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STUDY ON THE METHANOLIC EXTRACT OF PLANT, *NYCTANTHES ARBOR-TRITIS* (HARSINGAR) ON PROMASTIGOTE FORM OF LEISHMANIA DONOVANI IN IN-VITRO CONDITION.

Biotechnology

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ABSTRACT

Leishmaniasis is a group of disease caused by kinetoplast protozoa of the genus *Leishmania*, comprising a group of unicellular organisms which are intracellular parasites of macrophage and other phagocytic cells of the reticuloendothelial system. Visceral leishmaniasis (VL) or Kala-azar a vector-borne parasitic disease (Chang, KP.; et.al, 1985). In India since ancient period the traditional methods of treatment are based on the principles of ayurveda and naturopathy. Considering the aforesaid facts, I have selected some medicinal plant for the herbal treatment of Kala-azar. The plant products or plant extracts will certainly be economical and easy to reach than the allopathic drugs. Therefore, aim is to search a plant which is locally available and without any side effect for the treatment to Kala-azar (Mishra P. 2008; W.H.O. Tropical diseases 2008. Effects of *Nyctanthes arbor-tritis* plant is already reported as antibacterial property therefore I have tested extracts of such plant on promastigote form of *Leishmania donovani* in in-vitro condition. Phytochemical screening of the plant extract showed the presence of alkaloid, flavonoid, fixed oil and fats, saponin, tannin and phenolic compounds. The selected plant is freely available every where known as Harsingar, obtained from Gaya, Bihar region & their methanolic extracts has been prepared in the laboratory through Soxhlet apparatus in methanol solvent to use during experiment. The effects of plant extracts on the parasite will be studied by providing variable doses to the parasite. The plant extracts at varied concentration will be added into media containing the parasite in the ratio between extract & media determined during experimentation in the laboratory. After incubation at different time intervals of 24, 48 and 72 hours the effect of extracts on the parasite had been studied in-vitro condition. The plant extracts exhibit that with the increasing duration of time the efficacy of the plant extracts also increases significantly in NA_M taken into the consideration.

KEYWORDS

Visceral leishmaniasis, *Nyctanthes arbor-tritis* (Harsingar), M199, MTT assay, PBS (Phosphate buffer saline),

INTRODUCTION

Leishmaniasis are a group of multiorgan, vector-borne disease caused by intracellular protozoan parasite of genus *Leishmania* and transmitted by sandflies.

Visceral leishmaniasis [VL] also called as "Kala-azar" is featured by skin pigmentation, irregular fever, weight loss, lymphadenopathy, hepatosplenomegaly (both the spleen and liver are enlarged than normal), pancytopenia and anemia. The visceral leishmaniasis is most common in India, East Africa and in Brazil. According to WHO report more than 90% of the cases reported in 2019 were from India, Brazil, Iraq, Ethiopia, Kenya, Somalia, Nepal, and Sudan (Piscopo and Mallia, 2007; WHO 2021). India alone shares nearly 25 % of the global load of kala-azar. This is a serious public health issue in the eastern part of the country specially in the states of Bihar, eastern Uttar Pradesh, Jharkhand, West Bengal, and areas adjoining to Nepal. In term of districts, 54 districts in the four states., 33 districts of Bihar, 4 districts of Jharkhand, 6 districts of Uttar Pradesh and 11 districts of West Bengal are affected. Sporadic cases also appear from some other states like Uttarakhand, Assam, Himachal Pradesh, Gujarat, Jammu & Kashmir, Madhya Pradesh, Kerala, Haryana, Sikkim, Pondicherry, and Tamil Nadu (Talniya, 2016; Bhunia et al., 2013; Kumar et al., 2020).

Bihar is one of the most severely affected state of India. More than 50 % of all the cases of leishmaniasis are reported from Bihar. VL is still common in the 458 blocks of Bihar. Some VL affected blocks have persistently remained extremely endemic for many years (Kumar et al., 2020; WHO-NVBDCP, 2017). .

For Leishmaniasis, synthetic drugs are effectual but with lethal side effects along with its high cost. As a consequence of these (40-41) complications, scientists are in constant exploration to bargain new drugs against these parasites. Since the ages, significant attention has been given to the discovery of novel, less- or non-toxic agents of plant origin having antileishmanial capacity. A broad spectrum of different plants from around 14 species have been documented to contain potentially effective leishmanicidal compounds. With this knowledge in background, the present investigation has been carried out to explore the antileishmanial activity of methanolic extract of *Nyctanthes arbor-tritis* plant.

