

Research Article

Study on developmental biochemical characteristics of *Leishmania donovani* promastigote and inhibitory effect of *Achyranthes aspera* Linn plant extract on it

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Abstract

Leishmania donovani is an obligatory intracellular digenetic parasite transmitted by insects, causing serious global health problems as it is endemic to most developing countries. Extensive use of antimony compounds as drugs poses high toxicity and cost; therefore, herbal medicine has identified a position. This study explored the developmental and biochemical characteristics of *L. donovani* promastigote and the effect of ethanolic extract of *Achyranthes aspera* Linn (*Amaranthaceae*) plant on it. The parasites were incubated at 2.5×10^6 cells/well for 72 h at 23 °C in the presence of various concentrations of extract ($\mu\text{g/mL}$) dissolved in 1% dimethyl sulfoxide (DMSO) with sterile phosphate-buffered saline and 1% DMSO as negative controls and meglumine antimoniate as positive control. Friedman's repeated measures analysis showed that 96hr of development is the junction point in promastigotes ontogeny. Post 96hr, it grows with a long stationary phase with higher enzymatic activities of acid phosphatase, superoxide dismutase and glutathione (oxidized and reduced). Total protein estimated showed a linear relationship ($R^2 = 0.999$). Phytochemical screening of the plant extract showed the presence of alkaloid, flavonoid, fixed oil and fats, saponin, tannin and phenolic compounds. It showed an effectual free radical scavenging in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with an inhibitory concentration IC_{50} value of 61.70 $\mu\text{g/mL}$. At a concentration of 250 $\mu\text{g/mL}$, the plant extract completely inhibited the promastigotes *in vitro* while at 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$, the survival level declined by 25-50%. These findings corroborate the ethnopharmacological use of this plant for the treatment of leishmaniasis caused by *L. donovani*.

Keywords: *Achyranthes aspera* Linn, Inhibitory concentration, *Leishmania donovani*, Phytochemicals, Promastigote

INTRODUCTION

Leishmaniasis are a group of diseases caused by dimorphic protozoan parasites from more than 20 *Leishmania* species (protozoa; kinetoplastida). There are three main forms of the disease: *cutaneous leishmaniasis* (most common form), *mucocutaneous leishmaniasis* (most severe form) and *visceral leishmaniasis* (most disabling form) which is also known as kala-azar.

These parasites are transmitted to humans by biting an infected female phlebotomine sandfly, a tiny 2 to 3 mm long insect vector. It is prevalent throughout the tropical and sub-tropical regions of Africa, Asia and Europe and has been considered one of the six entities on the World Health Organization tropical disease research (WHO-TDR) list of most disabling diseases (Thakur, 2020). In 2018, about 90 countries or territories were considered endemic for, or had previously reported

cutaneous leishmaniasis and *visceral leishmaniasis*, respectively. More than ninety per cent of the world's cases are in India, Bangladesh, Nepal, Sudan and Brazil. At present, more than 1 billion people live in areas endemic to leishmaniasis and are at risk of infection. Ninety per cent of all the cases in India are reported from Bihar State alone. Around the world, an estimated 30, 000 new cases of *visceral leishmaniasis* and more than 1 million new cases of *cutaneous leishmaniasis* occur annually (<https://www.who.int/news-room/factsheets/detail/leishmaniasis>). The DALY (disability-adjusted life year) burden for leishmaniasis was calculated to be 860 000 for men and 1.2 million for women (Thakur, 2020).

For visceral leishmaniasis treatment, the universally recommended drugs, the pentavalent antimonials were first used about 60 years ago. Over recent decades standard alternative medicines have become available (Kumar *et al.*, 2022). Treatment failure to conventional drugs in kala-azar in endemic areas of Bihar has been surfaced since late 1970s and is becoming a common problem in many hyper endemic areas of the state, and now accounts for the treatment failure in about forty percent cases. Treatment of the Kala-azar patient is done by providing them to pentavalent antimonial drugs like amphoterricin B, pentamidine miltefosine and paramomycin which control the development of the Leishmania parasite. But these drugs have severe side effects and excessive use develops a resistant strain (Sundar *et al.*, 2008). All these conditions have directed to search for novel alternative therapies, particularly during its promastigote development may be helpful for designing chemotherapeutic approaches to multiple cellular targets and thereby successfully treating leishmaniasis (Kopec *et al.*, 2013; Nikmehr *et al.*, 2014).

