**Original Research Article****DOI:10.26479/2019.0501.56*****IN VITRO* ANTILEISHMANIAL ACTIVITY OF *DIPLAZIUM ESCULENTUM*****R Jyoti¹, Kakuli Chakraborty¹, Rajen Haldar², Bikramjit Raychaudhury^{1*}**

1. Department of Physiology, Ananda Chandra College, Jalpaiguri, W.B., India.

2. Department of Physiology, University Colleges of Science and Technology,
University of Calcutta, Kolkata, India.

ABSTRACT: Many reports are available to believe that flora and fauna are being used by local people in India for treatment of diseases like leprosy, blood pressure, rheumatic disorders and also for several ailments but practically there is no scientific evidence to believe the miraculous cure that being taken place. The treatment of visceral leishmaniasis with available drugs is limited. Moreover no vaccine has been identified till date. The existing drugs are very much toxic and none of them are free from side effects. To make the situation worse some of the parasite strains have developed resistance against the classical antimonial drugs. Resources from North Bengal is more or less unexplored to discover alternative medicines which may be successfully applied to treat diseases like leishmaniasis for which there is a need for new drug development. In an attempt to develop new indigenous drugs against leishmaniasis, we have screened aqueous leaf extract of *Diplazium esculentum* and tested *in vitro* to assess its potential. Aqueous extracts showed 100% inhibition in growth at a concentration of 80 mg/ml. However at a lower concentration of 50 to 60 mg/ml, promastigote growth was inhibited by 60-75%. with a IC_{50} of 40 mg/ml. The action of *Diplazium esculentum* as a chemotherapeutic agent is found to be mediated through inhibition of superoxide dismutase and simultaneous release of toxic superoxide radical. We propose that *Diplazium esculentum* may be considered as a prospective candidate to establish a better line of therapeutic process against visceral leishmaniasis.

KEYWORDS: Medicinal plant, *Leishmania*, *Diplazium esculentum*, Superoxide dismutase.**Corresponding Author: Dr. Bikramjit Raychaudhury* Ph.D.**

Department of Physiology, Ananda Chandra College, Jalpaiguri, W.B., India.

Email Address: glycosome@yahoo.com

1.INTRODUCTION

Leishmaniasis are tropical and subtropical parasitic diseases which affect nearly 12 million people with an annual incidence of two million and is responsible for considerable morbidity and mortality [1-2]. The disease is transmitted by sandflies. In human, the disease occurs in at least four major forms: cutaneous, diffuse cutaneous, mucocutaneous and visceral. *Leishmania* epidemics have been reported in Northern India-the junction of Eritrea, Ethiopia, Sudan and southern Europe [3]. The leishmaniasis cause disabling and sometimes highly mutilating lesions. Visceral leishmaniasis is the most severe form and attacks the spleen, liver and lymph nodes. 90% of all visceral leishmaniasis cases occur in India, Bangladesh, Brazil and the Sudan [4]. Within these countries, the disease claims its victims among the most impoverished members of the community. Left untreated, the disease is usually fatal. Clinical treatment is almost totally dependent upon the use of pentavalent antimonial drugs such as sodium stibogluconate (Pentostam) as well as N-methylglucamine antimonite (Glucantime). Both antimonial drugs require high dose regimens and long courses of treatment using parenteral administration making them unreliable for wide scale use [5-6]. Moreover, resistance to antimonial drugs has been reported in the fatal form of the visceral leishmaniasis diseases [7]. Second line of drugs like pentamidine and amphotericin B have been clinically used but possess serious toxic side effects [8]. Several other drugs showed varying degrees of efficacy as well as with high level of toxicity [9-10]. Vaccination efforts have also been unsuccessful. Therefore, there is an urgent need to search for improved drug therapy against leishmaniasis. Dooars region in West Bengal covers a vast area of 8,800 sq. km in India with its unique ecosystem. Perhaps, the micro details of ecosystem is yet to be explored. People in this area have started living here hundreds of years back fighting with the odds of nature. Through this process they have gathered the wisdom to cure themselves from different types of diseases using resources available around them. There are many traditional knowledge resource persons who are just getting vanished in this age of globalization. But indigenous or traditional knowledge of human health and medicine can form a strong base for primary health care. In recent years, there has been renewed interest in plant medicine for the treatment against different diseases as herbal drugs are generally out of toxic effect reported from research work conducted on experimental model animal [7]. The use of medicinal plants for the treatment of parasitic diseases is well known & documented since ancient times. The present study deals with the assessment of *Diplazium esculentum* to establish its antileishmanial activity and mode of action for a potent chemotherapeutic agent against *Leishmania* pathogen. *Diplazium esculentum*, commonly known as Dhenkir Shaak, is an edible fern found in North Bengal. The plant is often harvested from the wild for its edible young leaves (croziers). The plant is used in treating headache, pain, fever, wounds, dysentery, glandular swellings, diarrhea, and various skin infections [11].

