5.1. Literature review

Leishmaniasis is a zoonotic disease, caused by 20 different species of an obligate protozoan parasite of the genus *Leishmania*. The disease is vector-borne, and all species of *Leishmania* are transmitted by at least 70 different types of phlebotomine sand flies. Mammalian species, mainly humans, play the role of host. Leishmaniasis is responsible for the second-highest number of deaths due to parasitic infection globally and is overwhelmingly associated with poverty. It has an estimated prevalence of 12 million humans infected and causes a burden estimated at 2,357,000 disability-adjusted life in a year (Annual Report 2009, WHO, Geneva). It is a neglected infectious disease affecting nearly 98 countries worldwide (de Paula et al., 2019) with about 1.7 billion people at risk of contracting the infection (Sosa et al., 2019). The manifestation of the disease shows a vast range froms an asymptomatic localized skin lesion, to life-threatening progressive visceral form (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis).

5.1.1. History

Long before identification, leishmaniasis was reported as early as the first century AD, in pre-Incan pottery from Ecuador to Peru as skin lesions and facial deformities. From the 15th to 16th century in Spain, it was known as 'White Leprosy', resembling a leprosy lesion. It was marked as 'Andean Sickness', 'Valley Sickness', and many other names. In Africa and India, in the mid-18th century, it was known as Kala-azar or Black fever. In 1776, Alexander Russel made an important advance in this field and called visceral leishmaniasis 'Allepo Boil'. The disease got its name after William Leishman, who worked as a doctor with the British army in India, and identified and named the strain in the splenic smear of a patient, who died of 'Dum-Dum' fever in 1901. In 1903, Charles Donovan for first time characterized the causative agent as protozoan and morphologically related to Trypanosomes (Cox, 2002; Akhoundi et al., 2016). Later, in 1930, Sir Ronald Ross named the parasite *L. donovani*, and eventually, thus, established the link between the very old disease and the causative organism. Species belonging to the genus *Leishmania* are the

etiological agent of a variety of manifestations of the disease collectively known as leishmaniasis. The common species of disease causing *Leishmania* are *L. donovani, L. infantum* and *L. archibedi* in the old world, and *L. chagasi* in the new world. Sandfly was identified as a vector by Swaminath et al. (1942) by using human volunteers and humans, wild animals, and domestic animals were known to act as reservoir hosts. Later it was established that *Lutzomyia longipalpis* is the only sand fly vector species that transmit visceral leishmaniasis and reservoir host is a wild or domestic dog (WHO expert committee report 1991, https://apps.who.int/iris/handle/10665/37432;de Paula et al., 2019).

5.1.2. Clinical manifestations and classification of leishmaniasis

The range of clinical manifestations of leishmaniasis is wide and is specified by the type of invading parasite and also the genetic determinants present in the human host which influences the overall immune response. These clinical features collectively called leishmaniasis are classified as (i) visceral leishmaniasis (VL) or 'kala-azar' (ii) cutaneous leishmaniasis (CL), diffused and localised; (iii) post kala-azar dermal leishmaniasis (PKDL), (iv) mucocutaneous leishmaniasis (MCL) and (v) leishmaniasisrecidiva. (Torres-Guerrero et al., 2017; Valpedo et al., 2021).

Visceral leishmaniasis

This is one of the most deadliest kind of the leishmaniasis commonly known as kala-azar and caused by *L. donovani* in India and Africa, *L. donovani chagasi* in South America and *L. donovani infantum* in Mediterranean areas. *L. tropica* is also marked as causative agent of VL in recent studies. If remain untreated, VL can emerges in fatal form with 100% morality. VL is characterised by long term low-grade fever, hepatosplenomegaly, that is enlarged spleen and liver with biological signs of anaemia and weight loss (Woodruff, 1972; Rajagopala et al., 2008; Singh and Sundar, 2015), affecting the organs and tissues like liver, spleen, lymph nodes, bone marrow, lung, intestine, skin and rarely oral mucosa, placenta and thymus. In the year 2020, 90% cases of VL have been reported in 10 countries, those are Brazil, China, Ethiopia, Eritrea, India,

Kenya, Somalia, South Sudan, Sudan and Yemen (WHO, 2020, https://www.who.int).

Post Kala-azar dermal leishmaniasis (PKDL)

PKDL is characterised by non-ulcerative cutaneous lesion developed in post kala-azar subject mainly in India and Sudan following drug treatment (WHO, 2020). It is proven to be due to accumulation of heavily infected macrophage under the skin (Zijlstsa et al., 2003) and manifesting nodules, papules, hypopigmented macules mainly on face first then a spread on other parts of body are seen.

Localised cutaneous leishmaniasis

This is the most common form of leishmaniasis worldwide, with approximately one million new cases that are diagnosed every year, and about 90% of the cases are found in developing countries (Badirzadeh et al., 2020). It is caused by *L. tropica, L. aethiopic*and *L. major* in old world and by *L. mexicana, L. guyanesis, L. braziliensis* and *L. amazonensis* in new world. CL is characterised by development of self-healing benign lesions on skin without any mucosal involvement. In *L. mexicana* infections, lesions (Chiclero's Ulcer) develop on pinna of ear (Saito and Murray, 2020). Sometimes, the disease exists in host body for a long time (*L. tropica*), leading to disfigured body parts.

Diffuse cutaneous leishmaniasis (DCL)

DCL is the rare form of the disease caused by *L. mexicana*, *L. aethiopica* and *L. amazonesis* reported from Venezuela, Brazil, Ethiopia and Dominic republic. The disease is featured by non-ulcerating nodules like lepromatous leprosy over the entire body without pain which can be healed only by treatment (Volpedo et al., 2021).

Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis is caused by *L. braziliensis braziliensis*. The disease occurs mainly (90%) in South America. MCL is characterised by two phases, primary cutaneous lesion followed by mucosal involvement (metastatic

infected macrophages). The primary lesions are spontaneously cured still infection may remain inside the host and without effective early treatment it may cause permanent disfigurement (Volpedo et al., 2021).

Leishmaniasis recidiva

Chronic recurrence of *L. tropica* causes a hypersensitive dermal response with nodular lesion and satellite lesions at margins of primary lesion of DCL which have already been healed. It is mainly found in people of Middle East (Volpedo et al., 2021).

5.1.3. Mode of transmission of disease

Leishmaniasis may be transmitted from vector to host, host to non-infected subject in many ways among which the most common mode of transmission worldwide is vector borne transmission. Other modes are parenteral, congenital, sexual and occupational from host to non-infected person (Fig. 5.1).

5.1.4. Epidemiology

As the world's most neglected disease, leishmaniasis is still affecting the poor people of developing and underdeveloped countries; prevalent throughout the tropical and sub-tropical regions of Asia, Africa, Southern Europe, South, and Central America. Moreover, many cases go misdiagnosed and unreported, and therefore the exact scale of impact on public health cannot be predicted. According to WHO (2022), most cases occur in Brazil, East Africa and India (Fig. 5.2). An estimated 50,000 to 90,000 new cases occur worldwide annually, with only 25% to 45% reported to WHO. In 2019, more than 90% of new cases reported to WHO occurred in 10 countries viz. Brazil, Eritrea, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan. Visceral leishmaniasis (VL) brings India on focus in the world and in India, mainly the state of Bihar, eastern Uttar Pradesh (Marwaha et al., 1991) and West Bengal (Chandra et al., 1995) are heavily affected. According to a recent report of WHO (2022), the four states of India where kala-azar is still a threat to public health are: Bihar (33 districts, 458 blocks), Jharkhand (4 districts, 33 blocks), West Bengal