Natural products are a source of a variety of substances with biological activity, thus the search for active pharmacological compounds from medicinal plants for the treatment of diseases has been prioritized by the World Health Organization (WHO) (Pan American Health Organization, 2020). The active phyto-molecules or compounds of plants origin against microorganisms may perhaps inhibit their growth by different mechanisms than those of synthetic antimicrobial chemicals and possibly will have momentous clinical importance in the management of resistant microorganisms (Jaiswal *et al.*, 2018; Bharti *et al.*, 2012 and 2016). Extracts isolated from several plants have been reported to have a biological activity such as antimicrobial, anti-inflammatory and antioxidant activities (Bharti *et al.*, 2013 and 2018). It has been documented that various plants like *Ageratum conyzoides*, *Bidens pilosa*, *Chenopodium ambrosioides*, *Eugenia uniflora* etc, have a potential anti-leishmanial effect. Their effects are related, at least in part, to the presence of coumarin and/or terpenoids. These plant extracts would be helpful in con-

trolling the parasite and also in the treatment of kala-azar. Subsequently, these plant extracts could be used where kala-azar is more prominent to control the promastigote form of *Leishmania* (Silveira *et al.*, 2020).

There are several reports on effective antimicrobial activity and their subsequent secondary complications. Several small drugs and medicinal plant extracts have a wide range of useful pharmacological properties (Nikmehr *et al.*, 2014; Kopec *et al.*, 2013). *A. aspera* Linn, is an erect, annual or perennial herb which grows as a ruderal weed throughout India (Patil, 2016). Conventionally, the plant is used in skin eruptions, piles, whooping cough, dropsy, asthma, leukoderma and colic diseases. It is also used as an astringent, as a diuretic, as laxative and as purgative, in addition to, as an antidote to snake bite, in bone fractures and in hydrophobia (Kumar *et al.*, 2022). Different plant parts (root, stem, leaf, inflorescence, and seeds) are used individually for treating different illnesses (Shendkar *et al.*, 2011; Dangi *et al.*, 2012). Based on a literature survey, the present investigation has been carried out to explore the mode of action of extracts of a medicinal plant *Achyranthes aspera* Linn (family *Amaranthaceae*) plant on promastigote form of *Leishmania donovani* to provide its effective treatment.

MATERIALS AND METHODS

Materials

The *Achyranthes aspera* Linn plant, commonly known as Apamarga, Chirchira, Latjira and Prickly Chaff-flower (Sinan *et al.*, 2020), was collected in February 2018 from the Botanical Garden of the University campus, Magadh University, Bodh Gaya, Bihar, India and identified according to the relevant monographs of Indian Pharmacopoeia (2016). A voucher specimen (BD-MU-1-20) of the plant was deposited in the Herbarium of the aforesaid.

Methodology of extract preparation

The collected parts of plant were separated, cleaned from dust, and placed in the shade inside a well-ventilated room until they were completely dried and weighed. Dried parts of plant were grounded to obtain a fine powder in a mortar. Fifty grams of freshly prepared plant material was extracted with 500 ml of ethanol by soaking for 48 h followed by filtration *via* Whatman filter paper No. 1. The filtrate was then centrifuged at 12000 rpm for 10 min followed by the supernatant refiltration under strict aseptic conditions. The filtrates were then vacuum concentrated below 40⁰ C using a rotary evaporator (Buchi, Switzerland). The residue obtained was stored in the freezer at minus 20⁰ C until further testing.

Phytochemical screening of plant extracts

The screening of chemical constituents was carried out

qualitatively and quantitatively with ethanolic extracts by using chemical methods. The ethanolic plant extract was analysed for the presence of alkaloid, saponin, flavonoid, fixed oils and fats, tannins and phenolic compounds according to standard methods adopted by Kumar *et al.* (2022).

Total phenol was determined by Folin–Ciocalteu reagent and expressed as mg gallic acid (GAL) equivalent/g dry weight (McDonald *et al.*, 2001). Briefly, the diluted plant extract (0.5 ml of 1:10 g/ml) or the standard phenolic compound, gallic acid was mixed with 5 ml of Folin–Ciocalteu reagent (1:10 diluted with distilled water) and aqueous sodium carbonate (Na_2CO_3) (4 ml, 1M). After 15 min, the total phenol from the mixture was determined by Colorimeter at 550 nm and expressed in terms of gallic acid equivalent (mg/g of dry mass). The standard curve was prepared using 0, 50, 100, 150, 200 and 250 mg/l solutions of gallic acid in a methanol: water mixture (50:50, v/v).