The universally recommended drugs used for the treatment of Visceral leishmaniasis are pentavalent antimonial. Recently, Emergence of (MDR) multiple drug resistance to human pathogenic organisms particularly the hemoflagellates like *Leishmania* species along with severe side effects has compelled to search for new antimicrobial substances from other sources including plant. It has been established that plants are one of the vital sources and the phyto-chemicals derived from them acts suitably & potentially against the parasites. It has also been reported that over 100 plants act suitably against various form of Leishmanial parasites (Hamid Eqbal et.al.2012). Different parts of the medicinal plant extracts are being used as antileishmanial agents. Considering these facts, present study has been undertaken to evaluate the extracts of *Nyctanthes arbor-tritis* (Harsinghar) on promastigote form of *L.donovani* in in-vitro condition. *Nyctanthes arbor-tristis*, a medicinal plant belonging to family Oleaceae and commonly known as Night Jasmin (Vats et al., 2009; Meshram et al., 2012). The plant has some traditional as well as medicinal values. This plant also has some phytochemicals like flavonoids, glycosides, D-mannitol, nicotiflorin etc. (Bordoloi & Lahkar, 2018). The whole plant exhibits pharmacological effects and the leaves show anti-fungal, anti-inflammatory and antibacterial effects (Gulsan et al., 2015).

Therefore, this study asses the present information that has also been co-related with those of the findings as antileishmanial drugs derived from the plant origin. Keeping in view, I have started worked on the promastigote form of *L. donovani* with the *Nyctanthes arbor-tritis* plant extracts at its different concentrations administered @ 24 hrs, 48 hrs & 72 hours and found significant performance at the concentration of 200 µg/ml and 250 µg/ml at 72 hrs., exposure of methanolic extract of the aforesaid plant.

MATERIALS AND METHODS

Collection of Plant Materials

Leaves *of Nyctanthes arbor-tritis*, (Harsingar) was taken for the experiment at Post graduate department of Biotechnology, Magadh University, Bodh Gaya, Bihar, India and identified according to the relevant monographs of Indian Pharmacopoeia and kept in the sterile condition in the laboratory for further process. As it washed with distilled water followed by drying at 40°C in the hot air oven till completely dried, further it was ground into fine powder in a mortar. The plant extracts were obtained with methanolic treatment by using Soxhlet apparatus.

The powder form of plant material was weighed and kept in the thimble for at least 24 hours for methanolic treatment. After methanolic treatment, the extracts were eluted from thimble and come back to methanol. The extracts along with methanol taken out from Soxhlet apparatus were kept in hot air oven to evaporate the dissolved methanol. To remove complete methanol and obtain extracts in crystalline form rotatory evaporator were used. The crystalline forms of extracts were kept in refrigerator at 4°C for further use.

Collection of *Leishmania* parasite/primary isolation of parasite:

The promastigotes of (strain MHOM/ IN/ AG/ *Leishmania donovani* 1003), originally acquired from Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna, Bihar. were obtained and routinely maintained in the laboratory, at the post Graduate department of Biotechnology, Magadh University, Bodh Gaya, Bihar, India. The mid log-phase parasites, promastigotes have been maintained weekly passage through the artificial culture medium, Medium 199 (M-199). Promastigotes were cultivated at 25±2°C in vitro in monophasic liquid medium, M-199 (Himedia), supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) and 25mM N-(2-hydroxyethyl) piperazine N'-2-ethane sulfonic acid (HEPES). The medium was then altered under sterilized conditions in culture room under laminar flow.

The culture medium was kept for 48 hours at room temperature to check for any contamination. 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 hemocytometer.

For viability testing, cells obtained from centrifugation had been mixed with 0.01M PBS and 2% Trypan Blue (Sigma, USA) sol 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 was used for further enzymatic analysis.

Maintenance of Promastigotes:

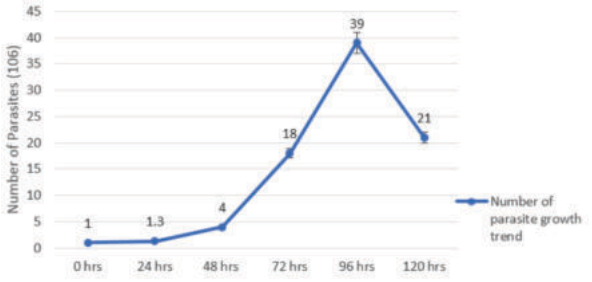
For maintenance of the parasites, the fresh M199 complete media was been taken in sterilized 5 ml culture bottle and primary isolated culture was transferred in aseptic condition every third day. The ʏ radiated test tube containing the primary isolates were taken for sub-culturing. These may be kept at ±4ºC for long time survival of the parasites.

