7.1. Literature review

Leishmaniasis is one of the main endemic parasitic infections worldwide, which is found in around 98 countries, especially in developing countries with about 1.7 billion people at risk of contracting the infection (Sosa et al., 2019). Leishmaniasis is considered as one of the six major neglected tropical diseases by WHO, due to its remarkable effect on global public health. The disease has great importance in HIV infected individuals as it is found as an opportunistic infection in those areas where both infections are endemic (Martínez et al., 2018).

A literature review reveals that several antileishmanial chemical compositions have already been reported (Murray, 2001; Marty and Rosenthal, 2002) but none of these proved to be the ultimate choice of drug due to varying degrees of efficacy and toxicity. Pentavalent antimonials, that are the first-line drugs in the leishmaniasis treatment, could result in severe toxic side effects including cardiotoxicity, pancreatitis, hepatotoxicity and nephrotoxicity. Discovery of antimony salt resistant pathogenic strains has made the situation worse to treat the patients against these parasites (Sundar, 2001). On the other hand, second-line drugs like pentamidine and miltefosine may cause diabetes when applied in high doses. Furthermore, the recently developed resistance of the pathogen against certain antileishmanial drugs has resulted in treatment failures. Therefore, new treatment approaches are immediately required (Badirzadeh et al., 2020). There is a need to identify new chemotherapeutic agents for effective therapy against all forms of leishmaniasis.

7.1.1. Experimental evidence of antileishmanial activity using plant extracts

In search for effective treatment for leishmaniasis, natural products may offer unlimited source of chemical diversity for identification of new drug templates (Fournet and Munoz, 2002). In the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects (Zaffer et al., 2015). In rural areas, traditional medicine for

leishmaniasis largely remains the only source of treatment being handled by the local people without proper scientific information. Government of India has also started to focus more on the potential of herbal preparations (The Times of India, 2003). The interest in the plant products, especially medicinal plants or their extracts, surfaced all over the world due to the belief that many herbal medicines are known to be free from side effects. In a study, activity of *Haplophyllum myrtifolium* against *L. tropica* was screened, both *in vitro* and *in vivo*, where the efficacy of the medicinal plant was tested by measuring the lesion size (developed in cutaneous leishmaniasis) in infected animal but the study was devoid of any experiment to determine the probable mode of action or cytotoxic effect, if any (Ostan et al., 2007).

In another study, leishmanicidal activity of the extracts of five well known Turkish medicinal plants were evaluated against *L. tropica* infected mice, taking glucantime as reference drug. Promastigote solution was injected subcutaneously and after development of lesion, extracts of five plants (both in water and chloroform) were administrated. Effectivity of test substances were determined by measuring the lesion size on foot pad, showing good results but two of the plants among five had cytotoxic activities in therapeutic dose limits (Ozbilgin et al., 2014). The work was confined only to screen the antileishmanial activity *in vitro* and *in vivo* condition but no experiment was done to establish probable mode of action. Some studies were done on human subjects too. In a study, leishmanicidal activity of a medicinal plant having anti-malarial effect (*Artemisia annua* L.) was tested on human subjects with uncomplicated cutaneous leishmaniasis who were not taking any other medication and were cured 45 days after initiation of treatment (Mesa et al., 2017). In another work, the ethanolic root bark extract of *Berberis vulgaris* was examined for treatment of *L. major* infected mice, with cutaneous leishmaniasis. About 90% recovery was found by treatment with 20% root bark extract. No tests for cytotoxicity or other experiments were performed which might establish any immunological or enzymatic modifications in test animal causing recovery (Salehabadi et al., 2014).

7.1.2. Mode of action of leishmanicidal plant extracts

Several research works were performed to evaluate mode of actions behind leishmanicidal activities of different medicinal plants but most of the studies were done on specific enzyme activity or any other internal environmental situation created in laboratory set up (Zhai et al.,1999). Antileishmanial activity of extracts from *Allium sativum* along with a molecule purified from the plant (allicin) was reported by Foroutan-Rad et al. (2017). The author observed that the molecule interfered in the function of thiol-redox proteins like glutathione and trypanothione/trypanothione reductase which led to the damage of the *Leishmania* parasites. It also caused microtubule disruption. In another investigations, aqueous and ethanolic extracts of garlic as well as allicin inhibited the growth of *L. mexicana* and *L. chagasiin vitro* and it was found that, allicin caused disruption of the activity of cysteine proteases enzyme which might be the reason of its antileishmanial activity (Eslami et al., 2013). Essential oil from the plant *Tetradenia riparia* was evaluated for the antileishmanial activity against *L. amazonensis*. It was found to inhibit some of the most critical cytokines for parasite growth and the establishment of infection, including granulocyte-macrophage colony-stimulating factor, interleukin-4 (IL-4), IL-10, and tumour necrosis factor (Demarchi et al., 2015). It has been demonstrated that the iridoid glucosides isolated from *N. arbor-tristis* seeds promoted the increase of reactive oxygen species (ROS) in parasites, causing redox imbalance induced oxidative stress, cell membrane damage and apoptosis-like death in both promastigote and amastigote of *L. donovani* (Arraché Gonçalves et al., 2021).

7.1.3. Role of SOD in antileishmanial activity

Super oxide dismutase (SOD, EC 1.15.1.1) is an enzyme which acts as a component of first line defense systems in the cell. It is a major detoxification enzyme and the most powerful antioxidant in the cell (Ighodaro and Akinloye, 2018). It protects the living tissues from oxidative damages caused by reactive oxygen species (ROS) such as super oxide anions, hydroxyl radicals, or free radicals. These super oxides and free radicals are generated by the effect of toxins, ultraviolet ray and nuclear exposure or during body defence activation

(Wang et al., 2018). SOD is a metalloenzyme which requires a metal cofactor for its activity. Various forms of SOD are distributed in the living systems depending on the type of metal ion required as a cofactor. These forms include (i) Fe-SOD which is commonly found in prokaryotes and chloroplasts of some plants (ii) Mn-SOD which is present in prokaryotes and mitochondria of eukaryotes and (iii) Cu/Zn-SOD which is found mainly in eukaryotes (Ighodaro and Akinloye, 2018).

