ORIGINAL RESEARCH PAPER
Volume - 12 | Issue - 02 | February - 2023 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

EFFECTS OF NYCTANTHES ARBOR-TRITIS (HARSINGAR) PLANT EXTRACTS ON PROMASTIGOTE FORM OF LEISHMANIA DONOVANI IN IN-VITRO CONDITION.

Biotechnology

Sushma Kumari Ray* Post Graduate Department of Biotechnology, Magadh University, Bodh Gaya *Corresponding Author

Kumar Lav Kush Tarun Post Graduate Department of Biotechnology, Magadh University, Bodh Gaya

Birendra Kumar Post Graduate Department of Biotechnology, Magadh University, Bodh Gaya

Sarfaraz Ali Post Graduate Department of Biotechnology, Magadh University, Bodh Gaya

Santwana Rani Department of Botany, College of Commerce, Arts and Science, Patliputra University, Patna

ABSTRACT

Leishmaniasis is a group of disease caused by the members of kinetoplast protozoa of the genus *leishmania* comprising a group of unicellular organisms which are intracellular parasites in macrophage and other phagocytic cells of the reticuloendothelial system. Visceral leishmaniasis (VL) or Kala-azar a vector-borne parasitic disease caused by protozoan parasite (Chang, KP.; et.al, 1985). In India the traditional methods of treatment are based on the principles of ayurveda and naturopathy. Considering the aforesaid facts this project has been selected to search the plants for the treatment of Kala-azar. The plant products or plant extracts will certainly be cheaper than the allopathic drugs. Therefore, aim is to search a plant which is locally available and if not available, it can be purchased from the local market to provide cheaper and without side effect treatment to Kala-azar (Mishra P. 2008; W.H.O. Tropical diseases 2008. Effects of *Nyctanthes arbor-tritis* plant extracts 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 obtained from Gaya, Bihar region & their ethanolic extracts will be prepared in the laboratory by Soxhlet apparatus in Ethanol 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 will be studied. The sample of parasite (promastigote of *L. donovani*) used for experiment (in-vitro) will be routinely culture in laboratory at 25± 2° with in different culture media after collecting parasites (sample) from the blood and bone marrow of patients of Kala-azar in the hospitals or research institutes. The plant extracts exhibit that with the increasing duration of time the efficacy of the plant extracts also increases significantly in NA_E taken into the consideration.

KEYWORDS

Visceral leishmaniasis, *Nyctanthes arbor-tritis* (Harsingar) M199.

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 Uttaranchal, Assam, Himachal Pradesh, Gujarat, Jammu & Kashmir, Madhya Pradesh, Kerala, Haryana, Sikkim, Puducherry, 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 is 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).

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 of 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 addresses assessing the present information has also been co-related with those of the findings as antileishmanial drugs derived from the plant origin.

MATERIALS AND METHODS

Collection of Plant Materials

Plant samples *Nyctanthes arbor-tritis*, was taken for the experiment. *Nyctanthes arbor-tritis* leaves was taken from 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. After collection, the desired plant parts (leaves) were first cleaned from extra weeds and washed with distilled water. After then, they dried at 40°C in hot air oven till completely dried and grind into fine powder in a mortar. The plant extracts were obtained by ethanolic treatment by using Soxhlet apparatus. The powder form of plant material weighed and kept in the thimble for at least 24 hours ethanolic treatment. After ethanolic treatment, the extracts were eluted from thimble and come back to ethanol. The extracts along with

ethanol taken out from Soxhlet apparatus were kept in hot air oven to evaporate ethanol. To remove complete ethanol and obtained 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 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. It 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 had been used for further enzymatic analysis.

Maintenance of Promastigotes:
For maintenance of the parasites, the fresh M199 complete media has been taken in sterilized 5 ml culture bottle and primary isolated culture was transferred in aseptic condition at every third day. The radiated test tube containing the primary isolates has been 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 were 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 the nutrition deprivation. The maximum density reached 3.9×10^7/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 antileishmanial 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 the in vitro experiments were run in quintuple and the results were expressed as the percent viability in parasites number.

Statistical Analysis:-
Data 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 haemocytometer 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 ethanol 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 incorporated in table-1 under the heads of control which are untreated and ethanolic extracts of *Nyctanthes arbor-tritis* are summarized the results due to effects produced by NA_E extracts at varied concentration at 24 hours of exposure.

Apparently the results noted below after the treatments of parasite with ethanolic 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 same.

Further, with the increasing of treatment time 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 $^{NA}{}_E$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 (99, 97, 80, 57, 47 and 36), whereas with the same concentration for 48hrs., of exposure we found (98, 95, 74, 48, 38 and 32) 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 (97, 95 and 71) whereas at the concentration of 150 µg/ml &200 µg/ml shows significant and highly significant effect with (48 and 36) at the same time 250 µg/ml shows

outstanding result with 32 on promastigote forms of *L. donovani* respectively.