2. MATERIALS AND METHODS

Reagents

RPMI 1640 medium and sodium thioglycollate were obtained from GIBCO Laboratories, Grand Island, New York, USA. Sodium Stibogluconate was from Gluconate Health Ltd and Percoll from Pharmacia Fine Chemicals, Sweden. All other reagents were obtained from Sigma Chemical Company, St Louis, Missouri, USA.

Filter Unit

Drug solutions and liquid media were sterilized by filtration through millipore filter (0.22 μ m pore size, Millipore Corporation, USA).

Preparation of leaf extract of *Diplazium esculentum* (DE)

400 gms of dried leaves and small branches of *Diplazium esculentum* was taken in a jar of REMI Mixer Grinder. 1300 ml of distilled water was added to it. The mixture was grinded in the same grinder for 15 minutes at an interval of 2 minutes between two consecutive uses. The extract was filtered with Whatman #1 (125 mm diameter) filter paper. The residue was discarded and filtrate obtained at this stage was evaporated to dryness in a Flux Evaporator. The yield of the dried powder of *Diplazium esculentum* was 17% (w/v). The powder was stored at 4°C in a desiccant for use in various experiments.

Propagation of parasites

Leishmania donovani, strain MHOM/IN/83/AG was originally derived from an Indian kala-azar patient [12]. The strain was maintained by intracardial passage every 8 weeks in a laboratory animal model, Syrian golden hamsters. The amastigotes were isolated from the spleen of infected animals in Medium-199 (GIBCO BRL, Gaithersburg, MD, USA), pH 7.4, supplemented with 10% heat inactivated fetal bovine sera, 2mM glutamine, 25mM N-2-ethansulfonic acid (HEPES), 100U/ml penicillin G-Sodium, 100 μ g/ml streptomycin sulphate. These amastigotes were injected intracardially into hamsters for the maintenance of virulent strains. Promastigotes were obtained by transforming amastigotes isolated from infected spleen [13] *in vitro*, and sub-cultured in the same medium at 22°C, at an average density of 2×10^6 cells/ml [14].

In vitro growth of *Leishmania donovani* (AG83) promastigotes in presence of *Diplazium esculentum* extract (DE)

Leishmania donovani promastigotes were cultured in Medium-199 with or without *Diplazium esculentum* extract at 22°C. The crude *Diplazium esculentum* powder was dissolved in dimethylsulphoxide (DMSO) at a concentration of 0.1% after being assayed as non-toxic and without inhibitory effects on parasite growth. Crude extract was added to the culture medium (2.0 ml) with different concentrations ranging from 0 to 80mg/ml (serially diluted). Number of motile parasites was counted under microscope to monitor growth status in absence and presence of testing material. Initially, the parasite concentration was 1.27×10^6 cells/ml, and the culture was used in

exponential growth phase. The effect of *Diplazium esculentum* against promastigotes was evaluated after 4 days of inoculation, using a Neubauer Haemocytometric chamber and calculating the percentage of growth inhibition by the formula:

$$\%IC = [Tc - Tp] \times 100 / Tc$$

where

IC = percentage of growth inhibition for each period of time and for each dose of the tested product

Tc = Number of flagellate protozoa /ml present in the control tubes

Tp = Average number

Animal

Syrian golden hamsters were originally obtained from Haffkine Research Institute, Mumbai, India and bred in our animal house (CSIR-IICB, Jadavpur, India).

Collection of macrophage and preparation of macrophage monolayer:

Sterile 4% sodium thioglycollate (2-3 ml for hamsters) was injected intraperitoneally into golden hamsters. After 48 hr., sterile, ice-cold SR media [RPMI 1640 medium supplemented with streptomycin (100 ug/ml) and penicillin (100 IU/ml)] was injected (20 ml for hamsters) into the peritoneal cavity with a 20 gauge needle attached to a sterile disposable syringe. After gentle massage of the abdominal region, the peritoneal fluid was withdrawn with the needle. The peritoneal exudates were pooled, centrifuged at 400xg for 10 min at 4°C and washed twice in the same media and finally resuspended in the SR medium supplemented with 10%FCS (v/v). The macrophages were counted in a haemocytometer and the concentration was adjusted with the medium. Cell suspensions containing 2×10^5 cells in 0.2 ml were distributed to sterile coverslips (25 mm²) placed in sterile disposable petridishes and were incubated at 37°C for 2 hr for cell adhesion. Then the coverslips were rinsed with SR medium (57°C) to remove non-adherent cells and then incubated with the same medium supplemented with 10% (v/v) FCS at 37°C overnight for further growth. Those macrophage monolayers were challenged with parasites on the next day.