During microbial attack the human body releases super oxides to destroy the microbes and microbes use their own SOD to encounter and neutralize host's super oxides. SOD is an enzyme, which exists both in host and parasites and functions differentially and this attract the interest of scientists and pharmacologist from years (Nishikimi et al., 1972; Ghosh et al., 2003; Raychaudhury et al., 2005). Variation in genomic structures, activities and sensitivities have made this enzyme a potential diagnostic target. One recent study was conducted on different types of SODs (characterised by presence of different metallic co factor, $Mn^{2+,}$ Fe^{3+} Cu^{2+}/Zn^{2+}) which differ in sensitivity to cyanide, azide, hydrogen peroxide etc (Folgueira et al., 2019). In another study, it was found that a molecule momordicatin, isolated from fruits of *Momordica charantia*, acted as antileishmanial agent both *in vitro* and *in vivo* (Gupta et al., 2010). It was also observed that Fe^{3+} containing parasitic SOD was totally inhibited by both the fruit extract and molecule within therapeutic dose but Cu^{2+}-Zn^{2+} containing SOD, present in host cell remain unaffected and that emerged as a probable mechanism of leishmanicidal activity of the studied plant or molecule.

7.1.4. Antileishmanial medicinal plants change liver function in host

Previous studies showed parasitic infection caused toxicity of liver in animals which increased the levels of two important serum enzymes, viz. serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate Transaminase (SGOT) (Kaur et al., 2016). In a previous work, liver function test was performed to assess the toxicity effect of a molecule, momordicatin in *Leishmania* infected experimental animals. Serum alkaline phosphatase, SGPT

and SGOT were measured in normal as well as DMSO and momordicatin treated infected hamsters and it was found that all the enzyme levels were changed in *Leishmania* infected but untreated animals whereas the enzymes level reached to nearly normal level after the momordicatin treatment (Gupta et al., 2010).

7.2. Materials and methods

7.2.1. Preparation of leaf extract of *R. serpentina*

Methanolic leaf extract of *R. serpentina* was prepared following the procedure described in previous chapter (section 3.2.1).

7.2.2. Parasite isolation

L. donovani strain MHOM / IN /AG / 83 was obtained from Indian kala-azar patient (Ghosh et al., 1985) and maintained by intracardial passage every 8 weeks in Syrian golden hamsters. Promastigotes were obtained by transforming amastigotes isolated from infected spleen (Jaffe et al., 1984) and maintained in Medium – 199 supplemented with 10% fetal calf serum (FCS) *in vitro* (section 5.2.2).

7.2.3. *In vivo* antileishmanial activity determination

Estimation of the *in vivo* antileishmanial activity was done following the method of Raychaudhury et al. (2005). Golden hamster was obtained from Haffkine Research Institute, Mumbai, India and bred in animal house of CSIR-IICB, Jadavpur, India. Eight-week-old hamsters weighing between 100-120 gm were first infected individually with freshly purified *L. donovani* promastigotes (2×10^5), administered through cardiac route. Therapy with target plant extract and reference drug were started after 30 days following infection. The plant leaf extract was administrated in graded doses (50 mg/kg body weight (bw), 100 mg/ kg bw and 200 mg/kg bw) and the reference drug was sodium antimony gluconate (SAG) (40 mg/ kg bw). The animals were divided into 5 groups, with 5 animals in each group. Intramuscular injections of intended drug or extract (200 µl) were given in every alternative day for one month. All the compounds were dissolved in 0.1 % (v/v) DMSO, which was found nontoxic to animal cells. Animal of control group received 200 µl of 0.1% (v/v) DMSO in transcellular way. After one month of administration of last dose of therapy,

animals of all groups were sacrificed and parasitic burden of liver and spleen were determined from impression smears after GIEMSA staining. Before sacrificing, blood was withdrawn for SGPT and SGOT assay.

7.2.4. Determination of parasitic burden in liver and spleen

To observe the anti-leishmanial effect of *R. serpentina* leaf extract, the parasitic load in spleens and livers of infected hamsters were determined before and after treatment from impression smears after GIEMSA staining (Gupta et al., 2010). For preparing spleen/liver impression stamp smear on glass slide, first the glass slides were made grease free. The spleen and liver were collected from experimental animals. Weight of the organs were recorded. The organs were cut with scissors and forceps and putting the cut part of the organ downwards (by the help of forceps), a stamp was made over the slide (about 10-12 stamp on one slide) with just touching the organ over the slide. The slide was allowed to dry and then fixed by methanol wash (for 10 minutes). Then the slide was stained with GIEMSA (section 5.2.6.2.3) and observed under the microscope for calculation of parasitic burden. During measurement, at least 10 nucleated cells were examined for each set. The total parasitic burden was calculated using the formula as follows –

Parasitic burden = Organ weight (mg) x number of amastigotes per cell nucleus x 2 x 10^5

7.2.5. Animal serum enzyme assay

To test the toxic effect of the plant extract in animal, the blood sera of untreated normal hamsters, infected hamsters (with *L. donovani*), and infected hamsters receiving treatment were subjected to assay for liver enzymes, SGPT and SGOT (Gupta et al., 2010) using kit from Dr. Reddy's Laboratory, Hyderabad. Protocol was followed as per the manufacturer's instruction. SGPT and SGOT values were expressed as units/L (Mayne, 1994).