Flavonoids were determined by the aluminium chloride Colorimetric method (Kumar *et al.*, 2022). The plant extract (0.5 ml) was discretely mixed with methanol (1.5 ml), aluminium chloride (0.1 ml of 10%), potassium acetate (0.1 ml of 1 M) and 2.8 ml of distilled water. After 30 min of incubation at ambient room temperature, the absorbance was measured at 450 nm with a V-670 research grade UV-Vis spectrometer. Quercetin solutions at concentrations of 12.5–100 $\mu\text{g}/\text{ml}$ in methanol were meant for the calibration curve.

Culture of selected parasites

Promastigotes were cultivated axenically (Kar, 1997) *in vitro* at $\pm 30^\circ \text{C}$ in monophasic liquid medium, M-199 (Gibco BRL, USA), supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) (Gibco BRL, USA) and 25mM N-(2-hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES).

Determination of various enzymatic activities

The promastigotes of *L. donovani* (strain MHOM/IN/AG/1003), originally acquired from Indian Kala-azar patient (Ghosh *et al.*, 2003) and maintained in Leishmania strain bank at the Indian Institute of Chemical Biology, Kolkata was obtained and routinely maintained in the laboratory, at the Department of Botany, Magadh University, Bodh-Gaya, Bihar, India. The mid log-phase parasites, promastigotes have been weekly passage through the artificial culture medium, Medium-199 (M-199) (Kar *et al.*, 1997). Promastigotes were cultivated axenically *in vitro* at $\pm 30^\circ \text{C}$ in the monophasic liquid medium, M-199 (Gibco BRL, USA), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Gibco BRL, USA) and 25mM N-(2-hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES). The medium was then filtered under sterilized conditions in culture room under laminar flow. It was kept for 48 hours at

room temperature to check for any contamination, and then stored in the refrigerator for further use (Kar *et al.*, 1997).

The parasites were isolated from culture media by centrifugation at $1600\times g$ for 10 minutes and the pellet thus precipitated contains live cells. Pellets were washed thrice in 0.01M phosphate buffered saline (PBS), pH 7.2 at 25°C and re-suspended in the medium. Parasite's growth was assessed quantitatively by enumeration of promastigotes in a haemocytometer. For viability testing, cells obtained from centrifugation had been mixed with 0.01M PBS and 2% Trypan Blue (Sigma, USA) solⁿ 1:1 (v/v) and enumerated under the light microscope. The cells that took colour were dead cells and the colourless cells were live cells. Promastigotes were collected at different growth phases by centrifugation in $2000\times g$ for 10 minutes at 4°C , washed thrice in 0.01 M PBS, and pelleted. Then, the pelleted promastigotes were then solubilized in a lysis buffer (50 mM Tris + 0.1% Triton X-100; pH 8) and left for 20 minutes at 4°C . The extract was then centrifuged at $5000\times g$ for 20 minutes at 4°C . The extract was centrifuged at $5000\times g$ for 20 minutes at 4°C to remove cell debris, and the supernatant was used for further enzymatic analysis.

The different enzymatic activities were determined by various methods adopted by Kumar *et al.* (2022). The *acid phosphatase* activity was expressed as p-mole PNP/min/mg of protein. The *Superoxide dismutase* (SOD) activity was expressed as 50% inhibition of NBT/min/ mg protein. The abundance of reduced glutathione (GSH) activity was expressed as $\mu\text{g}/\text{mg}$ of protein while that of oxidized glutathione (GSSG) activity was expressed as $\mu\text{g}/\text{mg}$ of protein. The *Glutathione reductase* (GR) and *Glutathione peroxidase* (GPX) activities were expressed as nmol/ min /mg of protein. Total protein content was determined using a Folin phenol reagent by a modification to the established Lowry assay (Lowry *et al.*, 1951). According to the standard curve constructed using the results obtained for the dilution series of BSA (10 to 500 $\mu\text{g}/\text{ml}$), the two variables of the assay, the BSA concentration and absorbance value at 650 nm, showed a linear relationship where the squared correlation coefficient, R^2 , was 0.999. The absorbance values for selected concentrations of BSA (10, 30, 100, 150, 300, 500 $\mu\text{g}/\text{ml}$) at any given time were within the accepted limits. The optical density (absorbance) was measured at 650 nm on a Systronics UV-Vis Spectrophotometer, No.117, using a blank for zero measurements.