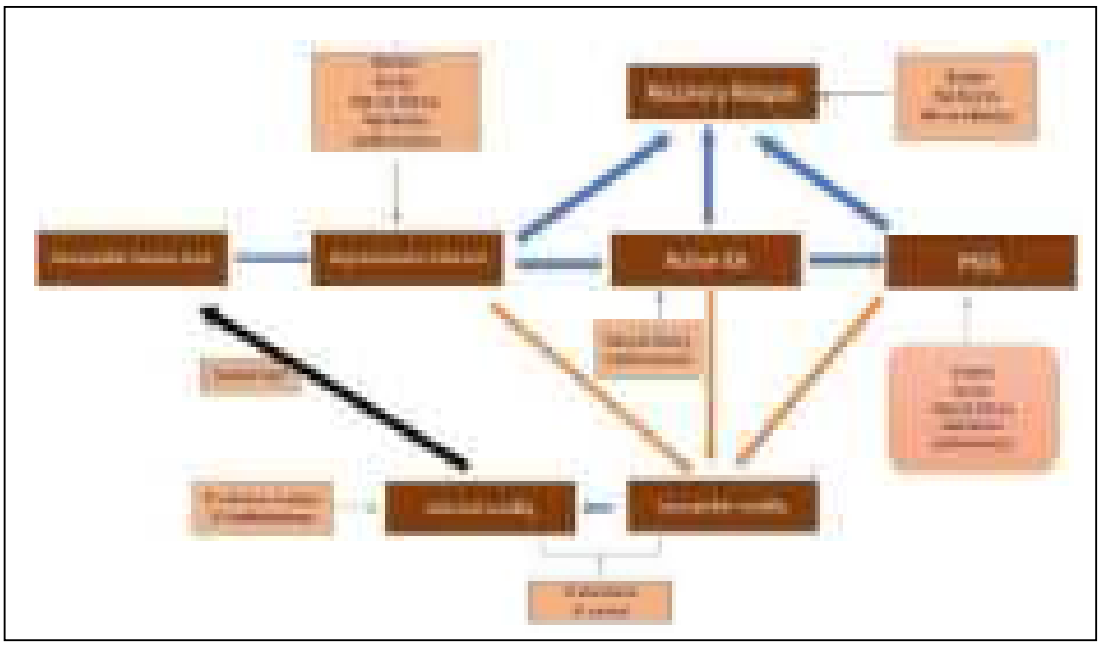

Figure:5.1. Schematic framework showing transmission of visceral leishmaniasis

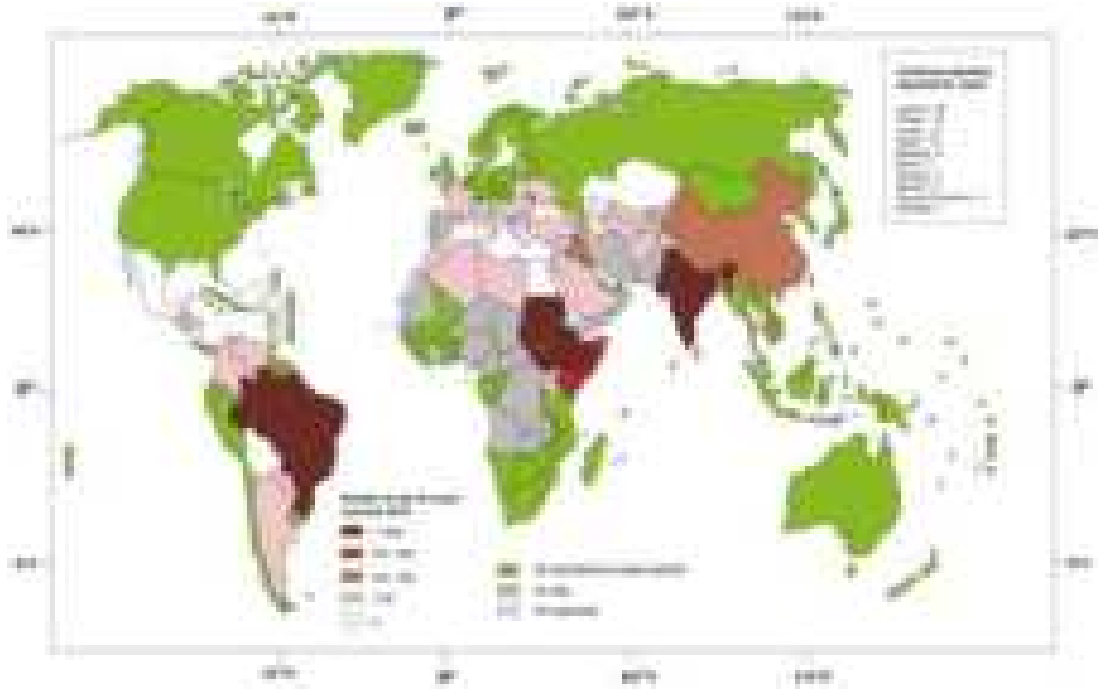

Figure: 5.2. Status of endemicity of visceral leishmaniasis Worldwide 2016 (WHO) (Prepared using ***ArcGIS 10.3.1*** *software*)

(11 districts, 120 blocks) and Uttar Pradesh (6 districts, 22 blocks). Recent news of leishmaniasis in some blocks of Malda districts, West Bengal, increases the concern (Uttar Banga Sambad, 18th Feb 2019) (Fig. 5.3). National Center for Vector Borne Diseases control, Ministry of Health and Family Welfare & Government of India keeps the record of cases of VL, post Kala-azar dermal leishmaniasis (PKDL) and AIDS -VL coinfection in India. They reported some cases from states other than above four, like Assam, Sikkim and Uttarakhand from the year 2014 to 2017**(**https://nvbdcp.gov.in**).**

5.1.5. Leishmaniasis and AIDS-a vicious cycle

Leishmaniasis is a well-recognized opportunistic infection in HIV infected people (Kooli et al., 2021). The first case of leishmaniasis associated with HIV infection was reported in 1985, and thereafter persistent rise in the number is seen worldwide. Studies said, in areas of endemicity, HIV infection increases the risk of developing VL by 100 to 2,320 times, reduces the likelihood of a therapeutic response, and increases the probability of relapse. At the same time, VL helps the clinical progression of HIV disease and the development of AIDS (Alvar et al., 2008). In the 1.5-9% of AIDS patients are reported to develop visceral leishmaniasis, and on the other side, 25-70% of people affected with leishmaniasis are related to HIV infection. For the last few decades, AIDS and VL are observed to work in a vicious cycle of mutual reinforcement (Fuzibet et al., 1988; Lindoso et al., 2009). According to WHO (2022), *Leishmania*-HIV coinfections have been reported from 45 countries. High *Leishmania*-HIV coinfection rates are reported from Brazil, Ethiopia and the state of Bihar in India. In a recent study, incidence of hemophagocytic lymphohistiocytosis (HLH) in HIV-positive person with visceral leishmaniasis has been reported (Kooli et al., 2021).

5.1.6. Morphology of parasite

Leishmania parasite, a protozoan, exists in two morphological states, and thus, is known as a dimorphic parasite. As a promastigote, it exists in sand fly and as an amastigote, it exists in the vertebrate host.

Figure: 5.3. Some articles published in leading newspapers depicting news of kala-azar in India and West Bengal in recent time

5.1.6.1. The promastigote forms

The promastigote form is the extracellular form of the parasite, seen in the insect vectors and also in laboratory culture(Herwaldt,1992). It is flagellated, having a spindle-like shape, 15-20 nm in length, and 1.5-3.5 nm in breadth (Fig: 5.4 A and B). When sandfly ingests the blood of an infected person or vertebrate host, the amastigotes are transformed into promastigotes within hours in the midgut of the sandfly and are transformed into different stages (Ashford, 1997). In metacyclic promastigotes, the nucleus is in the centre and the Kinetoplast in the anterior end. The flagellum of promastigote is 15-28 nm in length with a tubular and latticed structure.

5.1.6.2. The amastigote forms

The amastigote forms are seen within the body of vertebrate hosts; it resides in the reticuloendothelial cells of humans, dogs, or hamsters. It is a round or oval-shaped flagellated form with a delicate filament extending from the basal body. It is 2-4 nm long, with little or no motility (Fig: 5.4 A and C).