7.2.6. Superoxide dismutase (SOD) assay

The activity of SOD was assayed by measuring the inhibition of pyrogallol autooxidation rate following the protocol of Marklund and Marklund, (1974) with some modifications. Promastigotes of *L. donovani* strain maintained in

Ray's solid medium at 22°C were collected (12g cell) and taken in ice cold potassium phosphate buffer (50mM) containing 10 mM TES, 1 mM EDTA, 0.5 mM phenyl methyl sulphonyl fluoride and 0.25 leupeptin (pH 7.6). The mixture was then sonicated in a sonicator [Soniprep, Mes (UK) Ltd.] (5-6 strokes of 20 sec) at 4°C. The mixture was then centrifuged for 100000 g for 60 minutes and the supernatant was collected. The protein content of supernatant was measured following Lowry's method (Lowry et al., 1951) using BSA as standard.

Superoxide dismutase activity was measured by pyrogallol autooxidation assay. The assay mixture contained 0.2mM pyrogallol in air equilibrated 50mM tris-cacodylic acid buffer (pH 8.2) and 1mM diethylene triamine pentaacetic acid. The promastigote lysate and *R. serpentina* leaf extracts at different concentrations (10-40 mg/ml)were added to the reaction mixture either simultaneously or after 30 min preincubation of lysate with extract. The rate of autooxidation was obtained by monitoring the increasing absorbance at 420nm (spectrophotometer, (Hitachi, No U 2000). The enzymatic activity of SOD was determined by its ability to inhibit autooxidation. One unit of SOD was defined as the amount of enzyme which inhibited the pyrogallol autooxidation rate by 50% (Raychaudhury et al., 2005).

7.2.7. Determination of superoxide radical release

Superoxide radical release was determined following previously described method (Yasuka, 1978) with necessary modification. Promastigotes lysate was incubated for 30 min with different concentrations of crude leaf extract of *R. serpentina* (Raychaudhury et al., 2005). Release of superoxide radical was measured spectrophotometrically by measuring the formation of blue formazan (derived from reduction of NBT).

7.2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecryl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was performed to study the effect of crude leaf extract of *R. serpentina*on the Fe-SOD enzyme of *L. donovani*. Cell free extract of *L. donovani* promastigotes (50 µg protein) (section 7.2.6) was mixed with *R. serpentina* leaf extracts in

different concentrations (5-40 mg/ml) and incubated for 30 min. Next, the samples were loaded in each lane of polyacrylamide gel and electrophoresed at 4°C. Gel was made according to the method of Laemmli (1970). A 10% resolving gel was used for separating the denatured proteins (Appendix 2, No. 28). Electrophoresis was accomplished in electrode buffer at 30 mA for 4 h using a Bio-Rad Mini Protean Gel electrophoresis system. Following electrophoresis, the gel was stained with 0.1% Coomassie blue (in 50% methanol). Pure Fe^{3+}-SOD was taken as reference. Intensity of SOD protein band in presence of different concentrations plant extracts indicated the amount of active protein.

7.2.9. SOD on-gel activity staining

Leishmanial SOD (in cell free lysate of *L. donovani* promastigotes) was treated with different concentration of *R. serpentina* leaf extracts (10 mg/ml, 20 mg/ml, 30 mg/ml) and then separated in 10% non-denaturing polyacrylamide gels. For preparing the gels, the method described by Davis (1964) was followed with some modifications (Appendix 2). In each lane 60 µg of protein was loaded. For activity staining the gels were incubated first in nitroblue tetrazolium solution (Solution A: 20 mg nitroblue tetrazolium and 10 ml of distilled water, soaked for 20 mins) and next in solution B containing riboflavin, potassium phosphate and TEMED (tetramethylethylenediamine) (4 mg riboflavin, 0.4 g potassium phosphate and 600 µl TEMED in 50 ml glass distilled water soaked for 20 min). After staining, the gels were illuminated and white band appears on blue background.

7.2.10. Statistical analysis

Experiments were performed in replication and standard error was determined. Statistical analysis was conducted through Student's t-test as described (Mishra and Mishra, 1983).

7.3. Results

7.3.1. *In vivo* effect of *R. serpentina* extract on intracellular amastigotes

Responses of *R. serpentina* treatment on intracellular parasite burden within macrophages (per 10 macrophages) in *Leishmania* infected animals was

studied**.** Parasite burden in liver and spleen of *Leishmania*-infected hamsters were significantly decreased after treatment with *R. serpentina* leaf extract in a dose dependent manner (Table 7.1). Giemsa-stained micrographs showing the effect of the plant extract are given in Fig: 7.1 (liver) and Fig: 7.2 (spleen). At a concentration of 50mg/kg bw, intracellular amastigote counts in the infected hamster liver (Fig: 7.1B) and spleen (Fig: 7.2B) was reduced upto 70%, whereas with dose of 200 mg/kg bw, the parasite burden was diminished upto 94% in infected spleen (Fig: 7.1C) and liver (Fig: 7.2C). Control group did not show any detectable change (Fig: 7.1A and 7.2A).

Table: 7.1. Dose–dependent responses upon treatment with *R. serpentina* extract on intracellular parasite burden within macrophages during *Leishmania* infection.

Dose of *R. serpentina* (mg/kgbw*)	**Parasitic burden (Count) per 10 macrophages**		**% Reduction in parasitic burden**	
	Liver	**Spleen**	**Liver**	**Spleen**
0 (untreated)	52±4	65±5	-	-
50	15±2	23±2	71%	65%
100	9±1	12±1	83%	82%
200	3±1	5±1	94%	92%
SAG[#]	1±1	2±1	98%	97%

*bw=body weight;[#]SAG at a dose of 40 mg/kg bw.