Harvesting Promastigotes culture: -

For obtaining Logarithmic (Log) Phase, the culture media was centrifuged at 2500 rpm for 20-30 minutes at 4ºC in a capped centrifuge tube. After centrifugation, the supernatant was discarded and the pelleted material containing the parasites were resuspended in Phosphate buffer saline and centrifuged three times. The pellets were finally mixed with M199 media.

Growth curve of promastigote of *L.D* in M199 media:

In the M199 media, 10^6 cells of promastigote from *Leishmania donovani* were inoculated and their growth dynamics observed in different time intervals, at 24, 48, 72, 96 and 120 hrs. It is observed that the growth reached maximum at 96 hrs, there after it started to decrease sharply may be due to the nutrition deprivation. The maximum density reached 3.9 x10^7/ml.



Preparation of PBS (Phosphate buffer saline):-

Phosphate buffer saline is a salty solution that contains sodium chloride, potassium bihydrogen phosphate, sodium hydrogen phosphate. The buffer helps to maintain a constant pH.

PBS is used in culture because it is isotonic & non-toxic to the cells. It can be used to dilute substances.

Preparation of the Crude Extracts

The crystalline form of each plant extracts was weighed 100 mg and dissolved in 1 ml pure DMSO and thus preparation of stock solution was made in concentration of 100 mg/ml. From these stock solutions different concentration of each plant extracts i.e., 25, 50,100,150, 200 and 250 µg/ml were made in 5% DMSO solution (5 ml DMSO + 95 ml D.W.). These plant extracts were exposed to culture medium aiming to reach the concentration that would produce to decrease the cell growth of *Leishmania* parasites by 50 percent using percent of growth inhibition method.

Further In-vitro toxicity by MTT assay were done by Ghasemi M, et.al., November 2021., has been followed, to understand the cell viability.

Treatment of *L.donovani* promastigote with plant extracts

The anti-leishmanial activity of different concentration (25, 50,100,150,200 and 300µg) of these plant extracts was assessed on promastigote forms of *L. donovani* which was cultivated in media M199. Parasites at log phase were centrifuged at 3000 rpm for 10 minutes, washed with normal saline by centrifuge with same speed and time. Parasites were diluted to a final density of 1×10^6cells/ml with a fresh culture medium of M199. Eventually 100 µl of the culture was added in all screwed caped culture tubes. The tubes were manually shaken for 2 minutes. The drug activity values of ethanolic extracts were analyzed and assessed to determine the number of leishmanial parasites in non-treated control (5% DMSO without plant extracts) to control culture tubes after different time intervals of 24, 48 and 72 hours microscopically monitored by using hemocytometer (improved Neubauer-counting chamber, Germany). All in vitro experiments were run in quintuple and the results were expressed as the percent viability in parasites number.

Statistical Analysis:-

All the statistical analyses were performed using PC based 37-39 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 $p \leq 0.05$. The numbers of parasites were counted with a hemocytometer under a light microscope after 48 and 72 hours of incubation. P values < 0.05 were considered as statistically significant. In order to keep in line with previous works in the same field, 48 and 72 hours were chosen to express the effect of the extract.

RESULTS

According to the design of the experiment, as I have selected *Nyctanthes arbor-tritis* plants extracts which are having antimicrobial activity. Selected plant is being used with methanol solvents for the extraction.

Different concentration of extracts like 25µg/ml, 50µg/ml, 100µg/ml, 150µg/ml, 200µg/ml and 250µg/ml at different time interval of exposure likely 24 hours, 48 hours and 72 hours on promastigote form have been observed and results obtained are recorded in table.

The details are incorporated as in table-1 under the heads of control which are untreated and methanolic extracts of *Nyctanthes arbor-tritis* are summarized, the results due to effects produced by NA_M extracts at

varied concentration at 24 hours of exposure.

Apparently, the results noted below after the treatments of parasite with methanolic extracts of *Nyctanthes arbor-tritis* at the concentration of 25 µg/ml, 50 µg/ml,100 µg/ml,150 µg/ml,200 µg/ml and 250 µg/ml for 24 hours exhibit no any significant difference found treated for 24 hours duration, the potency of plant extracts in respect to the control are almost the same.

Further, with the increase of treatment time upto 48hrs., with the earlier concentration @ 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml and 250 µg/ml the values changed showing increasing potency of plant extracts at 48hrs., and 72hrs., of treatment showing in table 2 and 3.