Preparation of the stock solution of test extracts

The stock solution of the plant extract was prepared according to the method of Mutoro *et al.* (2018a). Briefly, for anti-leishmanial assay, the stock solution of the extract was prepared in Schneider's *Drosophila* culture

medium (Fisher Scientific, 21720024) and filtered through 0.22- μm filter under a Laminar flow hood and stored at 4°C for further bioassays.

Evaluation of minimum inhibitory concentration (MIC) and active ratio of test extract on *L. donovani* promastigotes

The MICs were evaluated according to the method of Wabwoba *et al.* (2010). Briefly, the metacyclic promastigotes of *L. donovani* (concⁿ; 1×10^6 promastigotes/ml of culture medium) were medicated with the test extract whose concentrations were 2000 $\mu\text{g/ml}$, 1000 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$. The *L. donovani* promastigote treated with the extract was stained with 100 μL of trypan blue dye on a microscope slide and observed under the light microscope (XSZ-107 Series Biological Microscope, Sam-Tech Diagnostics) to check their motility and viability compared to the standard Schneider's Drosophila medium as the negative control. The lowest concentration of the test extract in which no live promastigotes were observed was the MIC and active ratio for the test extract. The 50% inhibitory concentration (IC_{50} value) was also calculated by Probit test in SPSS software.

Anti-leishmanial activity of the plant extract

Before evaluating the anti-leishmanial activity, the cellular density of each species was calculated using light microscopy. When cellular density touched a threshold concentration of 10^6 cells/mL, the promastigotes were washed twice with phosphate-buffered saline (PBS) followed by centrifugation at 2500 rpm for 10 min. For assessing the anti-promastigote activity, the method of Et-Touys *et al.* (2016) and Dutta *et al.* (2005) was followed. Briefly, about 100 μL of parasite culture were resuspended in a 96-well tissue culture plate, in fresh culture medium at a concentration of 2.5×10^6 cells/well, followed by incubation for 72 h at 23 °C under different concentrations of extract ($\mu\text{g/mL}$) dissolved in 1% dimethyl sulfoxide (DMSO) (Essid *et al.*, 2015, Costa *et al.*, 2011). The sterile PBS and 1% DMSO (vehicle) were used as negative controls, while Meglumine antimoniate (Glucantime®) was used as positive control. Cell viability was evaluated by determining the extract concentrations which inhibit half of the cell population (IC_{50}). The inhibition percentage (*I*) was calculated using the following formula (Essid *et al.*, 2015):

$$\text{Inhibition percentage (I)} = 100 \times (\text{Absorbance of untreated cells} - \text{Absorbance of treated cells}) / \text{Absorbance of untreated cells} \quad \text{Eq. 1}$$

Data analysis

All the statistical analyses (after Zar, 1999; Field, 2005) were performed using PC based software, Sigma Stat® version 3.5 for Windows (©Systat-softwares Inc. USA,

2006) and SPSS® version 10.0 for Windows (©SPSS Inc. USA, 1989). The statistical analysis was performed by a one-way ANOVA analysis of variance. The difference is considered as significant for $p \leq 0.05$.

RESULTS

Phytochemical screening of plant extract

Phytochemical screening of the selected plant extract showed the presence of different percentage of active phytochemicals such as alkaloid, flavonoid, fixed oil and fats, saponin, tannin and phenolic compounds. The phenolic content of the extract was found to be 10.2 mg GAL/g per gram dry weight basis, while the flavonoid content in terms of quercetin equivalent was found to be 2.43 ± 0.12 mg/g. The differences in phenolic and flavonoid content were found to be statistically significant ($p < 0.05$). Further, the extract showed an effectual 82.8% free radical scavenging in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay at a concentration of 100 $\mu\text{g/ml}$ (Table 1). The IC_{50} value of the extract was found to be 61.70 $\mu\text{g/ml}$.