In vitro* infection of peritoneal macrophages by promastigotes of *Leishmania donovani

Macrophage (Mφ) monolayers adhered on coverslips, were challenged with stationary phase promastigotes suspended in RPMI 1640 media containing 10% FCS at the ratio of 1:10. *Diplazium esculentum* extract was then added at different dosages. Infected Mφs were washed after 48h fixed with cold methanol and stained with Giemsa to examine intracellular parasite load under microscope.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by discontinuous system of Laemmli [15] in 10% polyacrylamide gels using a BIORAD® mini gel apparatus at 80v and 25mA. Briefly, the proteins were lysed in a sample buffer consisting of 60mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 0.025% bromophenol blue (final concentrations). 5% 2-mercapto ethanol was then added to the samples which were boiled for 5 min and immediately subjected to electrophoresis. Separated gels of 10% acrylamide and 4%

acrylamide stacking gels were used with 1mm thick gels. The protein load per well is indicated in the subsequent figure legends. Myosin (Mr 205,000), β -galactosidase (Mr 116,000), phosphorylase B (Mr 97,000), bovine serum albumin (BSA, Mr 66,000), ovalalbumin (Mr 45,000) and carbonic anhydrase (Mr 29,000) were used as molecular weight standards [Sigma chemicals]. The gels were stained with 1% coomassie blue in 50% methanol and destained in 50% methanol containing 10% acetic acid and 40% water. The non-denaturing polyacrylamide gels were made by following the procedure of Laemmli [15] except that SDS was not used and the samples were neither treated with any reducing agent nor boiled.

Assay of Superoxide Dismutase (SOD)

SOD activity was determined by the method of Marklund and Marklund [16]. This method involves the measurement of autoxidation rate of 0.2 mM pyrogallol in air equilibrated 50 mM Tris-cacodylic acid buffer, pH 8.2, containing 1mM diethylenetriamine- pentaacetic acid. The rate of autoxidation was obtained by monitoring the increase in absorbance at 420nm in a Hitachi Spectrophotometer, No. U2000.

SOD has the ability to inhibit the autoxidation and the extent of inhibition is taken as the measure of the enzyme activity. One unit of SOD was defined as the amount of enzyme which inhibited the pyrogallol autoxidation rate by 50%.

Protein estimation

The protein content was determined using Folin Ciocalteu's reagent [17] after precipitating the protein with 2% sodium deoxycholate and 24% trichloroacetic acid [18]. BSA was used as standard.

Ultrafiltration

Concentration of samples by ultrafiltration was performed in an Amicon® stirred cell under pressure with nitrogen.

Statistical Analysis

This was performed by using SPSS for windows version 6.0 computer program to conduct standard deviation [19].

3. RESULTS AND DISCUSSION

Effect of *Diplazium esculentum* leaf extract (DE) on *in vitro* growth of *Leishmania donovani* (AG83) promastigotes

Leishmania donovani promastigotes were cultured individually *in vitro* upto 4 days at 22°C in presence of different concentrations of aqueous leaf extract of *Diplazium esculentum* (DE) dissolved in DMSO. Final concentration of DMSO was maintained at 0.1% (v/v). At this concentration DMSO has no effect on the growth of *Leishmania donovani* promastigotes. At a concentration of 80 mg/ml (DE), parasite growth was inhibited by 100% (Figure1). However at a lower concentration of 50 to 60 mg/ml, promastigotes growth was inhibited by 60-75%.