Table 1: Showing Ethanolic extract of NA_E 24 hrs of Exposure

Sl. No	Control	Experimental NAE					
		25µg/ml	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	99.5	96.7	80.3	57.3	47.5	35.8
2	100	98.6	97.8	80.4	56.9	46.7	35.7
3	100	99.3	96.3	80.2	56.8	47.6	36.2
4	100	99.2	97.4	79.5	56.9	46.4	36.4
5	100	98.4	96.8	79.4	57.1	46.8	35.9
Mean	100	99	97	80	57	47	36

Table 2: Showing Ethanolic extract of NA_E 48 hrs of Exposure

Sl. No	Control	Experimental NAE					
		25µg	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	98.4	95.4	74.1	47.6	37.9	32.1
2	100	98.5	94.7	74.2	48.5	38.5	32.3
3	100	97.5	95.3	73.7	47.4	37.7	31.7
4	100	97.7	94.5	74.2	48.3	38.3	31.5
5	100	97.9	95.1	73.8	48.2	37.6	32.4
Mean	100	98	95	74	48	38	32

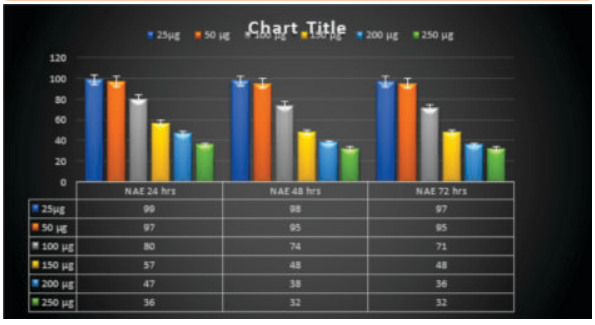
Table 3: Showing Ethanolic extract of NA_E 72 hrs of Exposure

Sl. No	Control	Experimental NAE					
		25µg	50 µg	100 µg	150 µg	200 µg	250 µg
1	100	98.3	95.3	70.8	48.4	35.9	31.8
2	100	98.1	94.8	70.7	47.7	35.8	32.2
3	100	98.4	95.4	71.3	47.6	36.4	32.4
4	100	97.5	94.4	70.9	48.2	35.6	31.9
5	100	97.7	94.1	71.3	48.1	36.3	31.7
Mean	100	97	95	71	48	36	32

Table 4: Showing Mean value, Ethanolic extract of NA_E 24,48 &72 hrs of Exposure

Hrs.	Control	Experimental NAE					
		25µg	50 µg	100 µg	150 µg	200 µg	250 µg
24	100	99	97	80	57	47	36
48	100	98	95	74	48	38	32
72	100	97	95	71	48	36	32
C.D. level at 0.5%		NS	NS	NS	*	**	***

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



Histogram Showing Ethanolic extract of NA_E 24,48 &72 hrs of Exposure

DISCUSSIONS

For Leishmaniasis, synthetic drugs are effectual but with lethal side effects along with its high cost. As a consequence 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. A broad spectrum of 14 Plants species has been documented having potentially effective leishmanicidal compounds. With this knowledge in background, the present investigation has been carried out to explore the leishmanicidal activity of ethanolic extract of *Nyctanthes arbor-tritis* plant.

Leishmaniasis along with sleeping sickness and chagas disease are considered as Neglected Tropical Diseases (NTDs) by the W.H.O and these threaten more than one billion people worldwide. Due to high increase of resistance against the drugs under the existing treatments (Ashutosh & Goyal, 2007) the search of new, safe natural therapies is urgently required (Akendengue et. al., 1999; Chan-Bacob et.al., 2001). Cos et. al, (2006) evaluated the anti-infective potential of different natural products and also advocated to promote this type of works in futures. Crofts and combs (2003), craft et. al, 2006 and Carter et. al, (2006) reviewed the current chemotherapeutic trends for leishmaniasis where they mentioned the need of novel drugs derived from natural resources. In search of naturally derived medicine several attempts were made by earlier workers (Montesino et. al., 2015, Aligiannis et.al., 2001. De Mesquita et.al., 2005; Ghosh et.al., 2011). Newman & Cragg (2007, 2012) reviewed the natural products as sources of new effective drugs evaluated during 1981 to 2010.

