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THE DEVELOPMENTAL AND BIOCHEMICAL FEATURES OF LEISHMANIA DONOVANI PROMASTIGOTE AND THEIR REPRESSION BY DATURA STRAMONIUM PLANT EXTRACT

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ABSTRACT

Leishmania is an intracellular digenetic obligate parasite spread by insects and poses grave health issues throughout the world. Extensive use of antimony compounds as drugs poses high toxicity and cost and therefore, a position has been identified for herbal medicine. This study has been carried out to explore the developmental biochemical characteristics of Leishmania donovani promastigote. In addition, the mode of action of Datura stramonium (Solanaceae) plant extract on promastigote form of leishmania have been examined. 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 viz., acid phosphatase, superoxide dismutase and glutathione (oxidized and reduced). Total protein estimated, showed a linear relationship (R2 = 0.999). Phytochemical screening of extracts showed the presence of alkaloid, flavonoid, fixed oil and fats, saponin, tannin and phenolic compounds etc, and showed an effectual free radical scavenging in the DPPH assay with an IC50 value of extract of D. stramonium (55.63 µg/ml). A concentration of 250 µg/mL of the plant extract completely inhibited the L. donovani promastigotes in vitro while concentrations of 50 µg/mL and 100 µg/mL decreased the survival level by 25-50%. Our findings corroborate the ethnopharmacological use of this plant for the treatment of Leishmaniasis. Also, our results are promoters as potential sources to search antileishmanial bioactive agents.

KEYWORDS

Leishmania donovani, promastigote, Datura stramonium, Phytochemicals, Inhibitory concentration.

INTRODUCTION

The leishmaniases are a group of diseases caused by dimorphic protozoan *Leishmania* parasites and transmitted to humans by the bite of an infected female phlebotomine sand-fly insect vector. It is prevalent throughout the tropical and sub-tropical regions of the world[1]. 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 for leishmaniasis and are at risk of infection. Ninety per cent of all the cases in India are reported from Bihar State alone. An estimated 30, 000 new cases of *visceral leishmaniasis* and more than 1 million new cases of *cutaneous leishmaniasis* occur annually[2]. The universally recommended drugs used for the treatment of visceral leishmaniasis, are pentavalent antimonials. Treatment failure to these conventional drugs along with severe side effects has been surfaced in endemic areas of Bihar[3,4]. This scenario has led to the search for novel alternative therapies, particularly during its promastigote development may be helpful for designing chemotherapeutic approaches of treatment[5,6].

The active compounds of plants origin have been reported to have biological activity such as antimicrobial, anti-inflammatory and antioxidant activities[7-16]. *Datura stramonium*, plant is an herbaceous annual plant from the nightshade family (Solanaceae) commonly known as black datura, green thorn apple, apple of Peru, jimson weed, devil's apple, devil's trumpet, or devil's snare[17]. The plant has been employed in traditional medicine as a hallucinogen, taken entheogenically for cough and chest complaints, severe cases of insect bites and stings and also on inflammations to allay the pain[5-6]. The plant is a known source of tropane alkaloids hyoscyamine, atropine, and scopolamine. The alkaloids of Datura are used as a spasmolytic, anti-asthmatic, and anticholinergic[8,19]. Based on literature survey, the present investigation has been carried out to explore the mode of action of ethanolic extracts of *D. stramonium* on promastigote form of leishmania to provide effective treatment against *L. donavani*.

Methodology

The *D. stramonium* plants was collected in February 2018 from Botanical Garden of University campus, Magadh University, Bodh Gaya, Bihar, India and identified according to the relevant monographs of Indian Pharmacopoeia[20]. A voucher specimen (BD-MU-2-20) of the plant was deposited in the Herbarium of the aforesaid. The collected parts of plants were cleaned from dust, and placed in the shade inside a well-ventilated room to dry and weight was obtained. Dried parts of plants were grounded to obtain a fine powder in a mortar. Fifty grams of each of the freshly prepared plant material was extracted with 500 ml of ethanol by soaking for 48 hrs. The extracts were filtered using Whatman filter paper No. 1, and the filtrate was centrifuged at 12000 rpm for 10 min. The supernatant was again filtered using Whatman filter paper No. 1 under strict aseptic conditions. The collected filtrates were concentrated in vacuum at temperature below 40^0C using a rotary evaporator (Buchi, Switzerland). The residue obtained was stored in freezer at minus 20^0C until further test.