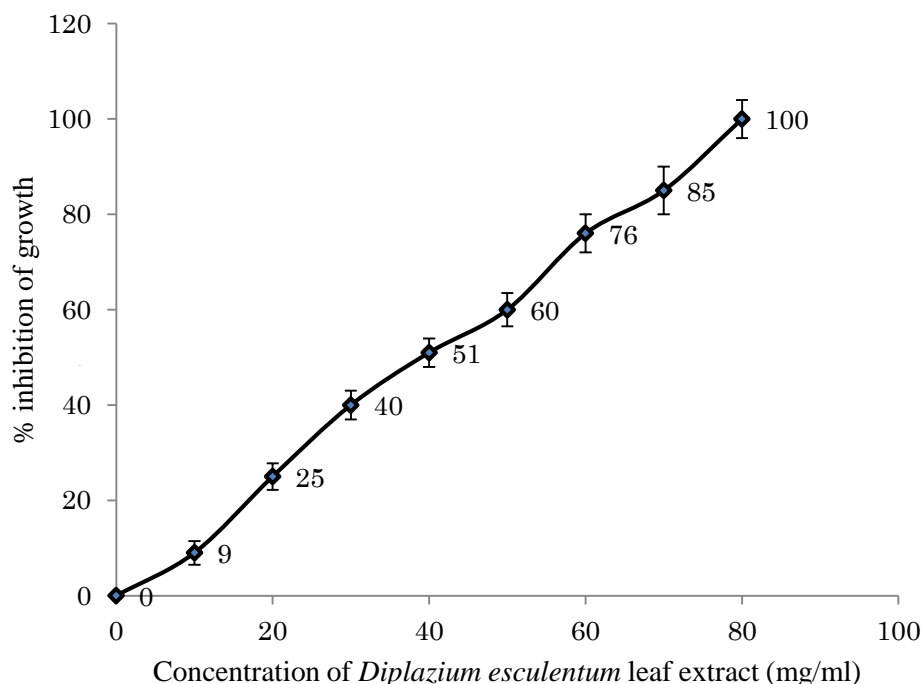


Figure 1. Effect of *Diplazium esculentum* extract on the growth rate of *Leishmania* promastigotes. An established second-line antileishmanial agent sodium antimony gluconate (SAG) was used as positive control to compare the efficacy of DE towards growth of *Leishmania* parasite. SAG at a concentration of 1.5 to 2.5 mg/ml inhibits 60-80% promastigotes growth (Figure2).

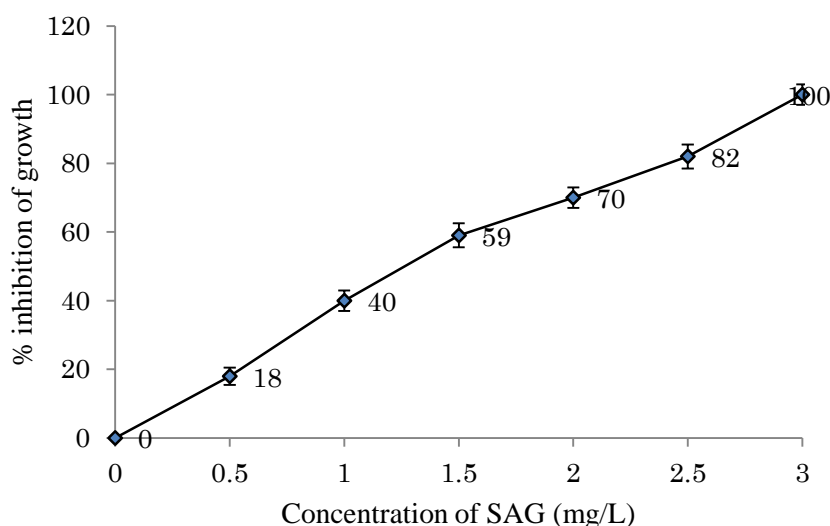


Figure 2. Effect of SAG on the growth rate of *Leishmania* promastigotes

Effect of *Diplazium esculentum* leaf extract (DE) on *Leishmania* pathogen after host-parasite interaction

Efficacy of DE was further tested on intracellular survival of *Leishmania donovani* in host macrophages. Parasite burden in *Leishmania*-infected hamster Mφs were significantly inhibited in a

dose depended manner (Figure 3). At a concentration of 140 mg/ml CE, intracellular amastigotes in the *Leishmania donovani* infected hamster Mφs could not be detected.

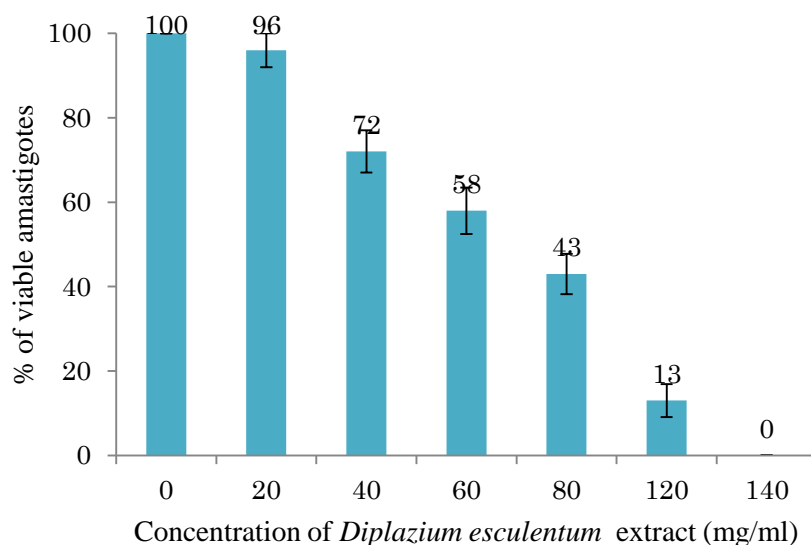


Figure 3. Dose dependent response of *Diplazium esculentum* extract on intracellular parasite burden within macrophages during leishmanial infection