5.1.7. Life cycle of parasite and the role of host

Leishmania parasite has dimorphic life cycle between primary reservoir (human) and secondary host (sand fly) (Fig: 5.5). When a female sand fly attacks *Leishmania* infected human host and feeds on blood, it ingests a number of amastigotes and these non-flagellated amastigotes are transformed into flagellated promastigotes in posterior midgut of female sand fly within hours. The promastigotes divide by binary fission and get converted into procyclic promastigotes, which are transformed into nectomonad form. Within 3 days of blood feeding, peritrophic membrane of gut of sand fly containing these parasitesbegins to break down and promastigotes are set free and move towards anterior midgut of the host (Ashford, 1997). In the anterior midgut, the parasite changes its form into nectomonad and then to haptomonads within the next five days and are attached to the stomodeal valve (Vickerman and Tetley, 1990). Thereafter, the highly motile metacyclic parasite localized

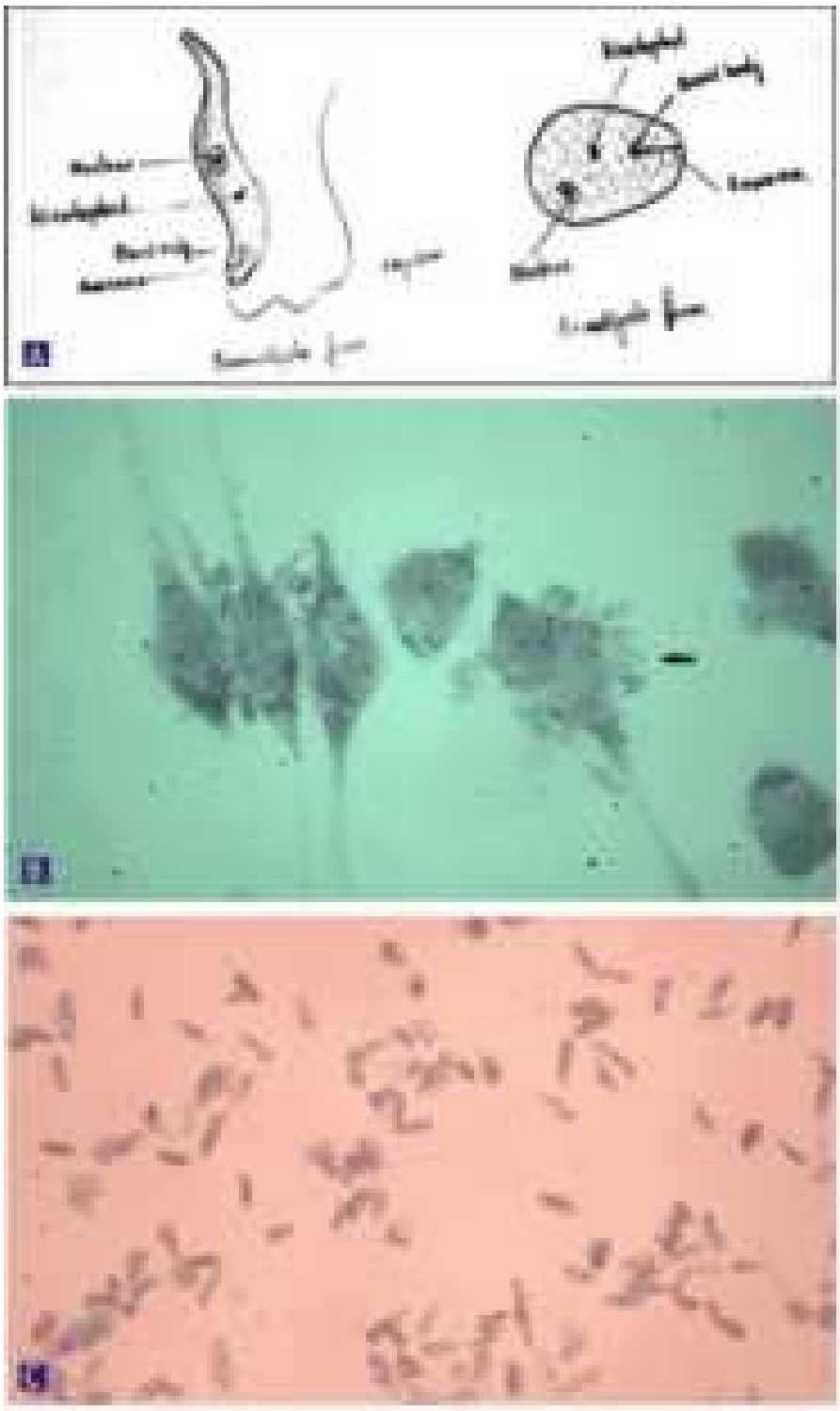

Figure: 5.4, A: Diagram of promastigote and amastigote form of *L. donovani*; B: Image of promastigotes in culture as seen under microscope; C: Image of amastigotes attacking macrophage in culture as seen under microscope

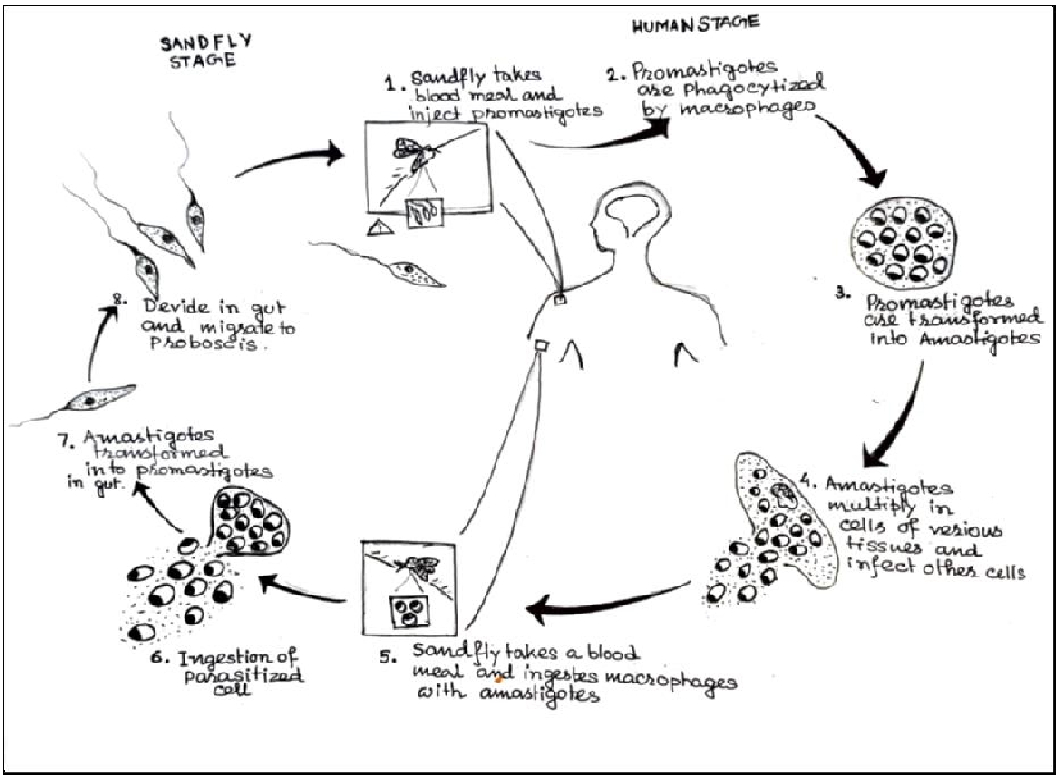

Figure: 5.5. Life cycle of *Leishmania* parasite

in the lumen of anterior midgut and foregut is ready for spreading into primary host during the next blood meal of the sand fly. The metacyclic promastigotes enter into vertebrate host, are taken up by macrophages, and transformed into amastigotes with in parasitophorus vacuoles (Kima, 2007). The manifestation of disease in various forms of leishmaniasis depends on the different tissues where the infected macrophages are present (Torres-Guerrero et al., 2017).