Phytochemical Screening Of Plant Extracts

The screening of chemical constituents was carried out qualitatively and quantitatively for the presence of alkaloid, saponin, flavonoid, fixed oils and fats, tannins and phenolic compounds according to standard methods[21]. Total phenol was determined by Folin–Ciocalteu reagent and expressed as mg Gallic acid (GAL) equivalent/g dry weight[22]. Flavonoids were determined by aluminium chloride colorimetric method[23].

Selected Parasite And Determination Of Various Enzymatic Activities

The promastigotes of *Leishmania donovani* (strain MHOM/ IN/ AG/ 1003), originally acquired from Indian Kala-azar patient[24] and maintained in Leishmania strain bank at Indian Institute of Chemical Biology, Kolkata were 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)[25]. Promastigotes were cultivated axenically *in vitro* at ± 30° 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). 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 refrigerator for further use[25].

The parasites were isolated from culture media by centrifugation at 1600×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) sol^{n} 1:1 (v/v) and enumerated under light microscope. The cells that took colour were dead cells and the colourless cells were live cells. Promastigotes were collected at different phases of growth by centrifugation in 2000×g for 10 minutes at 4°C and washed thrice in 0.01 M PBS. Pelleted promastigotes were solubilized in a lysis buffer containing 50 mM Tris and 0.1% Triton X-100, pH 8, and were left for 20 minutes at 4°C. The extract was then centrifuged at 5000×g for 20 minutes at 4°C to remove cell debris, and the supernatant had been used for further enzymatic analysis.

The *acid phosphatase* activity[26], *Superoxide dismutase* activity[27], reduced glutathione (GSH) and oxidized glutathione (GSSG) activity[28], *Glutathione reductase* (GR) activity[29], *Glutathione peroxidase* (GPX) activity[30] and total protein content[31] was determined. The optical density (absorbance) was measured at 650 nm on a Systronics UV-Vis Spectrophotometer, No.117 using blank for zero measurement. Stock solutions of the crude plant extracts were made as described by[32].

Minimum Inhibitory Concentration (MIC) And Antileishmanial Activity Of The Plant Extract

After, the cellular density of threshold concentration of 10^6 cells/mL, the promastigotes were washed twice with phosphate-buffered saline (PBS) and centrifuged at 2500 rpm for 10 min. To evaluate the anti-promastigote activity, 100 µL of parasites culture were resuspended in a 96-well tissue culture plate, in fresh culture medium[33-36]. The sterile PBS and 1% DMSO (vehicle) were used as negative controls, while Meglumine antimoniate (Glucantime®) has been used as positive control. Cell viability has also been evaluated by determining the extract concentrations which inhibit half of the cell population (IC_{50})[36].

Data Analysis

All the statistical analyses[37-39] 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 Extracts

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 etc. The phenolic content of the ethanolic extract of datura was found to be 15.34 ± 0.53 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 extract showed an effectual 77.7% free radical scavenging in the DPPH assay at concentration of 100 µg/ml (Table 1; Fig. 1). The IC_{50} value of the extract was found to be 55.63 µg/ml.

Table: 4.3: Free Radical Scavenging Effect Of Ethanol Extract Of *Datura Stramonium.*

Concentration (in µg/ml)	Absorbance Of Sample	Control	Ctrl-sample/ Ctrl	% Inhibition
10	0.218	0.256	0.148437	14.8437
20	0.204	0.256	0.203125	20.3125
30	0.202	0.256	0.210937	21.0937
40	0.180	0.256	0.296875	29.6875
50	0.158	0.256	0.382812	38.2812
60	0.135	0.256	0.472656	47.2656
70	0.108	0.256	0.578125	57.8125
80	0.077	0.256	0.69921	69.921
90	0.072	0.256	0.71875	71.875
100	0.057	0.256	0.777342	77.7342