Impact of *Diplazium esculentum* leaf extract(DE) on SOD activity and Superoxide radical release

Activity staining of native polyacrylamide gels clearly showed that SOD activity of *Leishmania* promastigotes was found to be inhibited in a dose dependent manner when treated with different concentrations of DE (Figure . 4). At higher concentration (150 mg/ml), SOD activity was totally lost.



A B C D E

Figure 4. Activity staining of DE treated Leishmanial SOD after separating by native PAGE in a 10% gel. 60 µgm protein was loaded in each lane. Gels were incubated in nitroblue tetrazolium and riboflavin solutions and finally illuminated. SOD activity which inhibits the formation of blue formazan, was visualised as white bands on a blue blue background. (A. Without DE; B.40mg/ml DE; C. 80mg/ml DE; D.120mg/ml DE ; E. 150mg/ml DE)

When commercially available Fe-SOD was treated with various concentrations of DE and then subjected to SDS-PAGE (Figure 5), intensity of the protein band gradually decreased. At 80mg/ml DE concentration, protein band for SOD could not be detected.

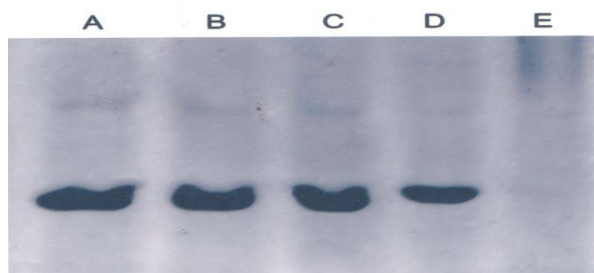


Figure 5. SDS-PAGE of Fe-SOD (*E.coli*) treated with increasing concentrations of DE. 10 μ gm protein sample was loaded in each lane to run on a 10% Polyacrylamide gel. The gels were stained with 0.1% coomassie blue in 50% methanol. (A. Without DE; B. 20 mg/ml; C. 40 mg/ml; D. 60 mg/ml; E. 80 mg/ml DE).

When promastigotes lysate and DE were added simultaneously (Figure 6) in the assay mixture to determine SOD activity, 38% inhibition was observed at 40 mg/ml. At the same concentration, however, 58% inhibition was found when promastigotes lysate was pre incubated with DE prior to SOD assay.

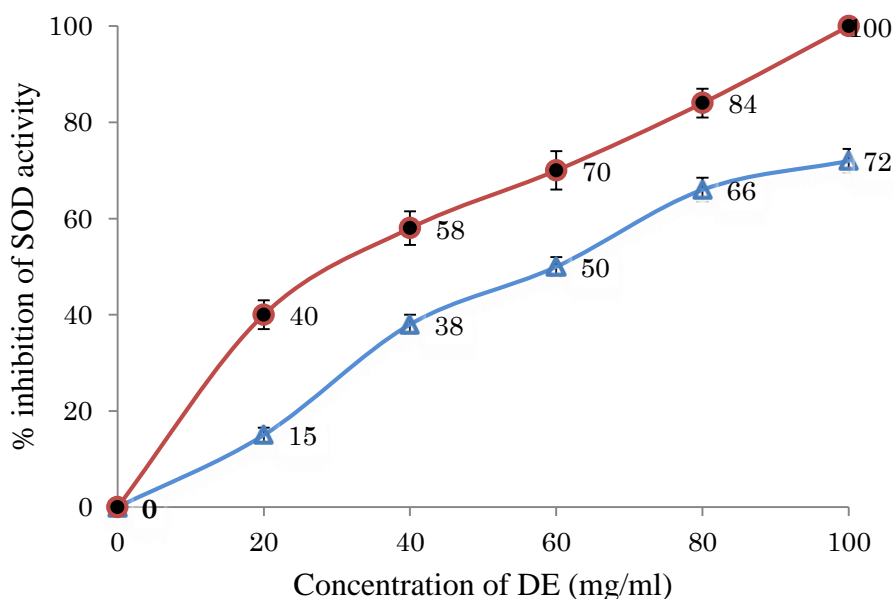


Figure 6. Status of Leishmanial SOD activity (Δ) when enzyme and DE were added simultaneously in the reaction mixture or (\bullet) enzyme and DE were pre-incubated for 30 minutes before adding to reaction mixture.