Growth pattern of parasites

Growth pattern of *L. donovani* (strain MHOM/IN/AG/1003) studied for six consecutive days, is tabulated in Table 2. Friedman's repeated measures analysis of variance on rank was performed to test whether the variations among different stages has been arisen randomly or followed a definite pattern. The differences in the values among the different developmental stages (24 hrs. to 144 hrs.) were greater than would be expected by chance ($p = < 0.005$). The 96 hour was observed as the junction point in promastigotes ontogeny which differ significantly from all preceding as well as subsequent stages. The promastigotes of *L. donovani* attained log phase of growth by the second day. A significant rise in growth rate was observed from 48 up to 96 hours, reaching the stationary phase at 96 hours. After 96 hours, *L. donovani* culture grew with a long stationary phase with a gradual decrease in population level. Promastigotes before 96 hours had a comparatively slender body and had little shorter flagella than that of stationary (metacyclic) forms.

Viability test (% survivability)

Cell viability is expressed as a percentage of survival from different culture hours and tabulated (Table 3). The differences in the median values among the treatment group (i.e., different developmental stages; 24 hrs. to 144 hrs.) are greater than would be expected by chance ($p = < 0.005$). Survival percentage did not vary much between the stages for the first four days (Table 3), but a decrease in viability in later stages may be due to the nutrient-depleted media.

Table 1. Free radical scavenging activity of ethanolic extract of *Achyranthes aspera* Linn.

Concentration (in µg/ml)	Absorbance of sample	Control	Ctrl-sample/ Ctrl	% Inhibition
10	0.213	0.256	0.167968	16.7978
20	0.203	0.256	0.207031	20.7031
30	0.174	0.256	0.320312	32.0312
40	0.156	0.256	0.390625	39.0625
50	0.147	0.256	0.425781	42.5721
60	0.128	0.256	0.50	50.0
70	0.101	0.256	0.605468	60.5468
80	0.72	0.256	0.71875	71.875
90	0.54	0.256	0.789062	78.9062
100	0.44	0.256	0.828125	82.8125

Table 2. Growth pattern of *Leishmania donovani* promastigotes in M-199 media

Hours in culture	Mean (density of cells ×10 ⁶ /ml)	Standard Deviation	Standard Error	Range (density of cells ×10 ⁶ /ml)	
				Maximum	Minimum
24 Hrs	4.597	0.307	0.088	4.705	3.750
48 Hrs	8.571	0.513	0.152	9.288	7.450
72 Hrs	11.509	0.243	0.078	11.850	11.100
96 Hrs	14.588	0.310	0.097	14.800	14.100
120 Hrs	13.761	0.309	0.093	14.100	12.750
144 Hrs	11.384	0.347	0.113	11.950	10.850

Various enzymatic activities

In promastigotes grown in M-199, differences in acid phosphatase activity were found between different developmental stages (Table 4). The present study observed that 96 hours of growth phase seemed to be an important junction concerning acid phosphatase activity in *L. donovani* promastigote development. After 96 hours, the enzyme Acid phosphatase started to differ significantly ($p \leq 0.05$) from earlier stages of development. The stationary phase promastigotes (96 hours and onwards) had higher enzyme activity than the log phase. The superoxide dismutase activity increased continually during promastigote development in *L. donovani*, and specific activity increased about 75% between 24-96 hours. The two alternative forms of glutathione-GSH and GSSG content varied significantly in the later stages from earlier stages of development. It might be due to the depletion of GSH and the accumulation of GSSG in mature promastigotes of *L. donovani*. During promastigote development, GSH: GSSG ratio had a reciprocal relationship with growth rate and the measure of infectivity in *L. donovani*. Thus, an active antioxidant system not only facilitated the growth in parasites but might also augment their chance of survival by increasing their virulence for successful transmission to the vertebrate host. Increased glutathione reductase activity in post 96 hours stages and subse-

quent decline in GSH : GSSG level in the respective stages indicates that glutathione reductase has been actively involved in reduction of GSSG to GSH. Glutathione peroxidase (GPX) did not exhibit much variability throughout the development (Table 4).

Total protein estimated

The total protein quantification by simplified Lowry assay was 100% repeatable, having less than 10% Coefficient of Variance. The absorbance of tested concentrations of BSA (10, 30, 100, 150, 300, 500 µg/ml) under defined standard conditions was found between lower and upper limits (Table 5).