Change in temperature in different host initiates the changes in form of the parasite. Amastigotes, when face a drastic change in temperature from 37° Celsius of mammalian body to 22-28°C in insect body, the onset of change in form starts. Not only temperature, the environment, motility of two types of hosts implies significant physiological difference in two forms of the parasite. All these findings give the possibility of *in vitro* transformation of promastigote in culture in laboratory. Transformation from amastigote to promastigote is associated with increase in respiratory rate, synthesis of cytochrome C, polyamines (Morrow et al., 1980), increase in cell volume and acquisition of antigen and expression of heat shock gene, enhancement in glycolytic enzyme activities and reduction in enzyme activities of fatty acid (Hart and Coombs, 1982).On the other hand, in amastigotes, glutathione peroxide, catalase and superoxide dismutase are active, which helps the parasite to survive in mammalian host's immuno-attack. There are changes in antigenic properties and gene expression.

The phlebotomine sand flies recognized as vectors of leishmaniasis causing parasites are of the order *Diptera,* Family *Psychodidae,* subfamily Phlebotominae. Among the 800 known species or subspecies of Phlebotomine only 40 species are responsible for transmission of leishmaniasis (Killick-Kendrick, 2002). The two main genera, *Lutzomyia* in new world and *Phlebotomus* in old world (Mann et al., 2021) are medically important as vectors. In India, *Phlebotomus argentipes*is prevalent. The parasite develops and multiplies in wide range of temperature (16°celsius to 34°celsius) in sand fly; and bites of sand fly are very common in Kala-azar endemic areas throughout the year. According to the observation, the number of flies reaches

a peak between February and July in Bihar, and July to October in West Bengal (Torres-Guerrero et al., 2017).

5.1.8. Pathogenesis of leishmaniasis and role of immune system in host body

Blood meal by a female sand fly caring *Leishmania* promastigote initiates the infection with *Leishmania* in vertebrate host or human. The saliva of sand fly is infused with virulent metacyclic promastigotes which are injected into skin and then into circulation during insect bite. The saliva also plays role in proper injection of parasite as it carries vasodilating molecule, substances having anticoagulant and immunomodulatory role (Sacks and Kamhawi, 2001). From the circulation in the host body, the promastigotes are ingested by phagocytes by complement aided opsonization and lectinlipophosphoglycan (LPG) – mediated phagocytosis (Wozencraft and Blackwell, 1987). Phagosome releases superoxides and hydroxyl radicals to encounter invading pathogen. But *Leishmania* parasite has several mechanisms to resist host immune attack and survive.

First the parasite produces acid phosphatase on their cell surface which blocks the production of superoxide (Glew et al., 1988). LPG of parasite also inhibits protein Kinase C which is essential for generating oxidative metabolites in host macrophage (McNeely et al., 1989). Growth factor withdrawal induced apoptosis of macrophage in host is also inhibited by invading parasite because viability of infected macrophages is required to be up taken by vector (sand fly) as much in numbers as possible (Delgado-Altamirano et al., 2017) (Fig: 5.6). Various studies reported the role of different components of acquired immunity of host body, such as interleukin (IL-10, IL-12), interferon (IFNγ) andtumour necrosis factors (TNFα) in disease development (Gazzinelli et al., 1996; Rittig and Bogdan, 2000; Maynard and Weaver, 2008; Volpedo et al., 2021) (Fig.5.6). Besides, the enzyme super oxide dismutase (SOD) which catalyses the dismutation of superoxides (O_2^-) into H_2O_2 and O_2, plays a vital role in the survival of parasites of leishmaniasis in host body. Its activity is also

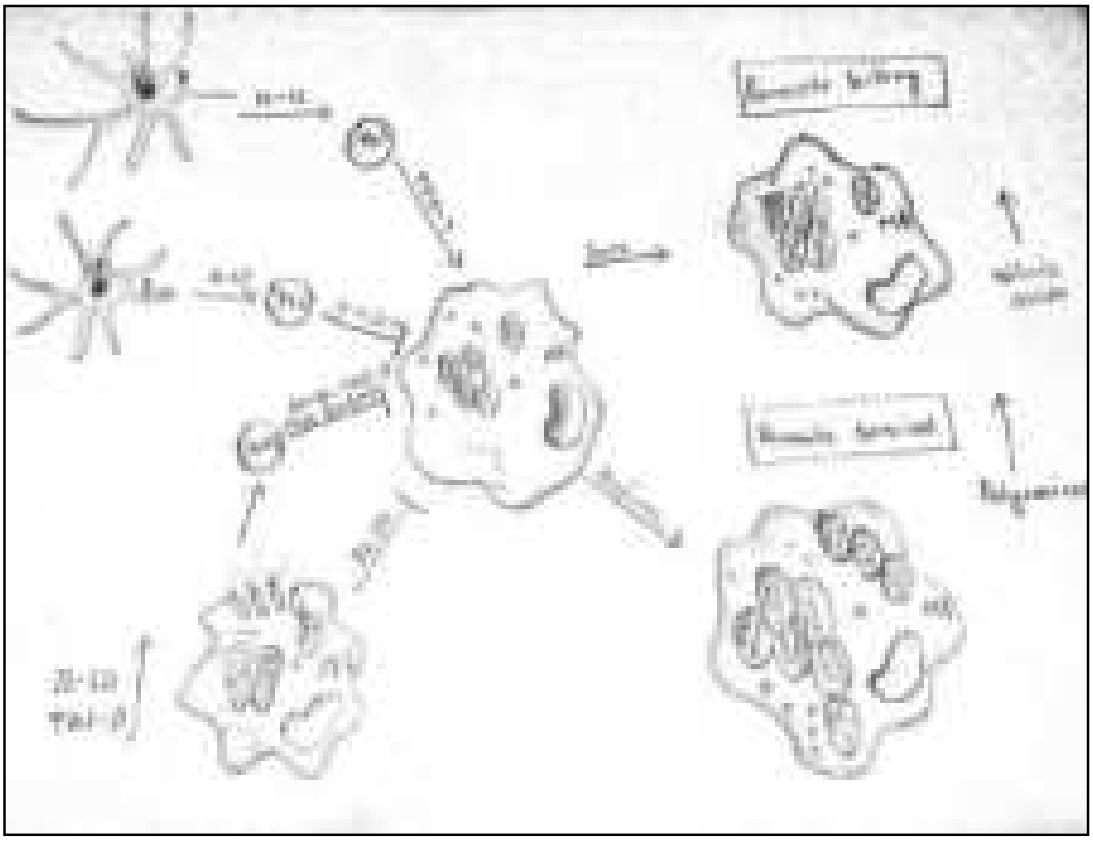

Figure: 5.6. Immunological response of host body towards invading pathogen of leishmaniasis

found to be elevated when the parasite is within the host (Dey et al., 1995; Ghosh et al., 2003).

5.1.9. Available line of treatment

Since 1940, antimonial drugs are first line of treatment of all forms of leishmaniasis worldwide. Meglumine antimoniate, (marketed as glucantine and prostib), sodium stiboglucanate or SSG (commercially available as pentastam, solustibosan and stibanate) are the widely used compositions. But with these compositions some complications are emerging in different studies, like requirement of high doses and drug resistance (Rees et al., 1980; Thakur et al., 2004). After the rise in SSG resistance, amphotericin ß is extensively used even as first line of drug in Bihar (India) with 98-100% positive results (Sundar et al., 2002). Newer drugs used include paromomycin, miltefosine and sitamaquine but these therapeutic agents are facing either the problem of drug resistance, inefficiency or adverse side effects (Sundar et al., 2002).