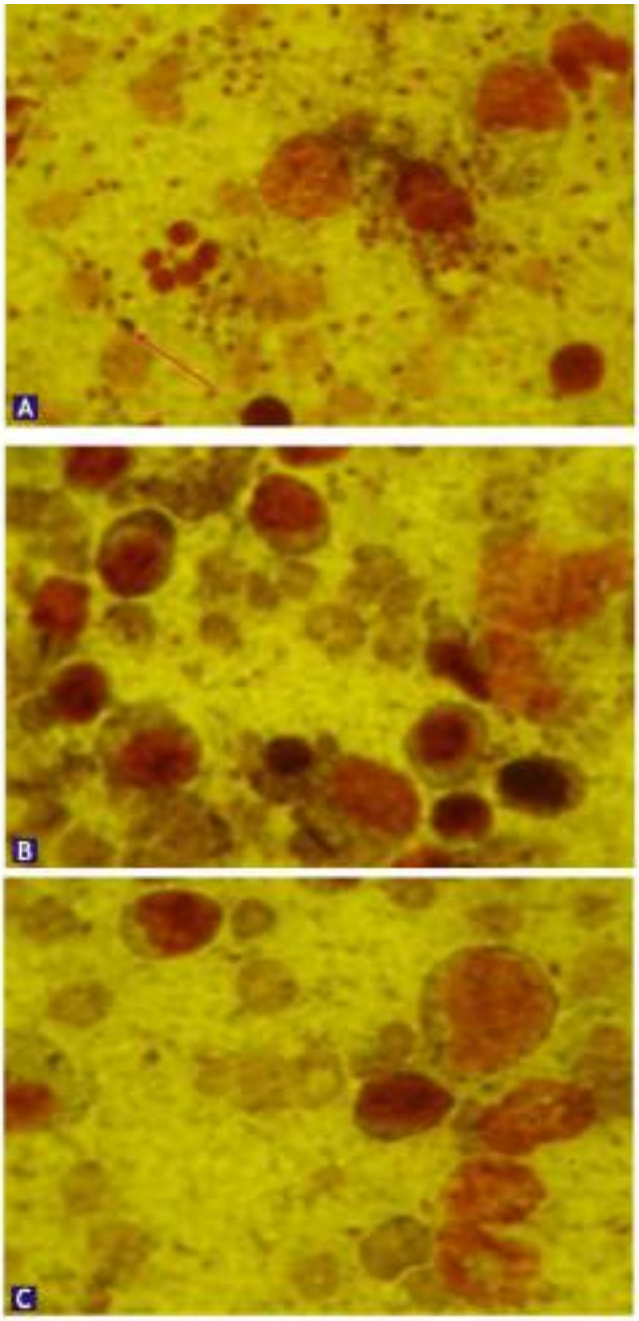

Figure: 7.1. GIEMSA-stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in liver. Black spots within the macrophage indicate amastigotes (arrow). [A] Untreated *Leishmania* infected macrophage. [B] *Leishmania* infected macrophages treated with 50 mg/kg body wt. leaf extract. [C] *Leishmania* infected macrophages treated with 200 mg/kg body wt. leaf extract.

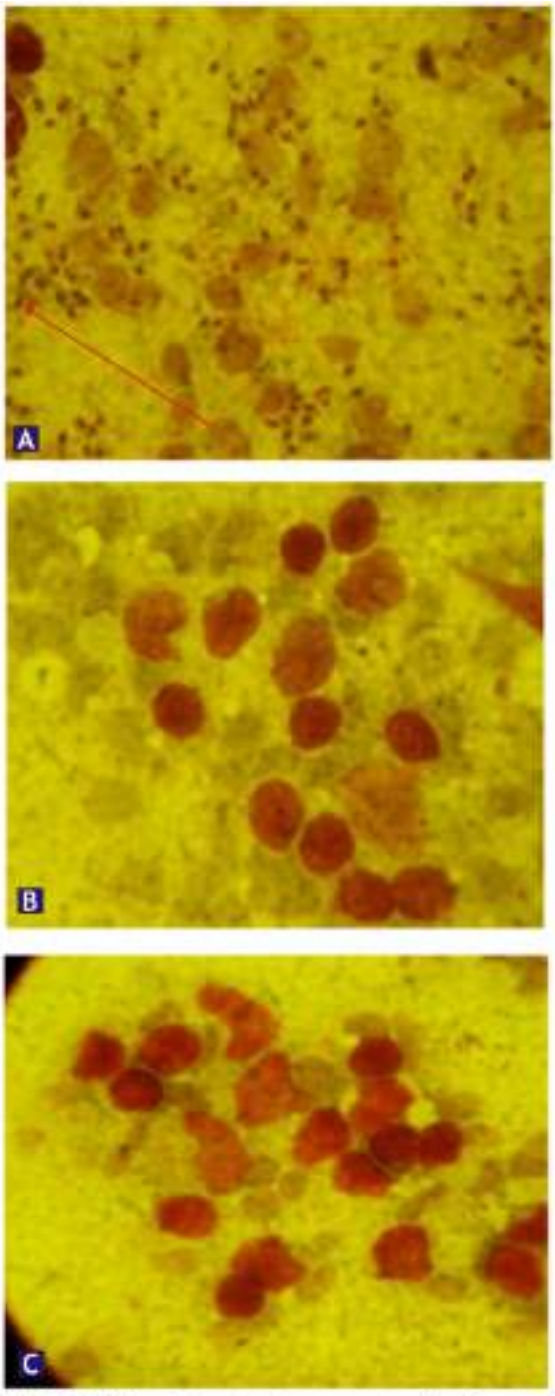

Figure: 7.2. GIEMSA-stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in spleen. Black spots within the macrophage indicate amastigotes (arrow). [A] Untreated *Leishmania* infected macrophage. [B] *Leishmania* infected macrophages treated with 50 mg/kg body wt. leaf extract. [C] *Leishmania* infected macrophages treated with 200 mg/kg body wt. leaf extract.

7.3.2. Animal serum enzyme assay

To check the liver function, the level of the specific serum enzymes and SGOT of hamster undergoing experimental visceral leishmaniasis were studied (section 7.2.3). The results are shown in table 7.2, and figure 7.3. The levels of SGOT and SGPT were markedly higher in *L. donovani* infected hamster compared to uninfected control. The level of both these enzymes decreased in infected hamster upon treatment with *R. serpentina* (at a dose of 200mg /kgbw). These results suggest that *R. serpentina* is non-toxic to liver within the limit of therapeutic doses.