Minimum inhibitory concentration of the extract on *L. donovani* promastigotes and their survival

The MICs for the extract were detected by looking at the motility and viability of the parasites in the 96-well tissue culture plate as compared to the Schneider's Drosophila medium (SIM) as the negative control. The IC₅₀ (µg/mL) values of the extract against the promastigote growth rate was 25 ± 2.85 with Meglumine antimoniate (Glucantime) as control drug. The survival levels of promastigotes after treatment with varying concentration of the extract were determined by comparing them with survival in the tested experiment. A concentration of 250 µg/mL of the plant extract inhibit-

ed the promastigotes *in vitro* 100 %. In contrast, a lower concentration of 50 µg/mL and 100 µg/mL decreased the promastigotes to a minimum survival level of 25% to a moderate survival level of 50% compared to control. Then again, the higher concentration of 500 µg/mL decreased the promastigotes to a moderate survival level of 50% only compared to controls. Therefore, 250 µg/mL of the extract was efficacious in inhibiting the growth of the parasite to maximum levels.

DISCUSSION

L. donovani is an obligatory intracellular digenetic parasite whose life cycle alternates between two stages: flagellated promastigotes, which develop in the midgut of the insect vector and amastigotes that multiply in the host macrophage (Alvar *et al.*, 2012). Major remedial regimens that are presently in use comprise meglumine antimoniate (Glucantime), sodium stibogluconate (Pentostam), amphotericin B, and pentamidine. These synthetic drugs are effectual but have lethal side effects and high costs (Kumar *et al.*, 2022; Corrêa *et al.*,

2011). As a consequence of these complications, scientists are in constant exploration to bargain new drugs against these parasites. From the ages, plants having medicinal capacities are being utilized for treating several ailments. Significant attention has been given to the discovery of novel, less- or non-toxic agents of plant origin having anti-leishmanial capacity (Bharti *et al.*, 2012 and 2013). Also, these days, public responsiveness is growing concerning natural products as medicines (Pratihast *et al.*, 2019; Bharti *et al.*, 2012, 2016 and 2018). A broad spectrum of plant species has been computed which enclose potentially effective leishmanicidal compounds. In India, leishmaniosis has been traditionally treated with folk medicine using native plants (Kumar *et al.*, 2022).

In a qualitative analysis of phytochemical screening, the extract showed the presence of different percentages of active phytochemicals such as alkaloid, flavonoid, fixed oil and fats, saponin, tannin and phenolic compounds, which shows a direct relationship with their free radical scavenging activities (Table 1). Free radicals and reactive oxygen species (ROS), counting su-

Table 3. Percentage of survival of *Leishmania donovani* promastigotes in M-199 media

Hours in culture	Mean % of survival	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	97.219	1.396	0.397	100.000	98.000
48 hrs	95.548	1.670	0.491	100.000	94.000
72 hrs	97.753	1.676	0.479	100.000	95.000
96 hrs	95.983	1.303	0.382	98.000	94.000
120 hrs	91.943	1.923	0.571	96.000	90.000
144 hrs	86.120	2.486	0.709	92.000	84.000

Table 4. Specific activity of acid phosphatase enzyme (µ-mole PNP/min/mg of protein) in *Leishmania donovani* promastigote at different developmental stages in M-199 media

Hours in culture	Mean Specific activity of various enzymes					
	Acid phosphatase (µ mole-PNP/min/mg of protein)	Superoxide dismutase (50% inhibition of NBT/min/mg of protein)	Mean abundance of glutathione (µg/ mg of protein)		Glutathione reductase (n mol/min/mg protein)	Glutathione peroxidase (GPX) enzyme (n mol/min/mg protein)
			Reduced	Oxidized		
24 hrs	0.519±0.021	0.593±0.024	12.271±0.841	3.462±0.261	0.294±0.031	0.059±0.003
48 hrs	0.710±0.027	0.728±0.031	11.346±0.783	3.289±0.233	0.327±0.033	0.067±0.007
72 hrs	1.409±0.038	1.104±0.033	11.013±0.672	3.763±0.372	0.364±0.073	0.079±0.006
96 hrs	1.468±0.035	1.121±0.042	10.359±0.589	3.968±0.309	0.461±0.079	0.095±0.009
120 hrs	1.721±0.119	1.217±0.048	9.641±0.511	4.021±0.378	0.494±0.088	0.129±0.012
144 hrs	1.937±0.127	1.352±0.072	8.676±0.475	4.225±0.385	0.437±0.084	0.151±0.016