Montesino et. al., (2015) noted the efficacy of marketed herbal medicinal products (HMP) as antiprotozonal activity where the extracts of salvia, valuriana, Hypericum, Sisbun, Arnica and Cucuma exhibited high IC_{50}. The present observations also agree in respect of IC_{50} value achieved by extracts of *Nyctanthes arbor-tritis* Similarly, Ghosh et. al., (2011) also noted the root extract of *valeriana wallichii* as antileishmanial activity but Glaser et. al., (2015) also observed the Cytotoxic compounds present in this extract. Ozbilign et. al., (2014) noted the antileishmanial activity of some Turkish medicinal plants in reference to *L. tropica* promastigotes in vitro. The findings are very much comparable to present observations.

Butler (2005) reported that plant derived products have been evaluated intensively in recent years for leishmanicidal compounds. Santosh et.al., (2008) pointed out the importance of new alternatives from microorganisms and plants for the treatment of leishmaniasis. In order to search the antileishmanial medicine a number of medicinal plants and its medicinal values have been evaluated in different countries, sauvain et.al 1994., Singha et.al., 1992., Martin et.al., 2009., Sale habaid et. al., 2014.

Al Jubouri and Al-Khan (2009) evaluated the effects of aqueous extracts of *Nirium sleander* and *Milla azederach* on growth of *Leishmania tropica* in vitro and obtained results are very much comparable to present findings. The ethanol, water and n-hexane extracts from the leaves of Arbutus unedo were tested in vitro against *L. tropica* promastigotes and the ethanol extract of A. unedo leaves at the concentrations of 100, 250, 500 µg/ml were found to be more effective (Kivcak et.al., 2009). The results obtained from the present investigation on the promastigotes of *L. donovani* also supports and agrees with the findings of Kiveak et. al., 2009. Thus, the present study constituents the first report on antileishmanial activities of plant extracts *Nyctanthes arbor-tritis*.

The prepared extracts, like the present investigation were used in various concentration against the promastigote cultures of *L. donovani* and IC_{50} values were calculated. Singh et. al., (2011) investigated the antileishmanial properties of several plants from visceral leishmanial endemic area of Bihar. In this study it was noted that inhibited growth of promastigote and significant promastigote killing were possible by the plant extracts of *Agave awercana* and *Azadirachta indica*.

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 treated @ 24 hrs, 48 hrs & 72 hours and found out standing performance at the concentration of 250 µg/ml with the 72hrs., exposure of ethanolic extract of said plant.

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., 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*.

Acknowledgement

The authors wish to express their profound gratitude to Rajendra Memorial Research Institute of Medical Sciences, Patna (ICMR) and Post Graduate Department of Biotechnology, Magadh University, Bodh Gaya. Our profound gratitude also goes to Dr. Santwana Rani, Dr. Sarfaraz Ali, Dr. Birendra Kumar and Dr. L.K. Tarun and Dr. S.N.P. Yadav "Deen" for their constant support & encouragement towards this work.

Conflict of interests

The authors declare that there is no competing interest.