The concept of vaccination against leishmaniasis was materialised a long time ago when intuitive knowledge of mother of Lebanese children motivated them to expose their children's arms to the bite of sand flies, which developed a self-healing single first lesion which would protect them from severe attack of the same disease in future (Vanloubbeeck and Jones, 2004). This practice inspired Professor Adler from Hebrew University of Jerusalem, Israel to develop first vaccine against leishmaniasis. At first scientifically an axenic culture of parasite was prepared, administration of which was known as leishmaniazation and it became usual in Israel and Russia, which was the first step in preparation of vaccine. Then killed parasites were used instead but the leishmaniazation process was discontinued due to uncontrolled long-lasting skin lesion. At present its use is limited worldwide (Murray, 2001). In past different attempts were made to discover an effective vaccine, such as live non-attenuated vaccines, live-attenuated vaccines, killed vaccines and DNA vaccines (Handman, 2001; Giunchetti et al., 2008) but none were found to be totally efficient or safe. Therefore, till today, there is no definite product which offers all-round protection against the disease (Badirzadeh et al., 2020).

In this context, natural remedies and novel molecules from natural sources provide an alternative to fight against leishmaniasis.

5.1.10. Natural remedies

From ages indigenous people of different countries are using vast resources of plant kingdom for medication without knowing the science behind it. Local people of leishmaniasis affected countries are using different plants or extracts of plant part like root, leaf, fruit etc to get rid of this endemic disease. Local literature and ayurveda have mentioned about these natural products. From ancient knowledge, scientists and researchers get inspirations to screen different referral plants or new ones against leishmania pathogen *in vitro* or in animal models (Table: 5.1).

In the present study, we have screened methanolic leaf extract of *R. serpentina* and *C. excavata* and the molecule excavarin-A for antileishmanial activity in search of a new therapeutic option.

Table: 5.1. Some studies on the efficacy and activity of herbal medicines used against leishmaniasis *in vitro* and *in vivo*.

Plant	Plant part/ extract used	Species of pathogen	Mode of action	Reference
Alkanna tictoria	Stem and root	*L. major*	IC_{50} 200µg/ml	Yousefi et al., 2009
Allium hirtifolium	Bulbs	*L. infantum*	Parasite growth inhibited in one day at 0.2 mg/cc	Amnzadeh et al., 2006
Artemisia annua	Aerial parts	*L. major*	IC_{50}400 µg/ml for ethanolic extract	Emami et al., 2012
Berberis vulgaris	Root	*L. tropica*	IC_{50}16.1 µg/ml for methanolic extract and 26.6 µg/ml for aqueous extract	Mahmoudvand et al., 2014
Berberis vulgaris	Whole plant	*L. major*	With 20% preparation, the lesions had decreased with complete healing in 5 mice(27.7%).	Kazemi et al., 2007
Calotropis gigantea	Aerial parts	*L. major*	IC_{50}0.18 mg/ml for methanolic extract and 0.17 mg/ml for hexane extract	Oskuee et al., 2012
Capsicum frutescens	Capsaicin from seeds	*L. infantum*	IC_{50} 5.01 µg/ml against promastigotes and 24.18 µg/ml against amastigotes	Vieira-Araújo et al., 2018
Ixora brachiate	Root	*L. major*	IC_{50} 0.078 mg/ml for ethanolic extract	Sadeghi-Nejad et al., 2014
Matricaria chamomilla	Flower	*L. major*	Treatment with chamomilla tea (for 5mins/day) for 2 weeks wound healing (skin ulcer) was 58.3%(in diameter)	Dashtpeima et al., 2015
Myrtus communis	Leaves	*L. tropica*	IC_{50} 28.9 µg/ml	Mahmoudvand et al., 2015
Peganum harmala	Seed	*L. major*	IC_{50}40 µg/ml	Yousefi et al., 2009
Phytolaca americana	Fruits	*L. major*	IC_{50}171.1 µg/ml against promastigotes	Jafroodi et al., 2015
Piper aduncum	Essential oil	*L. braziliensis*	IC_{50} 77.9 µg/ml against promastigotes	Ceole et al.,2017
Pistacia atlantica	Gum obtained from trunk and branches	*L. major*	Eight weeks treatment with gum (60 mg/kg body weight) decrease skin lesion size (mice) from 68.4 to 35.3 mm	Taran et al., 2010

Plant	Plant part/ extract used	Species of pathogen	Mode of action	Reference
Pistacia khinjuk	Stock	*L. tropica*	IC_{50} 58.6 µg/ml against promastigotes, IC_{50} 37.3 µg/ml against amastigotes	Ezatpour et al., 2015
Sature jakhuzestanica	Leaf	*L. major*	IC_{50}0.3 mg/ml for ethanolic extract and 0.6 mg/ ml for methanolic extract	Sadeghi-Nejad et al., 2011
Scrophulara striata	Whole plant	*L. major*	The parasites were killed at 20% concentration within two days.	Dalimi et al., 2013
Urtica dioica	Leaves	*L. major*	IC_{50} 4500 µg/ml against promastigotes	Badirzadeh et al.,2020

5.2. Materials and Methods

5.2.1. Preparation of leaf extract

Leaf extract of plants were prepared following the procedure described in previous chapter (section 3.2.1). The molecule Excavarin-A was isolated from the leaf extract of *C. excavata* following the procedure described in section 4.2.2.

5.2.2. Propagation of parasites

L. donovani, strain MHOM/IN/83/AG was originally derived from an Indian kala-azar patient (Ghosh et al., 1985). 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 foetal calf serum. Promastigotes were obtained by transforming amastigotes isolated from infected spleen (Jaffe et al., 1984) *in vitro* and sub-cultured in the same medium at 22°C, at an average density of $2x10^6$ cells/ml (Kar, 1990).

5.2.3. Maintenance of promastigotes

Solid Media: The promastigote form of *L. donovani* was maintained in modified Ray's solid medium at 22-25°C (Ray, 1932).

Liquid Media: Parasites were also grown in sterile Medium-199, as and when required. The medium was sterilized by passing through 0.22 µm sterile Millipore filters (Millipore Corporation, USA). Maintenance of *L. donovani* strain was done as described above. Promastigotes were obtained by transforming amastigotes ($1X10^7$cell concentration) isolated from infected spleen (Raychaudhury et al., 2005) and maintained in Medium-199 supplemented with 10% foetal calf serum (FCS) *in vitro*.

5.2.4. Model animal

Syrian golden hamsters were originally obtained from Haffkine Research Institute, Mumbai, India and bred in animal house of IICB, Jadavpur, Kolkata.

5.2.5. Isolation of amastigotes

Spleens of hamsters infected with *L. donovani* were suspended in liquid Medium-199, homogenized using a glass homogenizer and centrifuged at 1,000 rpm for 10 minutes. The supernatant was centrifuged at 2,000 rpm for 10 minutes and the pelleted parasites were washed in the same medium by centrifugation again at 2,000 rpm for 10 minutes and resuspended again in the same medium (Channon et al., 1984). These amastigotes were injected intracardially into hamsters for the maintenance of virulent strain and also used for further study. The animal ethics committee of the institute approved all animal experiments.

5.2.6. Screening for Leishmanicidal activity

The assessment of the *in vitro* leishmanicidal activities of leaf extracts of *R. serpentina* (RS), and *C. excavata* (CE)and a molecule excavarin-A were carried out as described below following the method of Gupta et al. (2010).

5.2.6.1. *In vitro* inhibitory assay of plant extracts against promastigote proliferation

All three samples were aseptically dissolved in 1% DMSO (dimethyl sulfoxide) and diluted appropriately with the growth medium. Promastigotes from the logarithmic phase were seeded into the wells of a 96-well plate (5×10^5 cells/ml) and kept for 24, 36 and 48 hrs. This was followed by the addition of the test compounds at different dilutions (in triplicate). The standard drug SAG

was used for comparison, whereas the medium and 1% DMSO was included as negative control. After treatment with extracts, the plates were incubated for an additional 48 hrs at 26°C. Following incubation, 10 µl MTT (4 mg/ml) was added and the cells were incubated for another 4 h at 37°C. Thereafter, the plates were centrifuged at 2,000 rpm for 5 min and the supernatant was removed. The insoluble formazan produced by the parasites was dissolved in DMSO (100 µl) and the absorbance was noted at 570 nm. The absorbance is a measure of living cell amount. Leishmanicidal effect of extracts was expressed as IC_{50} values which is the concentration of the extracts which caused death of 50% *Leishmania* pathogen cells. The result was computed as an average of three replications.