Enhancement in the rate of superoxide radical release was observed (Figure 7) due to DE treatment of *Leishmania* pathogen compared to normal parasite. At 80 mg/ml of DE, basal superoxide radical decay in parasites increased nearly two fold.

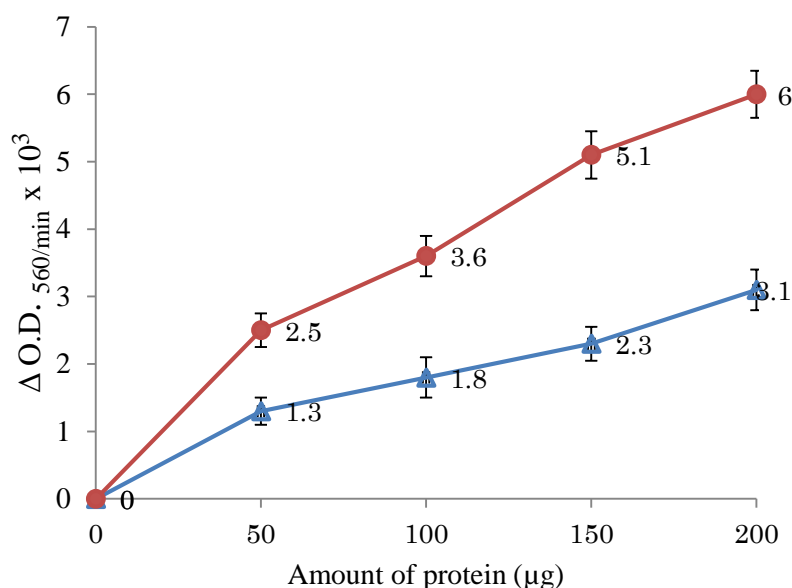


Figure 7. Rate of superoxide radical release by *Leishmania* promastigotes. Status of Leishmanial SOD activity (Δ) when enzyme and DE were added simultaneously in the reaction mixture or (●) enzyme and DE were pre-incubated for 30 minutes before adding to reaction mixture.

Leishmaniasis is a major protozoal health problem worldwide. It is highly endemic in many developing tropical and subtropical countries including India. The effort to get vaccine has not yet materialized. The existing first and second-line anti-leishmanial drugs have serious side effects and are expensive [20]. Resistance to existing drugs has also become a severe problem. Therefore, there is an urgent need for effective and inexpensive drugs and vaccine to replace or supplement those in current use. Plants can make important contributions as valuable sources of new medicinal agents [21]. In this regard scientific evaluation of the rich ethno-botanical heritage of India is expected to provide modern medicine with lead compounds for the development of new drugs. This study is a part of a continuing search for new drugs (with high activity and low side effects) to treat protozoa parasites, such as *Leishmania* and demonstrates the usefulness of the alkaloids/alkamides/phytochemical as a promising lead. Drug screening by the isolation of natural products seems to be an attractive approach which can result in the efficient elucidation of new lead compounds. This is a valuable option to standard screening of large compound libraries. Although a significant number of anti-leishmanial compounds have been investigated, the number of mechanistic studies is rather small. The actual target sites are unknown in most cases. It is of great importance to probe the active principles of anti-leishmanial agents for subsequent target-based drug design and employment of new screening assays. Genomic and proteomic approaches should be used to identify pathways that have already been targeted in other organisms. In primary visceral leishmaniasis, lack of response to sodium stibogluconate was observed in some geographical areas [22]. In this study *Diplazium esculentum* crude leaf extract was evaluated for antileishmanial activity *in vitro*. *In vitro* activity was