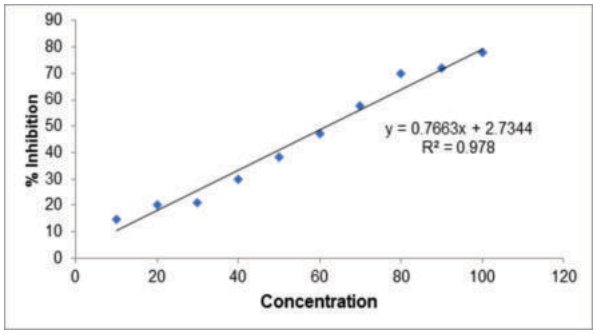

Fig. 1: Free radical (DPPH) Scavenging Effect Of Ethanol Extract Of *Datura Stramonium* At Different Concentrations.

Culture And Growth Pattern And Viability Of Parasite

Promastigotes were cultivated axenically[25] *in vitro* at ± 30° 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). Growth pattern of *Leishmania donovani* (strain MHOM/IN/AG/1003) was studied for six consecutive days (Fig 2).

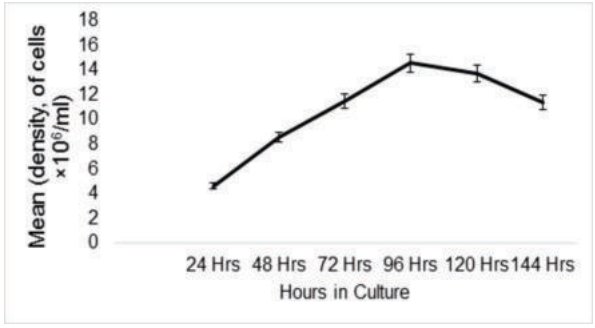

Fig 2: Growth Pattern Of *Leishmania Donovani* Promastigotes In M-199 Media At Different Developmental Stages (upper Error Bar = Standard Deviation; Lower Error Bar = Standard Error).

To test whether the variations among different stages has been arisen randomly or follow a definite pattern, Friedman's repeated measures analysis of variance on rank was performed for different developmental stages (24 hrs. to 144 hrs.). It has been observed that 96 hours is an important junction point in promastigotes ontogeny as it significantly differs from all preceding as well as subsequent stages. It is observed that the promastigotes of *L. donovani* attained log phase of growth by the second day. Post 96 hours, *L. donovani* culture grew with a long stationary phase with gradual decrease in population level. Promastigotes prior to 96 hours have a comparatively slender body and have little shorter flagella than that of stationary (metacyclic) forms. The viability (% survivability) of cells has been expressed as percentage of survival from different hours of culture table. Survival percentage did not vary much between the stages for first four days but decrease in viability in later stages (Fig 3).

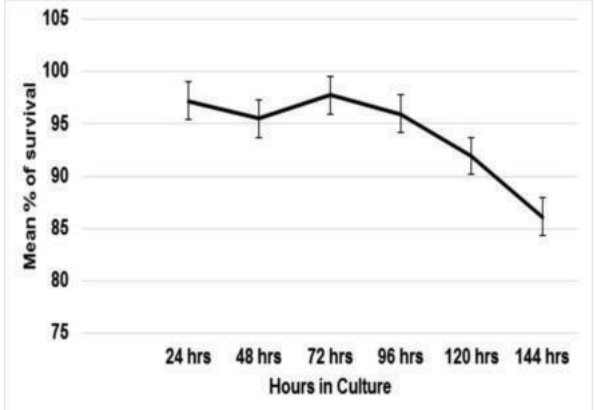

Fig 3: Percentage Of Survival Of *Leishmania Donovani* Promastigotes In M-199 Media At Different Developmental Stages.

Various Enzymatic Activities

In promastigotes grown in M-199, we observe that the stationary phase promastigotes (96 hours and onwards) have higher enzyme activity than that of log phase. The specific activity of superoxide dismutase increases about 75% between 24-96 hours. The GSH: GSSG ratio has a reciprocal relationship with both growth rate as well as measure of infectivity in *L. donovani*. Increased glutathione reductase activity in post 96 hours stages and subsequent 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 of variability throughout the development and their amount is found to be quite little in comparison to the H_2O_2 challenge, the *Leishmania* is exposed to (Table 2).

