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SHORT COMMUNICATION

Evaluation of antileishmanial potential of *Tinospora sinensis* against experimental visceral leishmaniasis

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Abstract The chemotherapeutic interventions against visceral leishmaniasis (VL) are limited and facing serious concerns of toxicity, high cost, and emerging drug resistance. There is a greater interest in new drug developments from traditionally used medicinal plants which offers unprecedented diversity in structures and bioactivity. With this rationale, ethanolic extract of *Tinospora sinensis* Linn and its four fractions were tested in vitro against promastigotes and intracellular amastigotes and in vivo in *Leishmania donovani* infected hamsters. Ethanolic extract exhibited an appreciable activity against promastigotes (IC_{50} 37.6±6.2 μg/ml) and intracellular amastigotes (IC_{50} 29.8±3.4 μg/ml). In hamsters, it resulted in 76.2±9.2% inhibition at 500 mg/kg/day×5 oral dose level. Among fractions, *n*-butanol imparted highest in vitro and in vivo activities. Ethanolic extract and butanol fraction also enhances reactive oxygen species (ROS) and nitric oxide (NO) release. The results indicate that *T. sinensis* may provide new lead molecules for the development of alternative drugs against VL.

Introduction

Visceral leishmaniasis (VL) or kala-azar is a fatal systemic infection caused by *Leishmania donovani*, an obligate intracellular protozoan parasite belonging to family Trypanosomatidae. It is endemic in 62 countries, primarily in the developing world, and the population at risk is estimated at 200 million (Guerin et al. 2002). Approximately 100,000 new cases of VL occur in India annually, and the state of Bihar accounts for 90% of these (Sundar 2001). If left untreated, the mortality rate for VL is near 100%. A major emerging problem is *Leishmania*/HIV coinfections, which present difficulty in diagnosis and treatment (Cruz et al. 2006). Resistance to sodium stibogluconate (SSG), the first line treatment, is increasing in Bihar state of India, where up to 60% previously untreated patients are unresponsive to its recommended dose regimen (Croft et al. 2006). Despite the development and registration of miltefosine, an oral drug for VL treatment in India (Sundar et al. 2002), search for new active compounds including those of natural origin is greatly encouraged, as there has been little progress toward development of vaccines against VL.

Tinospora sinensis (Lour.) Merrill (syn *Tinospora malabarica*) belonging to the family Menispermaceae, is commonly known as Gurch in Hindi and Sudarsana in Sanskrit (Jain and DeFilipps 1991). It is a large deciduous climber with rambling stems, bearing aerial roots from branches and is found almost throughout India, ascending to an altitude of 1,000 m. It has been used in traditional Ayurvedic medicine for treating debility, dyspepsia, fever, syphilis, bronchitis, jaundice, urinary, skin, and liver diseases (Wealth of India 1976; Chopra et al. 1956). It is also reported to possess anti-inflammatory (Li et al. 2003) and antidiabetic (Yonemitsu et al. 1993) activities. It also inhibits cyclophosphamide induced anemia, increase WBC count in mice, and possess immunomodulatory activity (Manjrekar et al. 2000). Stem of this plant is used as a brain tonic, in chronic rheumatism and for fumigation in piles and ulcerated wounds (Li et al. 2003). A white starchy

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Table 1 In vitro antileishmanial activity (IC_{50}) and cytotoxicity (IC_{50}) of *Tinospora sinensis* ethanol extract and its fractions against *L. donovani* promastigotes and intracellular amastigotes

Test sample/drug	Extracellular promastigotes IC_{50} (μg/ml)[a]	Intracellular amastigotes IC_{50} (μg/ml)[a]	Cytotoxicity IC_{50} (μg/ml)[b]	Selectivity index (SI)[c]
Ethanol extract	37.6±6.2	29.8±3.4	94.2±11.6	3.2
Hexane fraction	86.3±10.2	>100	>300	–
Chloroform fraction	>100	>100	16.7±3.3	–
Butanol fraction	41.6±6.5	17.6±4.1	>300	> 17
Aqueous fraction	>100	>100	>300	–
Miltefosine	7.5	3.3	35	10.6

Results are expressed as mean±SD of three independent experiments.
[a] IC_{50} Concentration of test sample that resulted in 50% decrease in parasite growth
[b] IC_{50} Concentration of test sample that resulted in 50% decrease in cell growth
[c] *SI* IC_{50} of test sample against J774 cells/IC_{50} of test sample against intracellular amastigotes

substance extracted from stems and roots, known as galo is used for treating fever.