Table: 7.2. Effect of *R. serpentina* extracts on serum enzyme levels

Serum	SGOT(U/L)	SGPT(U/L)
Uninfected and untreated	75 ± 3.3	46 ± 3.3
Infected but untreated	107 ± 4.5	72 ± 3.1
Infected and treated	78 ± 3	49 ± 2.8

7.3.3. Superoxide dismutase (SOD) assay

When promastigote lysate and *R. serpentina* extracts (10-40 mg/ml) were added simultaneously in the pyrogallol assay mixture to determine SOD activity, 50.5% inhibition was observed at the concentration of 20 mg/ml. However, at the same concentration, 85% inhibition was found when promastigote lysate was pre-incubated with *R. serpentina* prior to SOD assay (**Fig: 7.4**).

7.3.4. Impact of *R. serpentina* on SOD activity and superoxide radical release

When cell free promastigote lysate was tested for superoxide free radical release assay, it was observed that the rate of superoxide radical release before and after treatment of *Leishmania* pathogen with *R. serpentina* leaf extract, varies. Fig: 7.5 shows the rate of superoxide free radical release from different amount of cell free lysate after treatment with *R. serpentina* leaf extract at a concentration of 20 mg/ml. Preincubation with same amount of plant extract caused increase in free radical release.

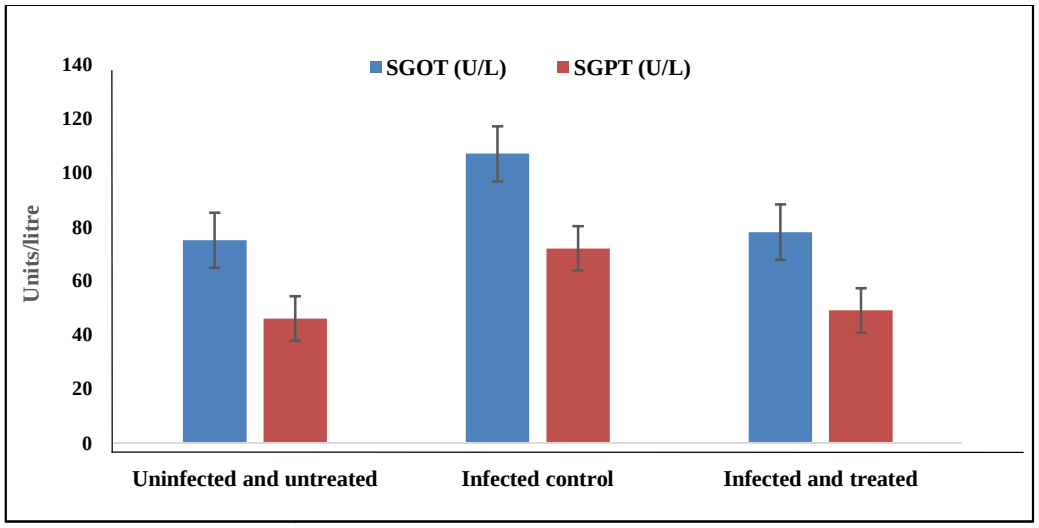

Figure: 7.3. Effect of *R. serpentina* on serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels (units/litre) in *Leishmania* infected hamsters.

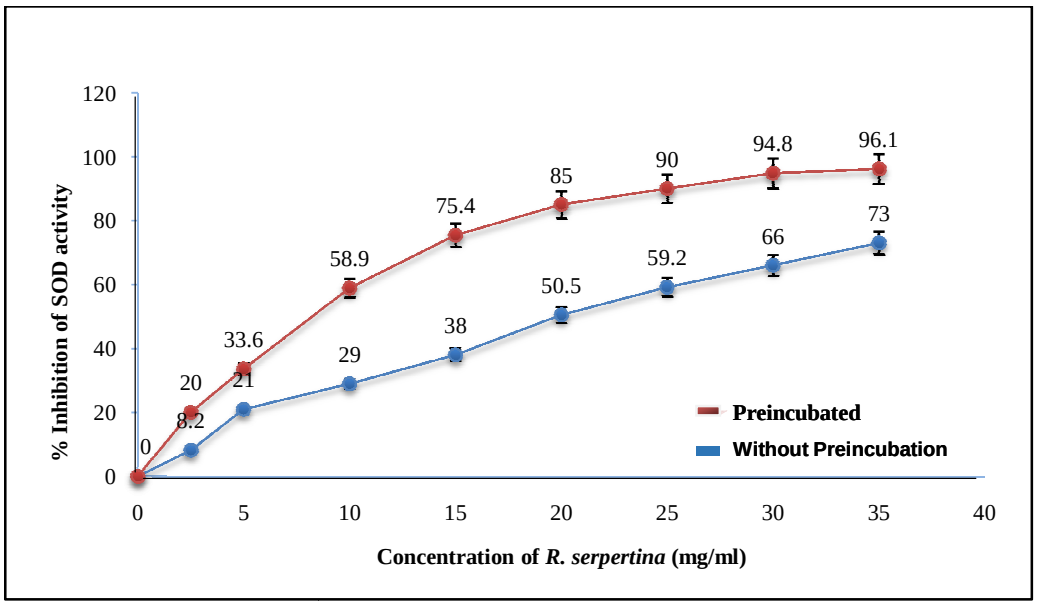

Figure: 7.4. Leishmanial SOD activity when enzyme (promastigote lysate) and *R. serpentina* leaf extracts were added to the reaction mixture either simultaneously (▬) or after preincubation for 30 minutes (▬)