Table 2: 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.

Specific activity of Acid Phosphatase enzyme					
Hours in culture	Mean specific activity µ mole-PNP/min/mg of protein	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	0.519	0.021	0.004	0.539	0.498
48 hrs	0.710	0.027	0.006	0.748	0.682
72 hrs	1.409	0.038	0.009	1.532	1.311
96 hrs	1.468	0.035	0.022	1.525	1.410
120 hrs	1.721	0.119	0.031	1.901	1.610
144 hrs	1.937	0.127	0.035	1.214	1.783
Specific activity of superoxide dismutase enzyme					
Hours in culture	Mean specific activity 50% inhibition of NBT/min/mg of protein	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	0.593	0.024	0.011	0.628	0.472
48 hrs	0.728	0.031	0.017	0.767	0.696
72 hrs	1.104	0.033	0.019	1.198	1.015
96 hrs	1.121	0.042	0.018	1.215	1.011
120 hrs	1.217	0.048	0.021	1.321	1.009
144 hrs	1.352	0.072	0.025	1.375	1.126
Abundance of reduced glutathione (µg/ mg of protein) in *Leishmania donovani* promastigote at different developmental stages					
Hours in culture	Mean abundance (µg/ mg of protein)	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	12.271	0.841	0.276	13.362	11. 724
48 hrs	11.346	0.783	0.197	12.762	10. 966
72 hrs	11.013	0.672	0.174	11.145	10.012
96 hrs	10.359	0.589	0.140	10.864	9.851
120 hrs	9.641	0.511	0.108	10.103	9.021
144 hrs	8.676	0.475	0.097	9.115	8.207
Abundance of oxidized glutathione, GSSG (µg/ mg of protein) in *Leishmania donovani* promastigote at different developmental stages					
Hours in culture	Mean abundance (µg/ mg of protein)	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	3.462	0.261	0.079	3.866	3.010
48 hrs	3.289	0.233	0.067	3.609	2.996
72 hrs	3.763	0.372	0.074	4.325	3.253
96 hrs	3.968	0.309	0.091	5.504	3.546
120 hrs	4.021	0.378	0.098	4.980	3.655
144 hrs	4.225	0.385	0.092	4.907	3.865
Specific activity of Glutathione reductase enzyme					
Hours in culture	Mean specific activity (n mol/min/mg protein)	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	0.294	0.031	0.009	0.321	0.252
48 hrs	0.327	0.033	0.007	0.381	0.299
72 hrs	0.364	0.073	0.014	0.412	0.319
96 hrs	0.461	0.079	0.011	0.510	0.408
120 hrs	0.494	0.088	0.013	0.575	0.410
144 hrs	0.437	0.084	0.019	0.588	0.397
Specific activity of Glutathione peroxidase (GPX) enzyme					
Hours in culture	Mean specific activity (n mol/min/mg protein)	Std Deviation	Std Error	Range	
				Maximum	Minimum
24 hrs	0.059	0.003	0.001	0.062	0.047
48 hrs	0.067	0.007	0.003	0.074	0.060
72 hrs	0.079	0.006	0.001	0.086	0.071
96 hrs	0.095	0.009	0.010	0.012	0.083
120 hrs	0.129	0.012	0.016	0.142	0.116
144 hrs	0.151	0.016	0.012	0.168	0.135

Total Protein Estimated

The absorbance values for selected concentrations of BSA (10, 30, 100, 150, 300, 500µg/ml) at any given time were within the accepted limits (between lower and upper limits as shown in Table 3).

Table 3: - Assessment Of Precision/ Accuracy Of The Assay

Absorbance values of different BSA (µg/ml)						
Replicates	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

Minimum Inhibitory Concentrations (MIC) Of The Extract On *L. Donovani* Promastigotes And Their Survival

The IC_{50} (µg/mL) values of the extract against the growth rate of promastigote was to be 28 ± 3.15. A concentration of 250 µg/mL of the plant extract inhibited the promastigotes *in vitro* by 100%. In contrast, a lower concentration of 50 µg/mL and 100 µg/mL decreased the promastigotes to minimum survival level of 25% to moderate survival level of 50% only. Then again, higher concentration of 500 µg/mL decreased the promastigotes to moderate survival level of 50%. Therefore, 250 µg/mL of the extract was found to be efficacious in inhibiting the parasites growth to maximum levels.

