antileishmanial effect of triterpenoid fraction from *T. dioica* root (CETD). **Materials and Methods:** The antileishmanial activity of CETD was evaluated against *Leishmania donovani* (strain MHOM/IN/83/AG83) promastigotes by *in vitro* promastigote cell toxicity assay by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide). Potassium antimonyl tartrate was used as reference. **Results:** Here, CETD markedly inhibited the growth of *L. donovani* promastigotes *in vitro* in a concentration dependent manner and demonstrated IC_{50} value of 18.75 µg/ml. The reference drug potassium antimonyl tartrate exhibited IC_{50} of 7.52 µg/ml. **Conclusion:** From the present study it can be inferred that the triterpenoid fraction of *T. dioica* root exhibited remarkable antileishmanial activity against *Leishmania donovani* promastigotes *in vitro.*

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INTRODUCTION

Leishmaniasis is a wide spread life-threatening disease caused by protozoa of genus *Leishmania* transmitted by female sandflies of the genera of Phlebotominae subfamily. According to available estimates of World Health Organization (WHO), the disease is spread across 88 countries causing serious health problems especially in developing countries with 350 million at risk of contracting the disease and with approximately 2 million new cases being reported each year. The three main manifestations of disease are visceral, cutaneous and muco-cutaneous leishmaniasis. Visceral leishmaniasis (VL), also commonly known as *kala-azar* is caused by *L. donovani*. More than 90% of world's cases of VL are reported in India, Bangladesh, Nepal, Sudan, Brazil and Ethiopia. In India, most of the leishmaniasis cases have been reported in Bihar, Orissa and Uttar Pradesh states. Cutaneous and muco-cutaneous leishmaniases are more prevalent in Afganistan, Saudi Arabia and some Latin American countries.[1–4]

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, pentamidine, and paromomycin.[5,6] The mentioned drugs have the disadvantages of high cost, lack of oral formulations (e. g., amphotericin B can be used only intravenously), or serious side effects that require close monitoring of the patients.[6] Also, rapid development of resistance by the parasites has been reported,[7–9] so that new therapies are needed to supplement or replace currently available therapies. More recently, emergence of coinfection of leishmaniasis with human immunodeficiency virus (HIV) has made the treatment even more challenging.[10]

Traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their therapeutic principles. The major merits of herbal medicine seem to be their perceived efficacy, low incidence of serious adverse effects and low cost.

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Trichosanthes dioica Roxb. (Cucurbitaceae), called pointed gourd in English, *Potol* in Bengali and *Patola* in Sanskrit, is a dioecious climber found wild throughout the plains of North and North-East India from Punjab to Assam and Tripura states of India. It is also grown and commercially cultivated in India, Pakistan, Bangladesh and Sri Lanka for its consumable fruits, a common culinary vegetable in the Indian subcontinent. In India, all parts of this plant have been traditionally used for various medicinal purposes. According to Ayurveda, the traditional system of Indian medicine, its root is a drastic purgative. The root has been traditionally used in India as purgative and as tonic, febrifuge, in treatment of jaundice, anasarca and ascites.[11–14]

In our previous course of studies, we have reported anthelmintic, antibacterial, antimitotic, antiproliferative, antitumor, analgesic, laxative, chemopreventive and arsenic toxicity ameliorative activities of the root of *T. dioica*.[15–27] As there are no experimental reports on antileishmanial activity on *T. dioica*, in the present study we found it necessary to evaluate the *in vitro* antileishmanial effect of triterpenoid enriched extract form *T. dioica* root extract against *Leishmania donovani* promastigotes.

MATERIALS AND METHODS

Collection and authentication of plant material

The mature tuberous roots of *T. dioica* were collected during December 2009 from Majdia, Nadia district, West Bengal, India. The species was identified by Dr. M. S. Mondal, at the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India, and a voucher specimen (CNH/I-I/57/2009/Tech.II/493) was deposited at the Pharmacognosy Research Laboratory, Bengal School of Technology, Delhi Road, Hooghly 712102, India for future reference.