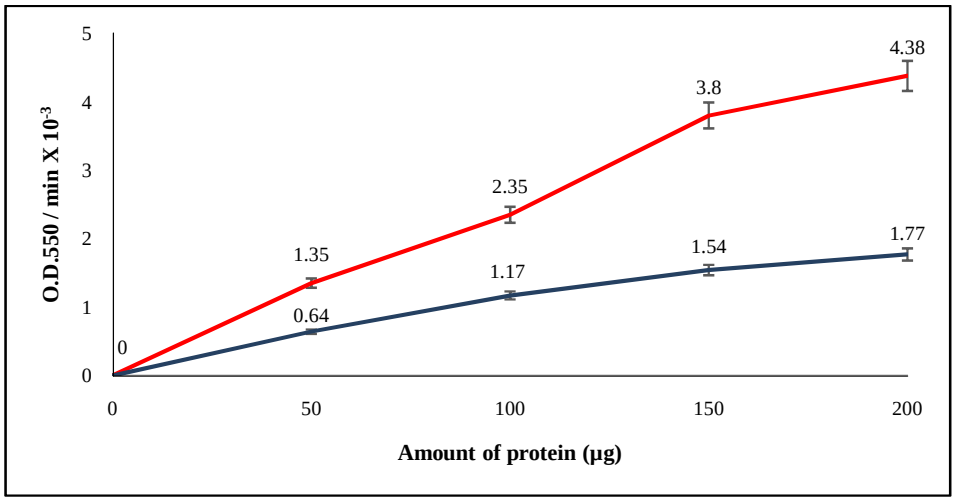

Figure: 7.5. Rate of superoxide radical release before (▬) and after (▬) treatment of *Leishmania* pathogen by *R. serpentina*. Formation of blue formazan derived from reduced nitroblue tetrazolium in the presence of superoxide radical was measured spectrophotometrically.

7.3.5. SDS-PAGE

When the cell free promastigote lysate was treated with different concentrations of leaf extracts of *R. serpentina,* and visualized on polyacrylamide gel,the intensity of the Fe^{3+}-SOD protein band gradually decreased. At 40mg/ml and 30 mg/ml concentration of *R. serpentina*, the SOD activity disappeared completely from the gel (Fig: 7.6).

7.3.6. SOD activity assay

In activity staining, leishmanial SOD activity was found to be inhibited with increasing concentration of *R. serpentina* leaf extract. SOD activity inhibited the formation of blue formazan which was visualised as white band on blue background (Fig: 7.7). In lane A, clear white band was seen where the sample without plant leaf extract was used. Then in lane B and C, the band size gradually decreased where leishmanial SOD was treated with increasing concentrations of leaf extracts (10 mg/ ml and 20 mg/ ml respectively). At the dose of 30 mg/ ml of leaf extract no activity band was observed.

7.4. Discussion

R. serpentina is known as an effective antihypertensive agent from long time. It is traditionally used in psychotic disorders like schizophrenia, anxiety, epilepsy, insomnia, insanity, and is also used as a sedative and a hypnotic drug (Bunkar, 2017).

The plant is mentioned in classic Indian medicinal text. Sarpagandha is included in AparajitGana indicating the use in mental disorder (susrutauttartantra 60/47), in EkasarGana (susrutakalpa 5/84) indicating its use against ‘visha’ and ‘musakavisha’ (susrutakalpa 7/29) (Bunkar, 2017). Its immense traditional use has inspired modern scientists to screen its extract for therapeutic value. Different studies showed its antihypertensive (Ranjini et al., 2015), antidiabetic (Azmi and Qureshi, 2013), antioxidant (Nair et al., 2012), antibacterial (Rathi et al., 2013), antidiarrhoeal (Ezeigbo et al., 2012) and hepatoprotective (Gupta et al., 2010) effects. Few works have been done on antimicrobial activity (Elizabeth, 2017) of *R. serpentina* but, no work has yet been done on its antileishmanial activity to the best of our knowledge.

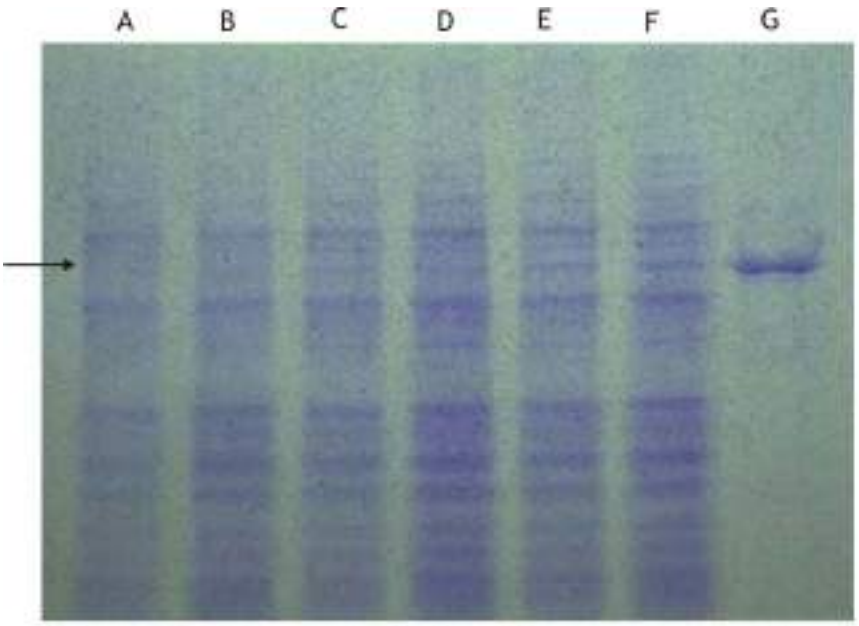

Figure: 7.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *Leishmania* cell free extract after treatment with *R. serpentina* leaf extract in different doses (50 µg protein was loaded in each lane). A. 40 mg/ml *R. serpentina*; B. 30 mg/ml *R. serpentina* C. 20 mg/ml *R. serpentina*; D. 10 mg/ml *R. serpentina*; E. 5 mg/ml *R. serpentina*; F. Without *R. serpentina*; G. Pure Fe^{2+}- SOD.