The plant was collected from the Paschim Midnapur district of West Bengal (India) in the month of September 2003 and preserved in the herbarium of our institute with voucher specimen number 4601. The powdered stems of the plant were extracted with ethanol resulting in crude extract which on successive fractionation provides hexane, chloroform, butanol, and aqueous fractions. Transgenic *Leishmania donovani* promastigotes expressing green fluorescent protein (GFP) were cultured in medium 199 (Sigma, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 26°C as described previously (Dube et al. 2007). J774 macrophage (Mϕ) cell line was maintained in DMEM medium (Sigma, USA) supplemented with 10% FBS in a humidified incubator at 37°C in the presence of 5% CO_2. Male Syrian golden hamsters (*Mesocricetus auratus*) weighing 45–50 g were used as the experimental hosts. Animal experiments were performed as per ethical guidelines laid down by the Institutional Animal Ethics Committee. *L. donovani* (MHOM/IN/80/Dd8) was maintained in hamster by serial passages using spleen-derived amastigotes as previously described (Singh et al. 2007 published online). Test samples were prepared and administered as described earlier (Dube et al. 2007).

In vitro antileishmanial activity against promastigotes was determined by putting 1×10^6 log phase GFP-expressing promastigotes into 48-well culture plates and exposing the cells to different concentrations of reference drug and as test samples for 72 h. The effect of extract and fractions was assessed by flow cytometry as described by Dube et al. (2007). The inhibition of parasite growth was determined by comparing the fluorescence levels of drug-treated parasites with that of untreated control parasites. Miltefosine served as reference control. In vitro antileishmanial activity against intra-macrophage amastigotes was determined as reported by Dube et al. (2005). IC_{50}, the concentration that produces 50% decrease in infected Mϕs compared to control was calculated.

The efficacy of *T. sinensis* extract and fractions was evaluated in hamsters having established infection of *L. donovani* (20 to 25 days old infection). Hamsters were divided into different groups of five animals each. Animals were administered by oral route at 25 to 500 mg/ml doses. Miltefosine was administered orally at a dose of 50 mg/kg×5. Splenic parasite loads of treated and untreated groups were determined on day 7 posttreatment. Percent parasite inhibition in treated animals was calculated using the formula

$$\%\ \text{inhibition} = \frac{\text{AT} \times 100}{\text{IT} \times \text{TI}}$$

Where AT the actual number of amastigotes/100 spleen cell nuclei in treated animals,
IT the initial number of amastigotes/100 spleen cell nuclei in treated animals and
TI is the times increase in untreated control animals.

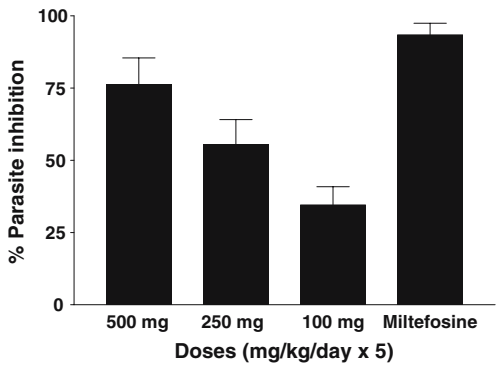

Fig. 1 In vivo efficacy of *T. sinensis* ethanol extract against established infection of *L. donovani* in golden hamsters. Results are expressed as mean±SD of three separate experiments.

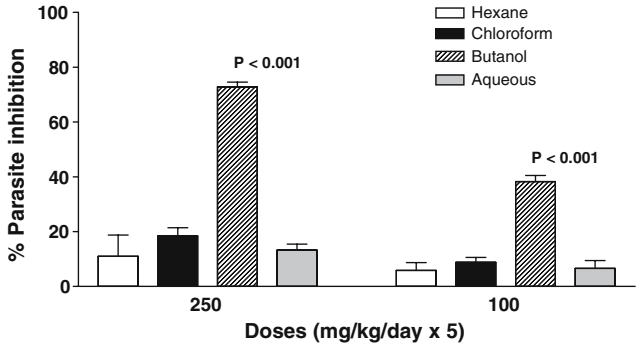

Fig. 2 In vivo efficacy of *T. sinensis* fractions against established infection of *L. donovani* in hamsters. Results are expressed as mean± SD of three separate experiments. Comparison among the experimental groups was made by Student's *t*-test. *P* values of 0.05 or less were considered as statistically significant.

The stimulation of ROS and NO generation by *T. sinensis* on J774 Mϕs was measured using fluorescent probes DCFH-DA and DAF-2DA, respectively. Cells were treated with extract and fractions for 12 h at 100 μg/ml concentration. Cells were washed with phosphate-buffered saline (PBS), resuspended in 500 μl PBS-containing fluorescent probes and incubated for 30 min at room temperature in dark. Cells were analyzed by FACSCalibur flow cytometer. Results were expressed as mean fluorescence intensity.