Preparation of triterpenoid fraction (CETD)

Just after collection, the fresh roots were washed thoroughly with water, cut into moderate pieces and immediately crushed thoroughly in tepid water (~50ºC) using a mechanical grinder. After cooling to room temperature (23 ± 2°C), the extract was separated from the remaining vegetable debris by pressing the material through muslin cloth. The resulting liquid is filtered and extracted once with *n*-hexane and the aqueous phase was further extracted successively with dichloromethane. The organic phases (dichloromethane extracts) were collected, pooled, and evaporated to dryness *in vacuo* (at 35°C and 0.8 MPa) in a Buchi evaporator, R-114. The dry extract i.e., triterpenoid enriched fraction (CETD, yield: 6.55%, w/w) was kept in a vacuum desiccator until use.

Standardization of CETD

Qualitative phytochemical analysis revealed the presence of triterpenoids in CETD.[28] Presence of cucurbitacin type triterpenoid aglycones in CETD was ascertained by planar chromatography on silica gel pre-coated high-performance thin layer chromatography (HPTLC) plates (Silica gel 60 F_{254} Merck, Germany) detected with vanillin-phosphoric acid reagent.[29] CETD was dispersed in 0.2% dimethyl sulfoxide as per required concentrations and sonicated for 10 min immediately prior to use in the study.

Reagents and chemicals

M-199 medium and fetal calf serum (FCS) were obtained from Gibco-BRL, dimethyl sulphoxide (DMSO) from Merck, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade obtained commercially.

Parasite culture

Promastigotes of *L. donovani* (strain no. MHOM/IN/83/AG83) were routinely cultured at 22°C in M-199 medium supplemented with 10% heat-inactivated FCS and gentamicin (100 μg/ml) and subcultured every 72 h.

Evaluation of antileishmanial activity

In vitro promastigote cell toxicity assay using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) cell proliferation assay was used to assess the antileishmanial activity *in vitro* as per reported methods.[30] Briefly, the exponential phases of promastigotes (2×10^6 cells/ml) were incubated with or without the test agents along with M-199 medium at 22°C. The test extract (CETD) was dissolved in 0.2% dimethyl sulfoxide (DMSO), and added to the culture in graded concentrations of 2.5, 5, 10, 20, 40 and 80 μg/ml. Similarly the reference drug potassium antimonyl tartrate was employed at the concentrations of 5, 10, 20 and 40 μg/ml. After 2 h of treatment, the tubes were centrifuged at 8000 g for about 10 min. The supernatant was decanted and the pellets were washed with 20 mM phosphate buffer saline (PBS). Each pellet was dissolved in 100 μl (2 mg/ml) of MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide) solution, and the tubes were incubated at 22°C for 4 h and then centrifuged at 8000 g for 10 min. The resulting pellets were dissolved in 500 μl of 0.2% DMSO and the absorbance was measured spectrophotometrically at 570 nm. Lysis of promastigotes (%) by the CETD was calculated by the formula as shown below.

$$\text{Lysis \%} = 100 - [(\text{test} - \text{positive control})/(\text{control} - \text{positive control})] \times 100$$

All the tests were carried out in triplicate and the results averaged. The IC_{50} value (50% inhibitory concentration) was

determined by plotting percentage lyses of promastigotes with respect to control against treatment concentrations.