tested on cultured promastigotes of a pathogenic strain of *Leishmania donovani*. Leaf extract of *Diplazium esculentum* has significant antileishmanial activity on cultured promastigotes. The novelty of this extract is that it inhibits 100% parasite growth in parasite growth in culture and the IC_{50} is 40 mg/ml. Superoxide dismutase (SOD) which is one of the key enzymes of oxygen defence system is known to be an essential factor in mediating normal cellular functions [23-24]. It is an enzymic scavenger of toxic oxygen radicals, which are responsible for cell damage [25]. The role of SOD in both eukaryotes and prokaryotes has been attributed to its catalysis of the dismutation of superoxide radicals (O_2^-), producing molecular oxygen and H_2O . As a result the enzyme has been found to be targeted for the treatment of several diseases [26-28]. *Leishmania* and other trypanosomatid protozoa require SOD for (O_2^-) reduction, suggesting that inhibition of this pathway could be targeted for effective chemotherapy. This possibility has been investigated using the metal complex. SOD also plays a vital role during host-parasite interaction [29]. Its activity is elevated when *Leishmania* parasite infects host cells [30]. In this study it has been found that SOD activity is inhibited to downregulate degree of parasite infection during DE treatment to a great extent. It is known that main form of Leishmanial SOD contains Fe as cofactor [31]. The enzyme activity is lost as shown by activity staining of non-denaturing gels. Appearance of pure Fe-SOD available from a heterologous source (*E. coli*) was found to be diminished during SDS-PAGE analysis in a dose dependent manner. It is presumed that inhibition of SOD and simultaneous release of superoxide radicals [32] impose toxic effect to destroy intracellular parasites during experimental visceral leishmaniasis. *Diplazium esculentum* crude leaf extract may be a good candidate to act as a chemotherapeutic agent against visceral leishmaniasis. This plant may be a suitable alternative if the antimonial agents are not effective or cannot be tolerated. Its ability to kill intracellular parasites *in vitro* seems to be more effective in lower concentration compared to toxic antimonials drugs tested.

4. CONCLUSION

Present study of aqueous leaf extract of *Diplazium esculentum* (DE) against visceral leishmaniasis suggests that the natural products isolated from *Diplazium esculentum* possess interesting antileishmanial activity. Data suggest that DE may be a better choice to act as an antileishmanial agent with better efficacy. These compounds could be synthesized in large scale and evaluation of their efficacy in *in vivo* model could provide a better understanding of their role as a drug candidate.

ACKNOWLEDGEMENT

Authors are thankful to Prof. Siddhartha Roy, Director, Indian Institute of Chemical Biology, Kolkata, for giving permission to work at CSIR-IICB, Jadavpur. The authors would like to thank the Institutional Animal Ethics Committee, CSIR-IICB for allocating animals to perform the study. Prof. Syamal Roy of CSIR-IICB is thankfully acknowledged for providing necessary arrangements and sincere help in conducting animal experiments at IICB. Financial support from Higher Education, Science & Technology and Bio-Technology, Govt. of West Bengal, is also gratefully

acknowledged. University Grants Commission, New Delhi is acknowledged for offering Senior Research Fellowship to R. Jyoti through Rajiv Gandhi National Fellowship (RGNF) Scheme. We are extremely grateful to Dr. Rajen Halder, Department of Physiology, University of Calcutta, for critically reviewing the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Adamczick C, Dierig A, Welzel T, Schifferli A, Blum J, Ritz N - Double trouble: visceral leishmaniasis in twins after traveling to Tuscany - a case report. BMC Infect Dis. 2018; 18: 495-99.
2. Gupta S, Raychaudhury B, Datta, SC - Host peroxisomal properties are not restored to normal after treatment of visceral leishmaniasis with sodium antimony gluconate. Experimental Parasitol. 2009; 123: 140-5.
3. Gelanew T, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G - Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 2010; 4: e889.
4. Bhattacharya SK, Dash AP – Elimination of Kala-Azar from the Southeast Region. Am J Trop Med Hyg. 2017; 96: 802-4.
5. Gupta S, Raychaudhury B, Banerjee S, Das B, Mukhopadhyaya S, Datta SC - Momordicin purified from fruits of *Momordica charantia* is effective to act as a potent antileishmanial agent. Parasitology International. 2010; 59 : 192–7.
6. Carvalho SH, Frézard F, Pereira NP, Moura AS, Ramos LMQC, Carvalho GB, Rocha MOC - American tegumentary leishmaniasis in Brazil: a critical review of the current therapeutic approach with systemic meglumine antimoniate and short-term possibilities for an alternative treatment. Trop Med Int Health. 2019; doi: 10.1111/tmi.13210. [Epub ahead of print]
7. Raychaudhury B - *In vitro* antileishmanial activity of *Nyctanthes arbor-tristis* – a medicinal tree. International Journal of Applied Biology and Pharmaceutical Technology. 2013; 4: 146-51.
8. Olliaro P, Bryceson AD - Practical progress and new drugs for changing patterns of leishmaniasis. Parasitology Today. 1993; 9: 323-8.
9. Ram VJ, Haque N, Guru PY - Chemotherapeutic agents XXV : Synthesis and leishmanicidal activity of carbazolpyrimidines. European Journal of Medicinal Chemistry. 1992; 27: 851-55.
10. Braconier JH, Miomer H - Recurrent episodes of thrombocytopenia during treatment with sodium stibogluconate. Journal of Antimicrobial Chemotherapy. 1993; 31: 187-8.
11. Subhrajyoti Roy S, Hazra B, Mandal N, Chaudhuri TK – Assessment of the antioxidant and free radical scavenging activities of methanolic extract of *Diplazium esculentum*. International