Table 5. Assessment of precision/ accuracy of the assay

Replicates	Absorbance values of different BSA ($\mu\text{g/ml}$)					
	10	30	100	150	300	500
Mean (M)	0.152	0.183	0.229	0.274	0.425	0.625
Standard deviation (SD)	0.006	0.006	0.011	0.015	0.028	0.044
2SD	0.012	0.013	0.022	0.030	0.056	0.088
Upper limit (M + 2SD)	0.163	0.197	0.250	0.302	0.480	0.711
Lower limit (M - 2SD)	0.139	0.171	0.207	0.242	0.369	0.537
Coefficient of variation (CV)	3.971	3.496	4.829	5.453	6.615	7.079

peroxide radicals, singlet oxygen, hydroxyl radical and hydrogen peroxide are the by-products produced during various physiological and biochemical processes (Bharti *et al.*, 2019; Murari *et al.*, 2016). The excessive production of ROS causes oxidative stress, which is responsible for several diseases (Bharti *et al.*, 2015). The tested plant extract of *A. aspera* Linn also showed a trend of an effectual free radical scavenging trend in the DPPH assay due to fairly dissimilar active phytochemicals (Bharti *et al.*, 2012, 2013 and 2015).

The present observations provide supporting evidence for the antioxidant potential, reducing power and hydroxyl radical scavenging activity of the plant extract. The IC_{50} value of the extract was found to be $61.70 \mu\text{g/ml}$. Similar observations were reported by Saeed *et al.*, (2012) and Rashid *et al.*, (2013) in their studies on extracts of *Torilis leptophylla* and *Fagonia olivieri* plants, respectively on *L. donovani* promastigote at different developmental stages.

It has been well documented that parasites growing in different media present different biological characteristics and distinct *in vitro* and *in vivo* infectivity (Santarém *et al.*, 2014). In the present study, the *Leishmania donovani* promastigotes were cultivated axenically *in vitro* at $\pm 30^\circ \text{C}$ in monophasic liquid medium, M-199 (Gibco BRL, USA), supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) (Gibco BRL, USA) and 25mM N-(2-hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES). This medium is ideal for the maintenance of parasites in the laboratory, diminishing the expected loss of virulence over time typical of parasite cultivation (Santarém *et al.*, 2014; Warburg *et al.*, 2008). In the present study, growth pattern of *L. donovani* (strain MHOM/IN/AG/1003) was studied for six consecutive days. In Friedman's repeated measures analysis of variance, the differences in the values among the different developmental stages (24 h to 144 h) were greater than would be expected by chance. It was also observed that the promastigotes of *L. donovani* attained a log phase of growth by the second day. There was a large increase in growth rate between 48 and 96 hours. The promastigotes reached the stationary phase at 96 hours. After 96 hours, *L. donovani* culture grew with a long stationary phase with a gradual

decrease in population level. Promastigotes prior to 96 hours had a comparatively slender body and had little shorter flagella than that of stationary (metacyclic) forms (Table 2).

The viability of cells is expressed as a percentage of survival from different hours of culture and is being tabulated in Table 3. The differences in the median values among the treatment group (i.e., different developmental stages; 24 h to 144 h) were greater than would be expected by chance. Survival percentage did not vary much between the stages for the first four days but the decrease in viability in later stages may be due to the nutrient-depleted media. Further, an appreciation of probable complex interactive factors can be best achieved through computer simulation models using the powerful simulation software that has recently become available (Jaiswal *et al.*, 2018).

Acid phosphatases are enzymes that hydrolyse phosphomonoesters in the acidic pH range with their active sites facing the extracellular medium. Extracellular acid phosphatase activity in *L. donovani* has been associated with the degree of promastigote virulence (Papadaki and Boleti, 2019). The differential acid phosphatase activity in different species of *Leishmania* or even different strains of the same species vary probably due to their different degree of virulence (Aragon *et al.*, 2001). The present study observed that 96 hours of growth phase seemed to be an important junction concerning acid phosphatase activity in *L. donovani* promastigote development. After 96 hours, the enzyme started to differ significantly ($p \leq 0.05$) from earlier stages of development (Table 4).