5.2.6.2. Test of efficacy of crude plant leaf extracts and the purified molecule excavarin-A on intracellular survival of *Leishmania*-infected hamster macrophages

5.2.6.2.1. Isolation of macrophages

The host body macrophages are the cells where parasites reside. To test the effect of plant extracts on parasites dwelling in the cells, the host macrophages were collected from host body, cultured over coverslips, infected with parasites and studied further. For collection of macrophages from host peritoneal cavity, RPMI-1640 [GIBCO Laboratories, Grand Island, New York, USA], supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS) was used. Syrian golden hamsters were injected intraperitoneally with 2ml of sterile 4% thioglycollate. After 72 hrs, ice cold RPMI sterile medium was again injected in a volume of 10 ml (with a 20-gauge needled syringe). After gentle massage at abdominal region, the fluid from peritoneal cavity was withdrawn. This fluid was then centrifuged at 400x g for 10 minutes at 4°C. After washing the pellet twice with same media, it was resuspended in fresh RPMI 1640 supplemented with 10% FCS with a cell concentration of $5x10^5$/ml.

5.2.6.2.2. Antileishmanial activity of plant extracts on amastigotes

The isolated macrophages were cultured on coverslips (20x25 cm^2) placed in sterile disposable petri dish. For this, the macrophages were counted in a haemocytometer and the concentration was adjusted with the medium. Cell suspensions containing $2x10^5$ cells in 0.2 ml were distributed to sterile coverslips and incubated for 2 hr at 37°C. Non-adherent cells were removed by wash using same media. Now again, the adhered macrophages were overlaid by same media and incubated overnight at 37°C in 5% CO_2 incubator. On the next day, these macrophages were infected with *L. donovani* promastigote (in stationary phase) suspended in media RPMI 1640 (with FCS) at the macrophage parasite ratio of 1:10 and incubated for 5h at 37°C in 5% CO_2. After incubation, cover slips were washed with RPMI 1640 medium containing 10% FCS to remove non infected promastigotes. Plant extracts and the standard drug were then added at different concentrations and incubated for 48h.Following incubation, the cover slips were washed with media fixed with cold methanol and stained with GIEMSA to examine intracellular parasite load under microscope. The experiment was performed in triplicate and number of viable amastigotes was determined in average.

5.2.6.2.3. GIEMSA staining

Initially the stock solution of GIEMSA stain was prepared and subsequently the working solution was prepared by diluting stock solution in 1:5 ratio with doubled distilled water. The cover slips with attached macrophages were fixed with ice-cold methanol for 5 minutes and allowed to dry. Then the working GIEMSA solution was poured over the film of macrophages and kept for next 5 minutes at room temperature. After that, with a gentle flow of tap water, the stain was flushed well and slides were examined under microscope, with oil immersion lens.

5.2.7. *In vitro* cytotoxicity assay

In a separate experiment, cellular viability of hamster macrophages after treatment with test substances, were determined by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to detect

living cells which have the ability to reduce yellow MTT to a blue formazan product (Mosmann, 1983). The experiment was conducted following the method of Gupta et al. (2010). Macrophage cells were maintained in 96-well plates using RMPI 1640 containing 10% FCS AT 37°C and 5% CO_2 for 24 h. After appropriate treatment for 30 minutes at 37°C with varying concentration of experimental plant leaf extracts,10 µlMTT (0.5 mg/ml) was added and plates were incubated for 4 h. *R. serpentina* and *C. excavata* were taken in the concentrations of 10 mg/ml, 20 mg/ml, 40 mg/ml, 80 mg/ml and 100 mg/ml. Excavarin-A was taken in the concentrations of 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 2.5 mg/ml 3 mg/ml and 3 mg/ml. Next 1N hydrogen chloride–isopropanol (1:24, v/v) was added, left for 15 minutes at room temperature and optical density was then read at 570 nm on an ELISA reader. The whole experiment was performed in triplicate and result was calculated as average of three separate experiments. IC_{50} was calculated by fitting the values to non-linear curve analysis.

5.3. Results

5.3.1. *In vitro* inhibitory assay of plant extracts against promastigote proliferation

The parasites in promastigote form were treated with leaf extracts of test plants and a molecule excavarin-A in different concentrations and percentage of viable promastigotes were determined as:

(Number of live cells after treatment/initial number of live cells) X 100

The percentage of growth inhibition of promastigotes were calculated for respective concentrations of test samples.

Excavarin-A was found to be most effective in inhibiting the growth of promastigotes. With application of graded doses of excavarin-A, the percent inhibition of promastigotes increased progressively and almost linearly. Its IC_{50} value was recorded as 1.24 mg/ml (Fig.5.9), which was found to be even less than the standard drug SAG which produced an IC_{50} value of 1.42 mg/ml (Fig.5.10).*R. serpentina* was found to be the most effective among crude

extracts and next to excavarin-A. It showed a different trend, maximum progression in the percent of growth inhibition of promastigotes was observed at lower concentrations.Thereafter, the increase in the % inhibition showed a declining trend. The IC_{50} was recorded as 4.04 mg/ml (Fig: 5.7). Much more concentration of leaf extract of *C. excavata* was required to inhibit the growth of promastigotes, and the IC_{50} value was found as 32.1 mg/ml (Fig: 5.8) (Table 5.2).

5.3.2. Antileishmanial activity of plant extracts on amastigotes

Study was done to test the effect of plant extracts on parasites, dwelling in the host macrophages in the amastigote form. Effectiveness of the test substances was determined by calculating percentage of viable amastigotes within macrophages after treatment. Excavarin-A emerged as most effective botanical in inhibiting the growth of internalized amastigotes. At 4 mg /ml concentration of excavarin-A, 52% viability of amastigotes was observed (Fig: 5.13). After treatment with leaf extracts of *R. serpentina*, a gradual decline in number of viable amastigotes with increasing concentration of leaf extract was observed (Fig: 5.11). At a concentration of 40mg/ml, there was 50% growth inhibition of amastigotes (Fig: 5.12). In case of *C. excavata* leaf extract, percentage of growth inhibition of amastigotes progressed with increasing concentration but the range of concentrations required were much higher than *R. serpentina*.

5.3.3 Comparative analysis between effective doses of tested extracts against promastigotes and amastigotes

All three botanicals showed growth inhibition of promastigotes *in vitro*. IC_{50} values of *R. serpentina* and *C. excavata* leaf extracts, and excavarin-A for growth inhibition of promastigotes were 4.04 mg/ml, 32.1 mg/ml and 1.24 mg/ml respectively (Table 5.2). Percentage of viable amastigotes within macrophages was also determined after treatment with graded doses of test samples and IC_{50} value was calculated by fitting the values to nonlinear curve analysis. IC_{50} values of *R. serpentina* and *C. excavata* leaf extracts, and