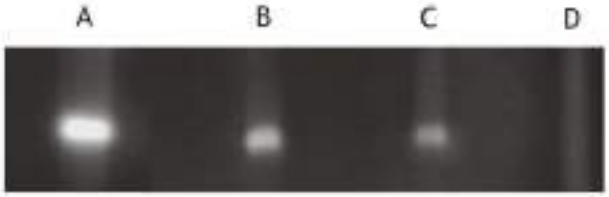

Figure: 7.7. Activity staining of *R. serpentina* leaf extract treated leishmanial SOD after separating by native polyacrylamide gel electrophoresis in a 10% gel. In each lane 60 µgm of protein was loaded. Gel was incubated in nitro blue tetrazolium and riboflavin solutions and finally illuminated. SOD activity which inhibits the formation of blue formazan, was visualized as white bands on a blue background. (A) Without extract B) treated with 10 mg/ml leaf extract (C) treated with 20 mg/ml leaf extract (D) treated with 30 mg/ml leaf extract

The present study screened the leaf extracts of *R. serpentina* for antioxidant, antifungal and antileishmanial activities both *in vitro* and *in vivo* models, and *R. serpentina* had emerged as the promising agent with no cytotoxic effect within therapeutic range.

In vivo studies for antileishmanial activity of *R. serpentina* on *L. donovani* infected golden hamsters showed positive results. *R. serpentina* reduced the parasite burden of liver and spleen of infected hamsters in a dose-dependent manner when administered intramuscularly. Our previous experiment showed that in the administered doses, the extract had no cytotoxic effect on hamster macrophages. Thus, the data suggested that *R. serpentina* can serve as an effective drug in reducing *L. donovani* infection in hamsters. *Leishmania* amastigotes resist phagosomal enzymes and are able to thrive and multiply within the acidic hydrolase rich parasitophorous vacuoles. Though the parasite is sensitive to humoral defense mechanisms, its intracellular habitat offers almost complete protection. Only if the macrophages are activated, the parasite may be killed and degraded by the host cell (Ram et al., 1992; Braconier and Miomer, 1993; Olliaro and Bryceson, 1993; Badirzadeh et al., 2020). Macrophage activation occurs both in natural and adaptive immune reactions in an immuno-competent host. It is induced by INF-γ, a cytokine released mainly by appropriately stimulated natural killer (NK) or T cells (Dasgupta et al., 2003; Volpedo et al., 2021). Activation of microbicidal mechanisms with macrophages may also be done by their exposures to immunomodulating agents. in humans, the protozoan *Leishmania* sp. is an obligated intracellular parasite of macrophages, (Rocha et al., 2005) this makes the treatment for leishmaniasis complicated.

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 (Fattman et al., 2002). Presently the enzyme has been targeted for the treatment of several diseases (Briedbach et al., 2002, Hortelano et al., 2017). SOD also plays a vital role during host–parasite interaction. Its activity is elevated when *Leishmania* parasite infects host cells (Dey et al., 1995). The

parasites use their own SOD to encounter host's superoxides and their own super oxides to get relief from oxygen stress. It is known that the main form of leishmanial SOD contains Fe^{3+} as a co-factor. In the present study the enzyme activity is lost following *R. serpentina* treatmentas shown by activity staining on non-denaturing gel. Leishmanial Fe-SOD was also found to diminished progressively with increasing concentrations of *R. serpentina* leaf extracts in SDS-PAGE. SOD activity completely disappeared at greater concentration of test extract.

It presumed that inhibition of enzymatic activity was caused by protein denaturing effect of the leaf extract (Raychaudhury et al., 2005). Due to deficiency in SOD activity, which is responsible to detoxify released superoxide radicals by the host immune system, toxic free radicals cannot be scavenged up to the maximum limit. The result of present work showing the rate of superoxide radical release in parasitic promastigote lysate before and after treatment with *R. serpentina* leaf extract established the fact clearly. This finding was also in agreement with the previous work of Martin-Montes et al. (2017). Super oxide dismutase has been demonstrated to be a key enzyme to play a vital role in the survival of intracellular parasites (Hortelano et al., 2017), where importance of this enzyme in the host–parasite interaction was established by generating SOD-deficient *L. donovani* (Ghosh et al., 2003). The results obtained from different experiments suggest that inhibition of SOD and simultaneous increase in release of superoxide radicals impose toxic effect to destroy intracellular parasites during experimental visceral leishmaniasis (Gupta et al., 2010). Parasitic infection causes toxicity of liver in animals which is manifested by increased levels of specific serum enzymes such as SGPT and SGOT (Kaur et al., 2016). The present study showed the levels of SGOT and SGPT were markedly higher in *L. donovani* infected hamster compared to uninfected control. The levels of both these enzymes decreased to normal level upon treatment with *R. serpentina*. The collected data suggests that *R. serpentina* may be a good choice to act as an antileishmanial agent with better efficacy. Results also reveal that *R. serpentina* seems to be non-toxic up to therapeutic dose.

The present study was undertaken with an aim to explore the indigenous knowledge on medicinal plants and ayurveda in order to evolve new compositions for fighting against leishmaniasis and fungal infections. The ethnobotanical studies were conducted in the remote areas of two districts of North Bengal, Jalpaiguri and Alipurduar. An intensive survey was done to gather information about medicinal plants used by the indigenous population and tribal communities inhabiting in the jungles of tarai – dooars and tea garden areas. Fifty plant species of 33 families were found to be used for medicinal purposes by various tribes of Jalpaiguri and Alipurduar districts. These plants are mostly (76%) collected from natural vegetation to treat cuts and wounds, cough and cold, fever, skin diseases, pain and inflammation, bone fracture, stomach ache and abdominal disorder, jaundice and liver problems. Leaf was found to be the most frequently used (33.9%) plant part followed by root (18.2%). Four very common and well-known medicinal plants, *R. serpentina* (sarpagandha), *M. oleifera* (drumstick), *N. arbor-tristis* (seuli or night jasmine) and *C. excavata* (agnijol) were selected for screening, as the utilizations of these plants were found to be vast and versatile among the studied population. Crude leaf extracts of *R. serpentina, M. oleifera, N. arbor-tristis* and *C. excavata* were analysed for their phytochemical contents and were also screened for antioxidant, antifungal and antileishmanial activities.