Results

Chloroform fraction of *T. sinensis* exerted cytotoxicity (IC_{50}-16.7 μg/ml) against J774 Mϕs as revealed by cell shrinkage and cell death (data not shown). Ethanol extract displayed moderate toxicity (IC_{50}-94.2 μg/ml), whereas hexane, butanol and aqueous fraction however, were, devoid of any cytotoxic effect (IC_{50} >100 μg/ml) Selectivity index for butanol (>17) was better than ethanol extract (SI 3.2). Ethanol extract displayed excellent antileishmanial activity with IC_{50} values of 37.6±6.2 and 29.8±3.4 μg/ml against promastigotes and intracellular amastigotes, respectively (Table 1). An appreciable activity was exhibited by butanol fraction with IC_{50} of 41.6±6.5 μg/ml and 17.6± 4.1 μg/ml against promastigotes and intracellular amastigotes, respectively. Other fractions were inactive against both the stages (Table 1).

Treatment of hamsters with ethanol extract at 500 mg/kg/day×5, p.o. resulted in 76.2±9.2% parasite inhibition (Fig. 1). At 250 and 100 mg/kg doses, it resulted in 55.4±

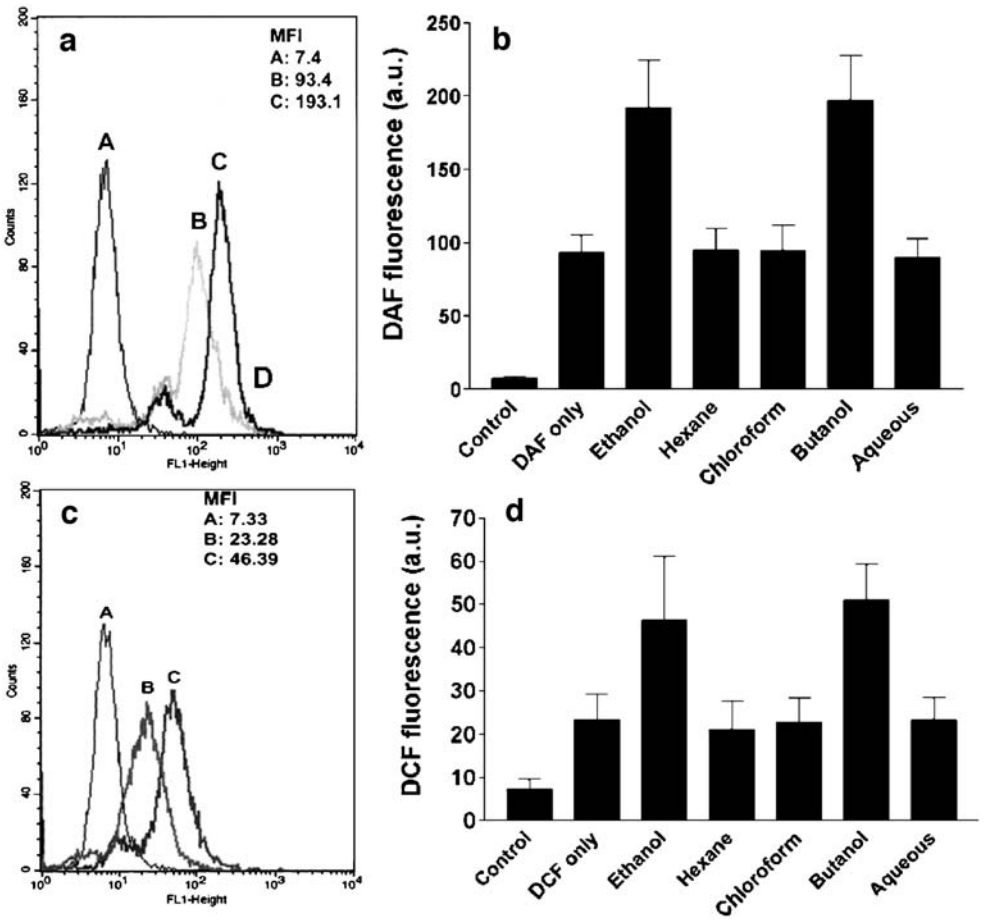

Fig. 3 Stimulation of ROS and NO generation in J774 macrophages by extract and fractions of *T. sinensis*. Cells pretreated with test samples (100 μg/ml) were analyzed by flow cytometry after DAF-2DA (for NO) and DCFH-DA (for ROS) staining. **a** and **b** represents flow cytometric analysis of effect of *T. sinensis* on DAF-mediated fluorescence, whereas **c** and **d** represents effect of *T. sinensis* on DCF-mediated fluorescence. Data represent the mean±SD of three separate experiments.