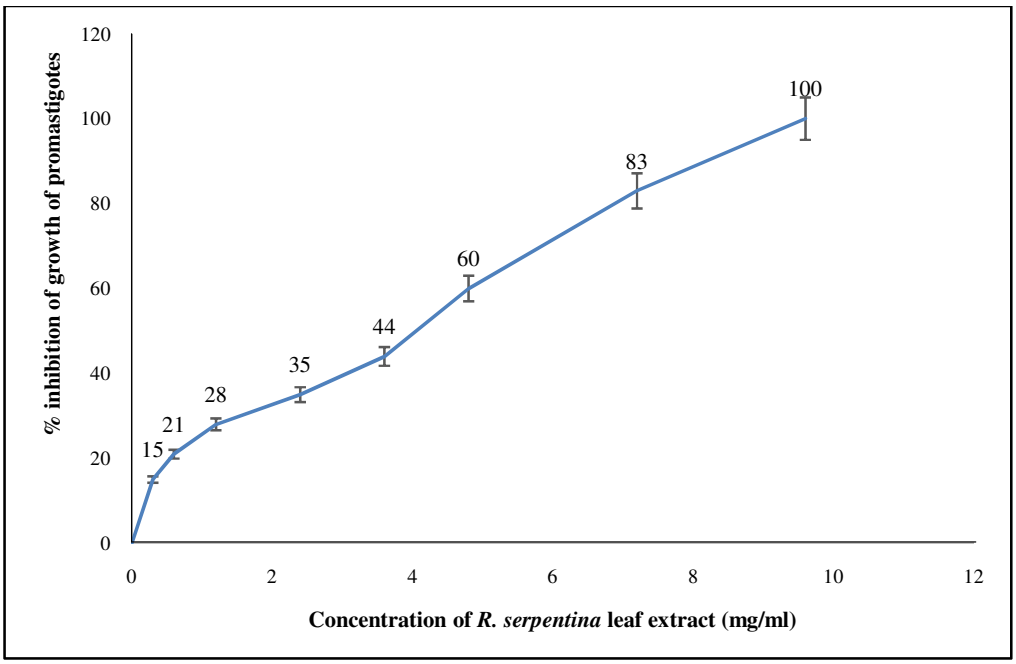

Figure: 5.7. Effect of *R. serpentina* leaf extract on growth of *Leishmania* promastigotes

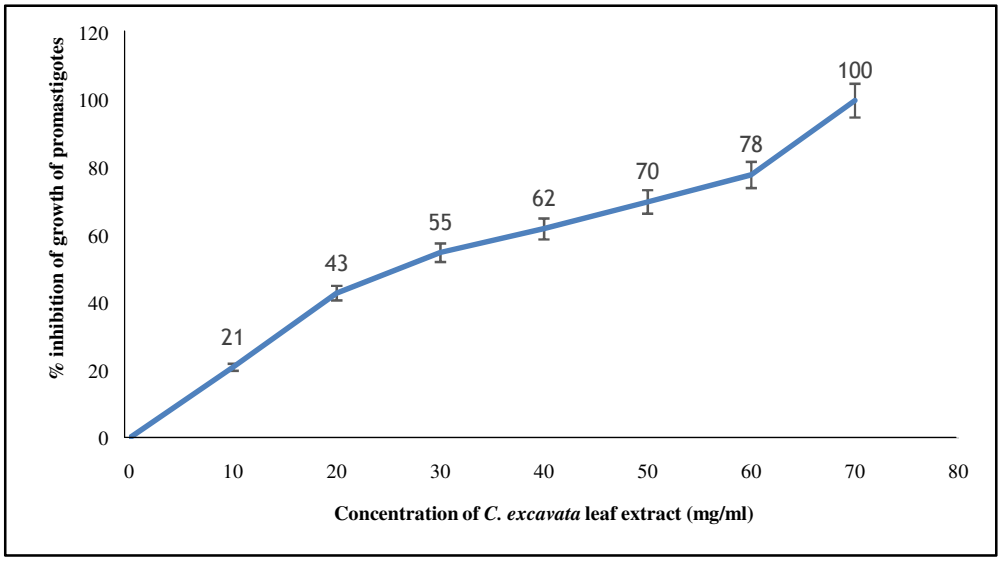

Figure: 5.8. Effect of *C. excavata* leaf extract on growth of *Leishmania* promastigotes

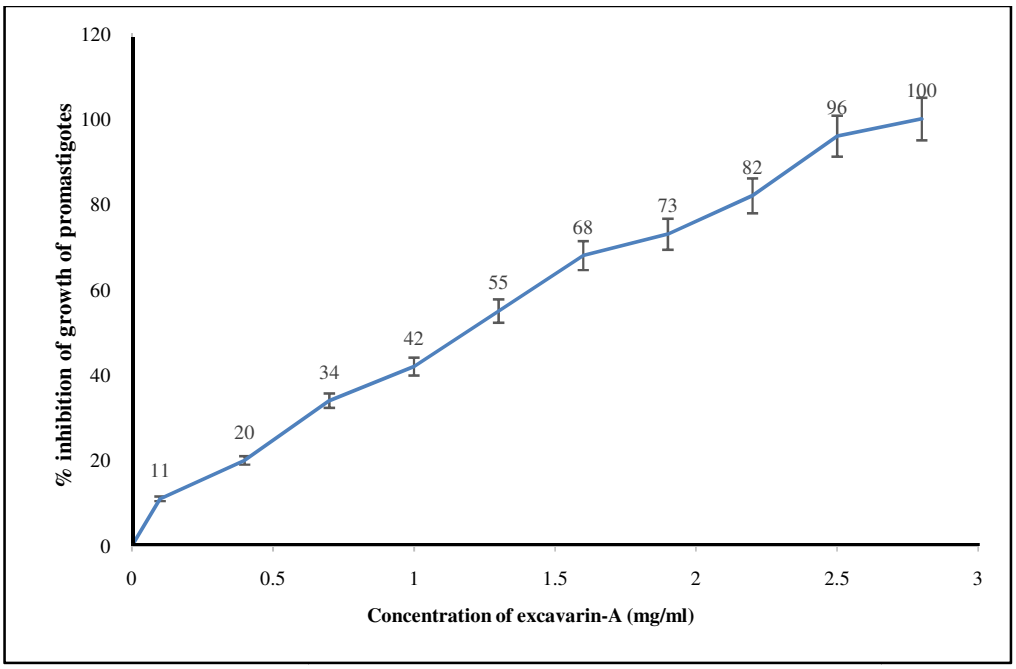

Figure: 5.9. Effect of excavarin-A purified from *C. excavata* on growth of *Leishmania* promastigotes

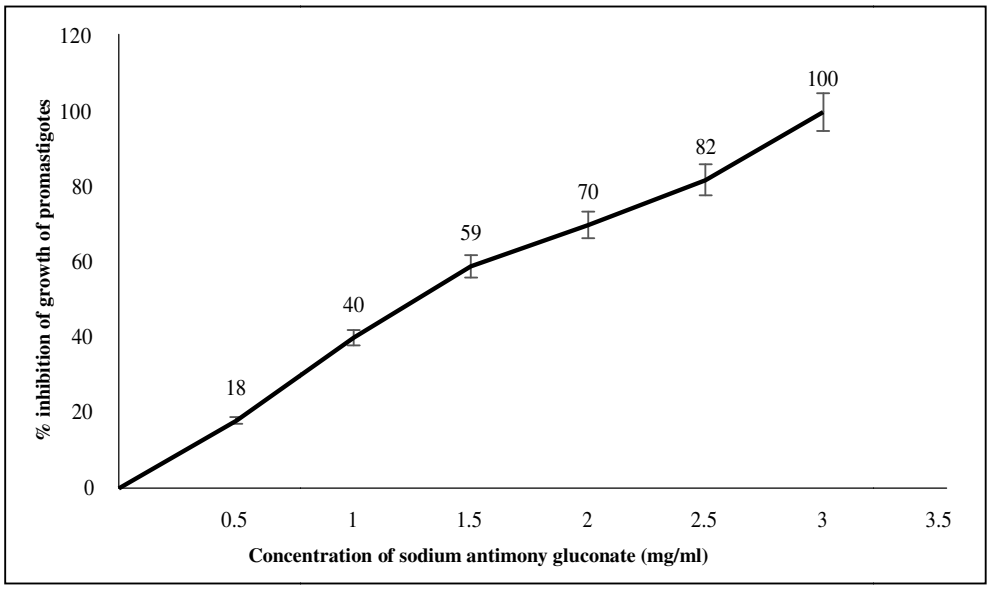

Figure: 5.10. Effect of sodium antimony gluconate (SAG) on the growth of *Leishmania* promastigotes