In phytochemical analysis, leaf extracts of each of the four plants showed positive result in qualitative test for alkaloid, flavonoid, terpenoid and tannin. In the quantitative assay, it was seen that the amount of phenolic compound in all four plant leaf extracts ranged from 22.22 to 42.65 mg GAE/gm extract. Flavonoid presence ranged from 9.39 to 31.15 mg QE/gm extract.

All the four plants were found to have free radical scavenging activities in DPPH assay. The percent inhibition of free radical neutralizing activities was determined. *C. excavata* showed the lowest IC_{50}, implying good antioxidant property, and *R. serpentina* showed the highest IC_{50} value. The relationship between phenolic and flavonoid content and antioxidant activities of respective plants were also quite evident. *N. arbor-tristis* had higher phenolic

content and lower IC_{50} value, whereas *M. oleifera* had low phenolic content and comparatively more IC_{50} value in percent inhibition of DPPH. In contrast, *C. excavata*, which showed lowest phenolic and flavonoid content but good antioxidant property (IC_{50} lowest), indicated the probability of presence of active compound of different polarity in its leaf extract.

The methanolic extracts of leaves of all the four plants showed good antifungal activities against human fungal pathogen *C. albicans in vitro*. *R. serpentina* leaf extract showed highest range of zone of inhibition (4.87cm) at the concentration of 100mg/ml. At the same concentration the size of ZOI for *M. oleifera, N. arbor-tristis*and *C. excavata* were 3.2cm, 1.6cm and 2.4cm respectively. But coumarin molecule, excavarin-A (isolated from *C. excavata* leaf extract) showed highest antifungal activity with a ZOI of 4.6cm at a concentration of 10mg/ml. The MIC value was lowest for excavarin-A (0.078 mg/ml) followed by *R. serpentina* (0.156 mg/ml), which was nearer to the known antifungal antibiotic nystatin having MIC of 0.039 mg/ml.

The methanolic leaf extracts of *R. serpentina* and *C. excavata,* and excavarin-A were screened for their antileishmanial activities *in vitro* on the promastigotes of *L. donovani* strain in graded doses. Result showed all the test substances had antileishmanial activities *in vitro*. The IC_{50} values for excavarin-A and the crude leaf extracts of *R. serpentina* and *C. excavata* was recorded as 1.24 mg/ml, 4.04 mg/ml and 32.1 mg/ml respectively. The purified compound was found to be much more effective than the crude extracts. The selected botanicals were also tested on amastigotes cultured in hamster macrophage in laboratory condition. Excavarin-A was found to be most efficacious followed by crude extracts of *R. serpentina* and *C. excavata.*

Superficial infections were created on experimental animals (male albino rats) with cell suspension of fungal strain of *C. albicans* ($1x10^5$cells/ml). Infection developed at the site of application after two days of pathogen application and the animals were treated superficially with diluted plant extracts on the wounds. Significant recovery after 8 days of treatment was evident in the treated animals in comparison to untreated group. The therapeutic action of

R. serpentina was found better than *M. oleifera*. Total count of WBC was increased in both cases of treated animals. This model of *in vivo* experiment using animal sample with open wounds and application of crude extracts superficially provides a preliminary but direct visible evidence of the efficacy of the plant extracts. Study with more pathophysiological parameters is needed to further evaluate the effectiveness and mode of actions of these botanicals.

The crude leaf extracts of *R. serpentina* and *C. excavata*, and the purified compound excavarin-A were checked for cytotoxicity by MTT assay, and tested for viability on macrophages isolated from hamster peritoneal cavity. *C. excavata* leaf extract and excavarin-A were found to be cytotoxic within effective therapeutic ranges. But *R. serpentina* leaf extracts were not found to be cytotoxic in cell viability test in its effective range of doses. So, on the basis of this observation, the crude methanolic leaf extract of this plant was studied further *in vivo*.

The parasitic burden of spleen and liver of golden hamsters experimentally infected with *L. donovani* decreased significantly following treatment with graded dose of leaf extracts of *R. serpentina*. Further an increase in serum SGOT and SGPT levels in infected animals, and the lowering of these levels by application of *R. serpentina* leaf extract to almost normal level indicated the therapeutic effect of the extract. Pyrogallol auto oxidation assay showed inhibition of activity of parasitic superoxide dismutase. The release of toxic superoxide radicals was also found to increase by the application of *R. serpentina* leaf extracts which indicated the probable mode of action of the active substances present in the applied sample. Polyacrylamide gel electrophoresis of *Leishmania* promastigote lysate confirmed these results. On gel activity staining through non-denaturing PAGE as well as SDS-PAGE showed degeneration of SOD bands with increasing concentration of *R. serpentina* extract.

The present study reported significant results of some preliminary screening tests of a few very common and widely used medicinal plants having anti-

oxidant, antifungal and antileishmanial properties. The study also proposed immunological modifications induced by application of test samples which may be helpful in further research to find the mode of action of therapeutic agents gained from natural resources. Some easy and effective protocols of experiments were also tried, which has very limited references so far. *R. serpentina* emerged as most effective plant with significant antimicrobial values and without cytotoxic properties. This plant is strongly recommended for further study and isolation of active substances to develop a new line of treatment. The present work is definitely a step forward in the utilization of natural resources of unexplored areas of North Bengal to elucidate the local knowledge scientifically to discover bioactive molecules related to new drug development against parasitic diseases, whose prevalence and impact is universal.