RESULTS AND DISCUSSION

The *in vivo* efficiencies of drugs have been reported to be under the control of different parameters, such as pharmacokinetic parameters,[31] so that for various reasons, including simplicity in *in vitro* culture maintenance, routine screenings of antileishmanial chemotherapeutic agents are often based on promastigote susceptibility assays.[32] In the present study, a relevant cell viability test (MTT assay) was used to investigate the inhibitory effect of CETD on the *in vitro* growth of *Leishmania donovani* promastigotes and the effects was compared with a trivalent antimonial reference drug. Here, the test extract CETD significantly and concentration dependently inhibited the growth of the promastigote forms of *L. donovani* (strain no. MHOM/IN/83/AG83) *in vitro* and demonstrated IC_{50} value of 18.75 μg/ml. Potassium antimonyl tartrate was used as reference which also concentration dependently inhibited the growth of the *L. donovani* promastigotes and exhibited IC_{50} value of 7.52 μg/ml [Table 1]. Here, the reference trivalent antimonial agent was found to be more active than CETD. It was quite obvious. However, CETD was also quite toxic and effective against *L. donovani* promastigotes.

Parasites of the genus *Leishmania* are transmitted by the female sandflies that ingest the parasite in the amastigote stage resident within macrophages, and then inoculate the promastigote stage into other hosts. There is a general lack of effective and inexpensive chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonials (Sb (III)) like potassium antimonyl tartrate or emetic tartar and pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious adverse effects and resistance has become a severe problem. Therefore, new drugs are urgently required. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans.[33] *T. dioica* root is a potent herbal drug demonstrating several important pharmacological properties (see the introduction section). Its main bioactive constituents were found to be triterpenoids and its antimicrobial (antibacterial) antiparasitic (anthelmintic), cytotoxic (antimitotic) and antiproliferative effects were found prominent in the triterpenoid enriched extracts.[15–18] This is the reason why the triterpenoid fraction of *T. dioica* root (CETD) was selected for the present study.

Table 1: Effect of CETD against *L. donovani* promastigotes (2×10⁶ cells/ml)

Test agents	Concentration (μg/ml)	Percentage lysis of promastigotes with respect to control (0.2% DMSO)*	IC_{50} value (μg/ml)
CETD	2.5	19.50	18.75
	5	30.34	
	10	37.27	
	20	53.84	
	40	68.08	
	80	76.73	
Potassium antimonyl tartrate	5	41.36	7.52
	10	54.71	
	20	67.38	
	40	92.36	

*Mean of three replicates, CETD= Triterpenoid fraction of *T. dioica* root

Being triterpenoid enriched extract, the abundance of triterpenoids especially cucurbitacin type triterpenoid aglycones was affirmed in CETD by qualitative phytochemical analysis and thin layer chromatography (HPTLC). Cucurbitacins are known to possess several biological activities including antimicrobial property.[34,35] The presence of putative cucurbitacin aglycones could provide the chemical basis of its antileishmanial efficacy *in vitro*. Previously the present authors have reported *in vitro* antibacterial potential of *T. dioica* root.[16] This notable property may be responsible for the promising antileishmanial activity of CETD. However, studying the exact mechanism of CETD behind its antileishmanial effect is beyond the scope of the present investigation.

Therapeutic evaluations for medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products. Natural products can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development, and the discovery of new therapeutic properties not yet attributed to known compounds.[36] Natural products have made, and are continuing to make, an important contribution to this area of therapeutics. Perhaps their future potential will be even greater. In this study we report the inhibitory effect of CETD on the *in vitro* growth of *Leishmania donovani* promastigotes. The observed activity represents an exciting advance in the search for novel antileishmanial agents from natural sources, since a significant and important effect against the promastigote form of the protozoan was demonstrated in the present study.

From the present investigation, it can be concluded that the triterpenoid fraction from *T. dioica* root demonstrated remarkable *in vitro* antileishmanial activity compared with potassium antimonyl tartrate, a trivalent antimonial against *Leishmania donovani* promastigotes. To the best of our knowledge, this is the first experimental report of the antileishmanial activity of *Trichosanthes dioica*. However, further definitive phytochemical and *in vivo* studies are

necessary in this context to ascertain the mechanism of action and in pursuit of a new effective antileishmanial agent from the plant kingdom.

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