DISCUSSIONS

For Leishmaniasis, synthetic drugs are effectual but with lethal side effects along with its high cost[40-41]. As a consequence of these complications, scientists are in constant exploration to bargain new drugs against these parasites. From the ages, significant attention has been given to the discovery of novel, less- or non-toxic agents of plant origin having antileishmanial capacity[10-14]. A broad spectrum of plant species has been documented having potentially effective leishmanicidal compounds[42-43]. With this knowledge in background, the present investigation has been carried out to explore the leishmanicidal activity of ethanolic extract of *Achyranthes aspera* Linn plant[44].

In qualitative analysis of screening of phytochemical, the extract showed the presence of different percentage of active phytochemicals. The extract also showed a trend of an effectual antioxidant potential *via* free radical scavenging in the DPPH assay due to presence of a fairly dissimilar active phytochemicals in line with the findings of other researchers[14,16,45]. The IC_{50} value of the extract was found to be 55.63 µg/ml in the line with other researchers[46].

It has been well documented that parasites growing in different media present different biological characteristics and distinct *in vitro* and *in vivo* infectivity[47-48]. In our study the Leishmania promastigotes were cultivated axenically *in vitro* in monophasic liquid medium, M-199, supplemented with 10% heat inactivated foetal bovine serum (FBS) and HEPES and the growth pattern was studied for six consecutive days. The promastigotes attained log phase by the second day with a large growth rate between 48 and 96 hours. Post 96 hours, *L. donovani* grew with a long stationary phase with gradual decrease in population level. Promastigotes prior to 96 hours have a comparatively slender body and have little shorter flagella than that of stationary (metacyclic) forms. Survival percentage did not vary much between the stages for first four days but decrease in viability in later stages may be due to the nutrient depleted media.

Further, extracellular acid phosphatase activity has been associated with the degree of promastigote virulence[13, 49]. It may also be related to their ability to survive and multiply in the insect host. We observe that after 96 hours, the enzyme acid phosphatase activity started to differ significantly with that of earlier stages of development. Our results also shows that superoxide dismutase activity increases continually during promastigote development. Specific activity increase ~75% between 24-96 hours[50]. The variation in GSH content in the later stages from that of earlier stages of development 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 both growth rate as well as measure of infectivity in *L. donovani*. Thus, active antioxidant system not only facilitated the growth in parasites but it might also augment it chance of survival by increasing its virulence for successful transmission to vertebrate host[51]. The specific activity of glutathione peroxidase did not exhibit much of variability throughout the development and it indicate towards the supplementary active role of other enzymes in H_2O_2 scavenging in *Leishmania donovani*. For protein quantification the standard curve constructed using the results obtained for the dilution series of BSA (10 to 500 µg/ml), the two variables of the assay, the BSA concentration and absorbance 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 µg/ml) at any given time were within the accepted limits. Similar results have been presented by another workers[52-54].

Determination of MIC involves exposing the test organism to serially diluted extract and determining the minimum concentration that inhibits growth[13, 54]. The survival levels of promastigotes after treatment with varying concentration of ethanolic extract of *D. stramonium* 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 to minimum survival level of 25% to moderate survival level of 50% only. Then again, higher concentration of 500 µg/mL decreased the promastigotes to moderate survival level of 50%. One of the factors affecting the efficacy of medicinal plant extracts is the environment in which the plant grows.

CONCLUSIONS

Natural products which have been found to possess antileishmanial activities can provide alternative treatment for antimonial-resistant Leishmania strains with improved efficacy and reduced resistance. The leishmanicidal activity of the extract of *Datura stramonium* plant has been found efficacious on promastigote form of leishmania to provide effective treatment against *L. donavani*. Our findings corroborate the ethnopharmacological use of this plant for the treatment of Leishmaniasis. These results are promoters as potential sources to search antileishmanial bioactive agents. However, further studies are needed to identify molecules that are responsible for these activities and to evaluate the mechanism effects on amastigote forms.

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Conflict Of Interest

The authors have declared no conflict of interest.

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