Whereas table 4 reveals average data of all three different exposure time 24, 48 and 72hrs., respectively. Table showing with the increasing duration of time the efficacy of the plant extracts also increases significantly in NA_M taken into the consideration, the concentration of 25 µg/ml, 50 µg/ml,100 µg/ml,150 µg/ml,200 µg/ml and 250 µg/ml for 24hours., exhibit (100, 98, 88, 69, 52 and 41), whereas with the same concentration for 48hrs., of exposure we found (99, 96, 76, 58, 43 and 36) but at the same concentrations when we increase exposure time 72hrs., data reveals no any significant effect at the concentration of 25 µg/ml, 50 µg/ml,100 µg/ml and 150 µg/ml (99, 95, 75 and 56) whereas at the concentration of 200 µg/ml and 250 µg/ml shows significant and highly significant effect with (43 and 36) on promastigote forms of *L. donovani* respectively.

Table 1: Showing % Cell Viability (Promastigote) in Methanolic extract of *Nyctanthes arbor-tristis* L (NA_M) 24 hrs of Exposure

Sl. No	Control	Experimental NA_M					
		25µg	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	100	98.2	87.8	69.2	51.9	41.0
2	100	100	98.2	87.9	69.1	52.2	41.2
3	100	100	98.1	88.2	69.1	51.8	41.1
4	100	100	97.7	88.1	68.7	52.2	40.9
5	100	100	97.8	88.0	68.9	51.9	40.8
Mean	100	100	98	88	69	52	41

Table 2: Showing % Cell Viability (Promastigote) in Methanolic extract of *Nyctanthes arbor-tristis* L (NA_M) 48 hrs of Exposure

Sl.No	Control	Experimental NA_M					
		25 µg	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	99.1	95.9	76.2	57.8	43.1	35.8
2	100	99.2	95.7	76.1	57.9	42.9	35.9
3	100	99.1	96.0	75.9	58.7	42.7	35.8
4	100	99.0	96.1	75.7	58.2	42.9	36.2
5	100	98.6	96.2	76.1	58.4	43.4	36.3
Mean	100	99	96	76	58	43	36

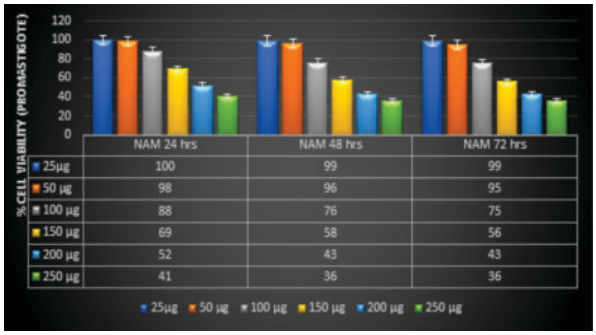
Table 3: Showing % Cell Viability (Promastigote) in Methanolic extract of *Nyctanthes arbor-tristis* L (NA_M) 72 hrs of Exposure

Sl.No	Control	Experimental NAM					
		25 µg	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	99.1	94.7	74.9	56	42.8	36.2
2	100	99.3	94.9	75.3	56	42.9	36.3
3	100	98.8	95.3	74.8	55	42.9	35.8
4	100	99.1	95.2	75.2	56	43.2	35.7
5	100	98.7	94.9	74.8	55	43.2	36.0
Mean	100	99	95	75	56	43	36

Table 4: Showing % Cell Viability (Promastigote) Mean value, in Methanolic extract of *Nyctanthes arbor-tristis* L (NA_M) 24,48 &72 hrs of Exposure

Hrs.	Control	Experimental NAM					
		25µg	50 µg	100 µg	150 µg	200 µg	250 µg
24	100	100	98	88	69	52	41
48	100	99	96	76	58	43	36
72	100	99	95	75	56	43	36
C.D. level at 0.5%		NS	NS	NS	NS	*	**

NS	:	Non Significant
*	:	Significant
**	:	Highly Significant
***	:	Outstanding



Histogram Showing % Cell Viability (Promastigote) Methanolic extract of *Nyctanthes arbor-tristis* L (NA_M) 24,48 &72 hrs of Exposure

CONCLUSION

The present study concluded that the leishmanicidal activity of the *Nyctanthes arbor-tritis* plant extract was efficacious at a concentration of 250 µg/ml for 72hrs., almost equally to the other standard drug (Glucantime) with an effective concentration of 25 ± 2.85 µg/ml on promastigote form of *L. donovani* has to provide for its effective treatment. The outcome of the present study advocates to explore the plant world to identify its active phyto-molecules as potential sources of safe, effective and economical anti-leishmanial bioactive agents. Natural products with anti-leishmanial activities can provide an alternative medicine for the treatment of antimonial resistant leishmania strains with improved efficacy and nonresistance. Further, studies are needed to evaluate their action mechanism against different forms of *L. donavani*.

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Conflict of interests

The authors declare that there is no competing interest.

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