Superoxide dismutase, a metal-containing enzyme is present both in *L. donovani* and in host macrophages, although the level was found much higher in the latter case. Moreover, a considerable inhibition of superoxide dismutase activity by NaCN in infected macrophages confirms the chemical nature of the increased superoxide dismutase to be of Cu-Zn type, usually found in host (Mukherjee *et al.*, 1988). The present results showed that superoxide dismutase activity increased continually during promastigote development. Specific activity increased $\sim 75\%$ between 24-96 hours (Table 4).

Its two alternative forms of glutathione, oxidized and reduced glutathione are important thiols involved in free radical scavenging and are studied in detail in the developing promastigote population (da Silva *et al.*, 2008). In the present study, the GSH content varies significantly in the later stages from earlier stages of development. It might be due to the depletion of GSH in mature promastigotes. It has been documented that during promastigote development, GSH: GSSG ratio has a reciprocal relationship with growth rate and measure of infectivity in *L. donovani*. Thus, active antioxidant system not only facilitated the growth of parasites but might also augment their chance of survival by increasing their virulence for successful transmission to vertebrate host (Colotti and Ilari, 2011).

The present study also showed the specific activity of Glutathione peroxidase at different hours of culture of *L. donovani* in M-199. Glutathione peroxidase did not exhibit much variability throughout the development. Their amount was found to be quite small compared to the H₂O₂ challenge the *L. donovani* is exposed to, apart from Leishmania glutathione peroxidase, have other H₂O₂ trapping enzymes *viz.* peroxidoxins, ovothiol etc. So, the lack of variability along the insufficient quantity of Glutathione peroxidase in the promastigote developmental stages might indicate towards the supplementary active role of other enzymes in H₂O₂ scavenging in *L. donovani*. Improvements in cost-effective techniques are necessary to increase the research outputs in resource-limited settings (Deepachandi *et al.*, 2020). Similar results have been presented by other workers while evaluating the serological markers to monitor the disease status of Indian post kala-azar dermal leishmaniasis (Mukhopadhyay *et al.*, 2012; Bhowmick and Ali, 2009).

The ancient approach of *Ayurveda* can be combined with modern logical approach to screen the traditional natural products for precise and effective drug discovery for superior antimicrobial potential. Therefore, the present study is important because it has demonstrated that taken plant extract of *A. aspera* Linn is effective against promastigote stage of leishmania. Determination of minimum inhibitory concentration (MIC) involves exposing the test organism to serially diluted extract and determining the minimum concentration that inhibits its growth (Bharti *et al.*, 2015; Bakri and Douglas, 2005). The micro-organism has been tested for their ability to produce visible growth in the presence of serially diluted plant extract as antimicrobial agent. The MICs were detected by looking at the motility and viability of the parasites in the wells compared to the Schneider's Drosophila medium (SIM) as the negative control.

The survival levels of promastigotes after treatment with varying concentration of ethanolic extract of *A.*

aspera Linn plant were determined by comparing with survival in the tested experiment. A concentration of 250 µg/mL of the extract inhibited the promastigotes *in vitro* by 100%. In contrast, lower concentrations of 50 µg/mL and 100 µg/mL decreased the promastigotes from minimum survival level of 25% to moderate survival level of 50% only. Then again, a higher concentration of 500 µg/mL decreased the promastigotes to moderate survival level of 50% (Table 3). It has been well documented that the environment in which the medicinal plant grows, affects the concentration of active phytoconstituents and hence its efficacy. To a greater extent, environmental factors model the chemical profiles of a plant qualitatively and quantitatively, subsequently resulting in the biological activity of the extracts. In addition to the pharmacological screening of plant extracts, phytochemical profiling offers an important source for explaining the often-heterogeneous activity of similar extracts obtained in dissimilar seasons or growing environments (Bharti *et al.*, 2013 and 2016).

Conclusion

The present study concluded that the leishmanicidal activity of the *A. aspera* Linn plant extract was efficacious at a concentration of 250 µg/mL compared to the standard drug Glucantime with an effective concentration of 25 ± 2.85 µg/mL on promastigote form of *L. donovani* to provide its effective treatment. The outcome of the present study advocates to explore the plant world to identify active phyto-molecules as potential sources of safe and effective anti-leishmanial bioactive agents. Natural products with anti-leishmanial activities can provide an alternative treatment for antimonial-resistant Leishmania strains with improved efficacy and reduced resistance. Further, studies are needed to evaluate their action mechanism against different forms of *L. donovani*.

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Conflict of interest

The authors declare that they have no conflict of interest.

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