- Journal of Food Properties. 2013; 16: 1351-70.
12. Ghosh AK, Bhattacharya FK, Ghosh DK - *Leishmania donovani*: amastigote inhibition and mode of action of berberine. Exp Parasitol. 1985; 60: 404–13.
 13. Jaffe C, Grimaldi G, McMahon-Pratt D. In: Morel, C.M. (Ed.), Genes and Antigens of Parasites: A Laboratory Manual, Fundacao Oswaldo Cruz, Rio de Janeiro, 1984. pp. 47-56.
 14. Kar K, Mukerji K, Naskar K, Bhattacharya A, Ghosh DK - *Leishmania donovani*: a chemically defined medium suitable for cultivation and cloning of promastigotes and transformation of amastigotes to promastigotes. J Protozool. 1990; 37: 277-9.
 15. Laemmli UK - Cleavage of structural proteins during the assembly of the head of bacteriophage T4 - Nature. 1970; 227: 680-5.
 16. Marklund S, Marklund G - Involvement of the Superoxide Anion Radical and a Convenient Assay for Superoxide Dismutase. Eur J Biochem. 1974; 47: 469-74.
 17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ - Protein measurement with Folin phenol reagent. J Biol Chem. 1951; 193: 265-75.
 18. Bensadoun A, Weinstein D - Assay of proteins in the presence of interfering materials. Anal Biochem. 1976; 70: 241-50.
 19. Mishra BN, Mishra MK - In Introductory Practical Biostatistics. Naya Prakash. 1983. pp 89.
 20. Ortega V, Giorgio S, de Paula E - Liposomal formulations in the pharmacological treatment of leishmaniasis: a review. J Liposome Res. 2017; 27: 234-48.
 21. Odeyemi S, Bradley G - Medicinal Plants Used for the Traditional Management of Diabetes in the Eastern Cape, South Africa: Pharmacology and Toxicology. Molecules. 2018; 23: e2759.
 22. Murray HW - Treatment of visceral leishmaniasis (kala-azar): a decade of progress and future approaches. Int J Infect Dis. 2000; 4: 158-77.
 23. Fattman CL, Scafer LM, Oury TD - Extracellular superoxide dismutase in biology and medicines. Free Radical Biol Med. 2002; 35: 236-56.
 24. Raychaudhury B, Jyoti R, Chakraborty K, Chakraborty A, Palroy M, Haldar R - Identification and purification of Fe containing superoxide dismutase from *Leishmania donovani*. European Journal of Pharmaceutical and Medical Research. 2017; 4: 336-40.
 25. McCord JM, Keele BB Jr, Fridovich I - An enzyme based theory of obligate anaerobiosis : the physiological function of superoxide dismutase. Proc Natl Acad Sci. USA. 1971; 68: 1024-7.
 26. Briedbach T, Scory S, Krauth-Siegel RL, Steverding D - Growth inhibition of bloodstream forms of *Trypanosoma brucei* by the iron chelator deferoxamine. Int J Parasitol. 2002; 32: 473-9.
 27. Yassien NA, Khalifa EA, el-Nouby KA - Role of anti-oxidant preparation (oxi-guard) on *Cryptosporidium parvum* infection in albino mice. J Egypt Soc Parasitol. 2001; 31: 95-106.
 28. Mohan S, Pradeep CG, Kumar AR, Javakumar K, Kaleysa RR - The adult-specific ubiquinone

- Q(8) functions as an antioxidant in the filarial parasite, *Setaria digitata*. *Biochem Biophys Commun.* 2001; 288: 949-53.
29. Raychaudhury B, Gupta S, Banerjee S, Das B, Datta, SC - Isolation of Leishmania glycosomes by a rapid method. *Analytical Biochemistry.* 2004; 332: 404-8.
30. Raychaudhury B, Banerjee S, Gupta S, Singh RV, Datta SC - Antiparasitic activity of a triphenyl tin complex against *Leishmania donovani* . *Acta Tropica.* 2005; 95: 1-8.
31. Dey R, Mitra S, Datta SC - Cyclic AMP mediates change in superoxide dismutase activity to monitor host-parasite interaction in *Leishmania donovani*. *J Parasitol.* 1995; 81: 683-6.
32. Sies H, de-Groot H - Role of reactive oxygen species in cell toxicity. *Toxicology Letters.* 1992; 64-65: 547-51.