REFERENCES

1. Akendengue B, Ngou-Milama E, Laurens A, Hocquemiller R (1999) Recent advances in the fight against leishmaniasis with natural products. *Parasite* 6:3–8.
2. Anonymous. (2012). *The Indian Pharmacopoeia*. 3rd edition. Government of India, New Delhi., Ministry of Health and family welfare.
3. Ashutosh, Sundar, S., Goyal, N., 2007. Molecular mechanisms of antimony resistance in Leishmania. *J. Med. Microbiol*. 56, 143–153.
4. Bordoloi, P.; Devi, T. and Lahkar, M. (2018). Evaluation of anti-inflammatory and anti-arthritic activity of ethanolic extract of leaves of Nyctanthes arbor-tristis an experimental animal mode. *Journal of Evolution Medicinal and Dental Sciences*, 7(10):1247-1251.
5. Carter KC, Hutchion S, Henriquez FL, Legare D, Oullette M, Roberts CW,2006.
6. Chang, KP., 1985. Biology of Leishmania and Leishmaniasis., Elsevier science publication, B.V. (Biomedical division). USA. Pp. 166-173.
7. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro'proof-of-concept'. *J Ethnopharmacol* 2006;**106**: 290-302.
8. Croft, S.L., Coombs, G.H., 2003. Leishmaniasis – current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol*. **19,** 502–508.
9. Croft, S.L., Seifert, K., Yardley, V., 2006. Current scenario of drug development for leishmaniasis. *Indian J. Med. Res*. **123**, 399–410.
10. De Mesquita, M.L.; Desrivot, J.; Bories, C.; Fournet, A.; de Paula, J.E.; Grellier, P.; Espindola, L.S. Antileishmanial and trypanocidal activity of Brazilian Cerrado plants. *Mem. Inst. Oswaldo Cruz* 2005, 100, 783–787.
11. Dutta, A., Bandyopadhyay, S., Mandal, C., Chatterjee, M. (2005). Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis.,119–122.
12. Et-Touys, A., Bouyahyal, A., Fellah, H., Mniouil, M., El-Bouryl, H., Dakka, N., Sadak, A.Bakri, Y. (2017), Antileishmanial activity of medicinal plants from Africa: a review. *Asian Pacific Journal of Tropical Disease*, 7, 826-840.
13. Field, A. (2005). Discovering Statistics using SPSS, 2 ed. Sage, London.Hamid Iqbal, Baharullah Khattak,Sultan Ayaz, Ali Rehman, Muhammad Ishfaq, Muhammad Naseer Abbas, Hameed ur Rehman, Saqib Waheed, Abdul Wahab Comparative Efficacy of Aloe veraand Tamarix aphylla against Cutaneous Leishmaniasis *International Journal of Basic Medical Sciences and Pharmacy (IJBMSP)*2012,Vol. 2, No.42-45.
14. Ghasemi M, Turnbull T, Sebastian S, Kempson I (November 2021). "The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis". *International Journal of Molecular Sciences*. **22** (23): 12827
15. Gottlieb, M., Dwyer, D.M. (1981): surface membrane acid Leishmania donovani phosphatase activity of promastigotes. (1),117-128.
16. Kar, K. (1997). Folic acid, the essential supplement to brain-heart infusion broth for cultivation and cloning of promastigotes. 231- *Leishmania donovani Parasitology*, M5, 235.
17. Kayser, O., Kiderlen, A.F. (2001). leishmanicidal activity of naturally occurring In vitro chalcones. (2), 148-152.
18. Kumar A, Saurabh S, Jamil S, Kumar V. Intensely clustered outbreak of visceral leishmaniasis (kala-azar) in a setting of seasonal migration in a village of Bihar, India. *BMC infectious diseases*. 2020b Dec; 20(1):1-3.
19. Montesino NL, Kaiser M, Brun R, Schmidt Thomas J "Search for Antiprotozal activity in Herbal Medicinal Preparations; New Leads against Neglected Tropical Disease" *Molecules* 2015, 20,14118-14138.
20. Montesino NL, Kaiser M, Brun R, Schmidt Thomas J "Search for Antiprotozal activity in Herbal Medicinal Preparations; New Leads against Neglected Tropical Disease" *Molecules* 2015, 20,14118-14138; doi:10.3390/molecules 200814118.
21. Mullen AB. Resistance of leishmania donovani ti sodium stibogluconate is related to the expression of host & parasiye gamma-glutamylcysteine synthetase. *Antimicrob agents Chemother* **50**:88-95.
22. Murari, K., Bharti, B.K., Bharti, S.K. (2013). Indian Plants in Medicine- Green Economics. (6), 864-865.
23. Newman DJ, Cragg DM, Snadder KM (2003) Natural products as source on new drugs 1981–2002, *J Nat Prod* 66:1022-1037.
24. Piscopo TV, Mallia Azzopardi C Leishmaniasis Postgrad Med J. 2007 Feb; 83(976): 649–65.
25. Rajani KS1*, Swasha Khandeparker Naik2, Subhadarshini Satapathy2 and Sweta Mishra2 Phytochemical analysis of the leaf extracts of Nyctanthes arbor-tritis *Plant Archives* Volume 21, No 1, 2021 pp. 1878-1881.
26. Santos, D, O., Coutinho, C.E., Madeira, M.F., Bottino, C.G., Vieira, R.T., Nascimento, S.B., Bernardino, A., Bourguignon, S, C., Corte-Real, S., Pinho, R, T., Rodrigues, C, R., Castro, H, C. (2008). Leishmaniasis treatment--a challenge that remains: a review. *Parasitology Research*, 103(1),1-10.
27. Shrikant Kumar, Puspaa Sinha, Akhtar Parwez, Birendra Kumar, Kumar Lav Kush Tarun and Sudhanshu Kumar Bharti* The development and biochemical features of *L.Donovani* promastigote and their repression by Datura stramonium plant extract. *Internatonal journal of scientific research*.Volume – 11, Issue – 09, September – 2022
28. Sundar, S., Rai, M., Chakravarty, J., Agarwal, D., Agrawal, N., Vaillant, M., Olliaro, P., Murray, H, W. (2008). New treatment approach in Indian visceral leishmaniasis: singledose liposomal amphotericin B followed by short-course oral miltefosine. *Clinical Infectious Diseases*, 47(8), 1000-1006.
29. Vats, M.; Sharma, N. and Sardana, S. (2009). Antimicrobial activity of stem bark extracts of Nyctanthesarbortristis Linn. (Oleaceae). Inter Nyctanthesarbortristis. *Journal of Pharmacognosy and Phytochemical Research*, 1: 12-14.