8.6 and 34.4±6.4% inhibition, respectively. Excellent antileishmanial activity was exhibited by butanol fraction resulting in 72.8±4% inhibition at a dose of 250 mg/kg (Fig. 2). At a lower dose (100 mg/kg), however, weaker (38.3±4.9%) antileishmanial activity was observed. Other three fractions have poor or no activities at the same dose levels. Reference drug, miltefosine, demonstrated 93.3±4.1% inhibition of *L. donovani* amastigotes at a dose of 50 mg/kg/day×5 p.o.

Stimulation of NO and ROS release in Mϕs by extract and fractions of *T. sinensis* was evaluated by FCM using fluorescent probes. Compared to MFI of 93.4±11.6 in untreated DAF loaded cells, treatment with ethanol extract (MFI 192.3±32.2) and butanol fraction (197.1±30.5) at 100 μg/ml significantly (twofold increase; $P<0.001$) enhanced the DAF-mediated fluorescence (Fig. 3). Other fractions were ineffective. Increase in DCF-mediated fluorescence after 12 h incubation with ethanol extract indicates activation of Mϕs manifested in an enhanced ROS production (Fig. 3). Compared to untreated cells (MFI 23.2±6), extract and butanol fraction strongly activated the Mϕs resulting in the increase in DCF fluorescence (46.4±14.7 and 51±8.3, respectively). Other fractions failed to enhance the ROS generation.

Discussion

From ancient times in India, plant products are clinically used for curing various ailments, but unfortunately, this treasure has not been fully explored for the containment of leishmaniasis. Therefore, more attention is desired for the exploration of plant species commonly used in traditional herbal medicine for treating parasitic diseases. In this study, ethanol extract and butanol fraction exhibited dose-dependent antileishmanial activity against promastigote and intracellular amastigote forms of *L. donovani*. Their high selective index (>3) indicates their safety towards Mϕs. Lack of antileishmanial activity in hexane, chloroform and aqueous fractions concludes that active constituents of *T. sinensis* are confined to butanol fraction. Good antileishmanial activity of this plant against clinical relevant (amastigote) stage is encouraging.

In animal studies, ethanol extract exhibited dose-dependent efficacy with highest activity being observed at 500 mg/kg dose. Butanol fraction showed efficacy similar to ethanol extract. It resulted in significantly higher ($P<0.001$) inhibition of parasite multiplication compared to other three fractions. Superior in vivo efficacy in butanol fraction firmly indicates that the antileishmanial principles of this plant are concentrated in it. These findings substantiate the in vitro experimental outcomes.

Next, we assessed the immunostimulating potential of this plant. Our findings indicates that ethanol extract and butanol fraction treatment substantially increased the DAF and DCF-mediated fluorescence in Mϕs as revealed by FCM. This confirms their ROS and NO enhancing ability towards Mϕs. ROS and NO are firmly established to play a role as antimicrobial effector molecules produced by activated Mϕs (Bogdan and Rollinghoff 1998; Stafford et al. 2002). These findings provide correlation between in vitro antileishmanial and immunostimulatory activities of ethanol extract and butanol fraction.

The presence of flavonoids and tannins etc. could be attributed for the immunostimulatory activities, as these constituents are reported to stimulate nonspecific macrophage functions. Similar findings are reported with *Desmodium gangeticum* (Singh et al. 2005) and *Ocimum gratissimum* (Ueda-Nakamura et al. 2006).

Phytochemical investigation on this plant revealed that its leaf and stem bark extracts contains steroids, flavonoids, alkaloids, triterpenes, polyphenols, and furano-diterpenes (Rastogi and Mehrotra 1998). The quinoline alkaloids, naphthylisoquinoline alkaloids, quinones, chalcones, terpenes, flavonoids are well known to exhibit leishmanicidal and immunomodulatory activity (Fournet and Munoz 2002; Rocha et al. 2005; Kayser et al. 2003). The presence of similar chemical constituents in *T. sinensis* might be responsible for its potent antileishmanial action.

In conclusion, the potent antileishmanial and significant immunostimulatory activity of *T. sinensis* indicates that the plant may provide promising leads for the development of new drugs against leishmaniasis. Further studies to elucidate the pure chemical entities of butanol fraction and detailed investigations of prophylactic and therapeutic in vivo immunostimulatory mechanisms of *T. sinensis* are in progress.

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