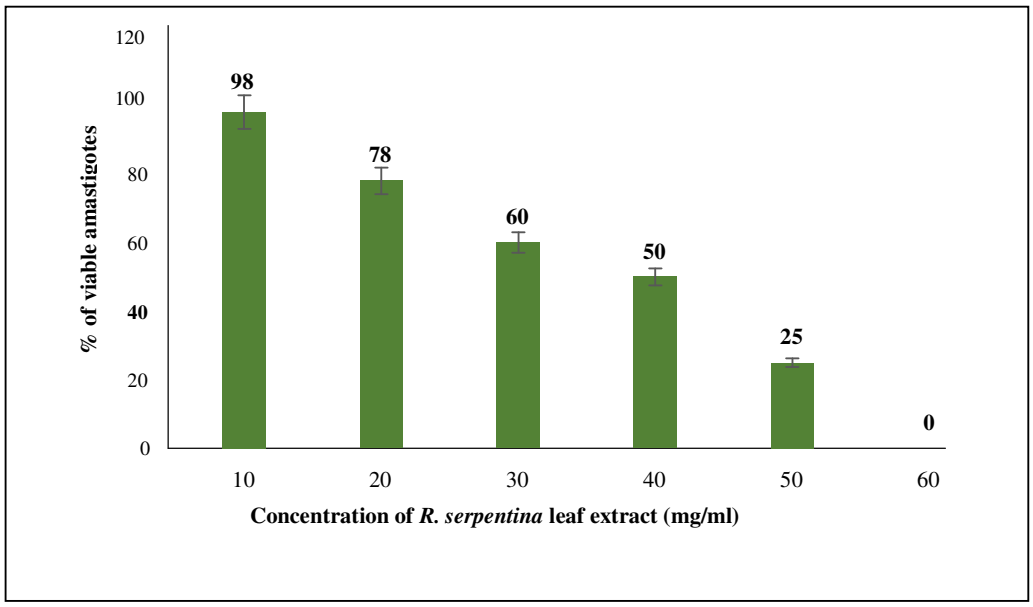

Figure: 5.11. Effect of *R. serpentina* leaf extract on growth of amastigotes within macrophages

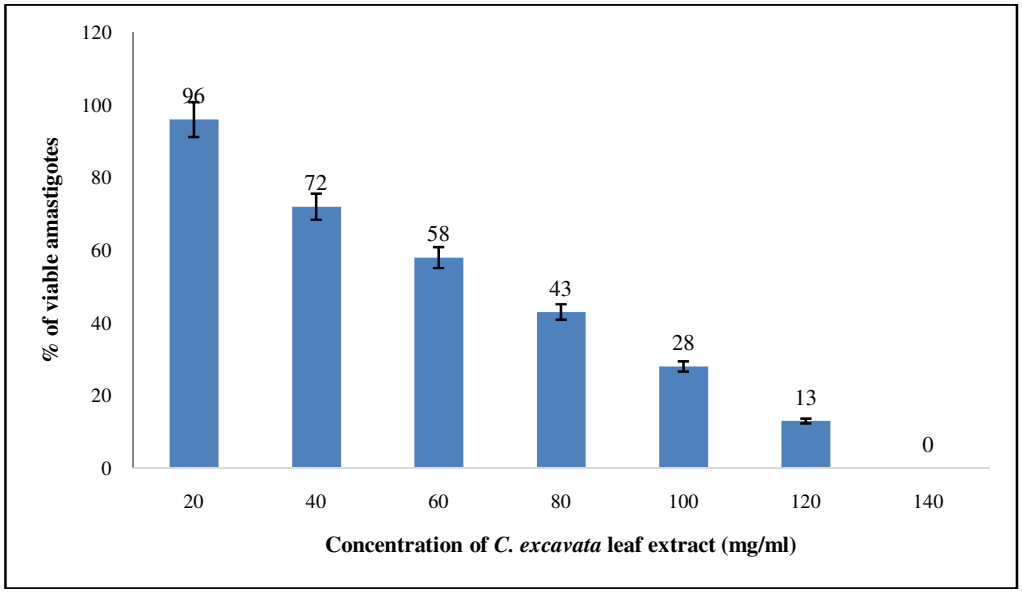

Figure: 5.12. Effect of *C. excavata* leaf extract on the growth of amastigotes within macrophages

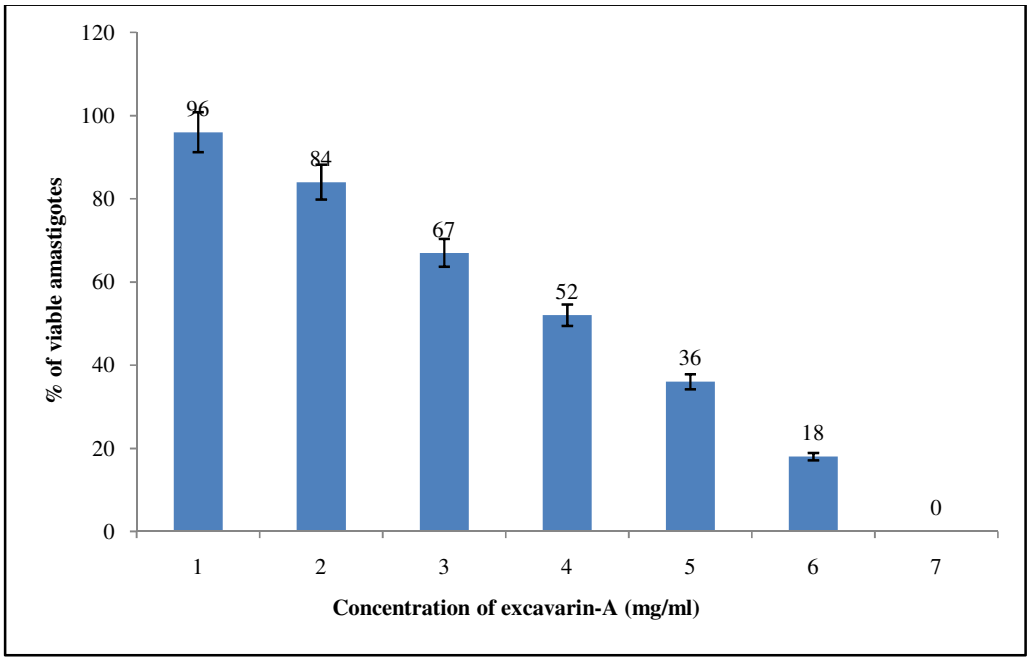

Figure: 5.13. Effect of excavarin-A on the growth of amastigotes within macrophages

excavarin-A for % of viable amastigotes were 40.0 mg/ml, 72.7 mg/ml and 4.8mg/ml respectively. It was found that concentration of plant extracts required for growth inhibition of internalized amastigotes were higher than that of promastigotes growth inhibition in case of all three test samples.

Table 5.2. Comparative analysis between effective doses of tested extracts against promastigotes and amastigotes

Plant extracts/Drug	**IC_{50} value for growth inhibition of promastigotes (mg/ml)**	**IC_{50} value for growth inhibition of amastigotes in macrophages (mg/ml)**
Excavarin-A	1.24	4.8
R. serpentina leaf extract	4.04	40
C. excavata leaf extract	32.1	72.7

5.3.4. *In vitro* cytotoxicity assay

R. serpentina and *C. excavata* were taken in a concentration range of 10 to 100 mg/ml as they were found to be antileishmanial in these range of concentrations. The coumarin molecule excavarin-A showed antileishmanial activity against promastigotes and amastigotes in the range of 0.5 mg/ml to 3mg/ml, therefore, it was tested for its cytotoxicity effect in that range of concentrations. Percentages of viable macrophages after treatment with test substances in different concentrations is shown in Fig: 5.14, 5.15 and 5.16. *R. serpentina* was found to be nontoxic at the IC_{50} value of 4.04 mg/ml (for promastigotes), and continued to show low toxicity even at much higher doses and the maximum cell viability was 52% at the highest dose of 100 mg/ml. On the other hand, *C. excavata* and excavarin-A were found to be cytotoxic to hamster macrophages in the range of effective doses.

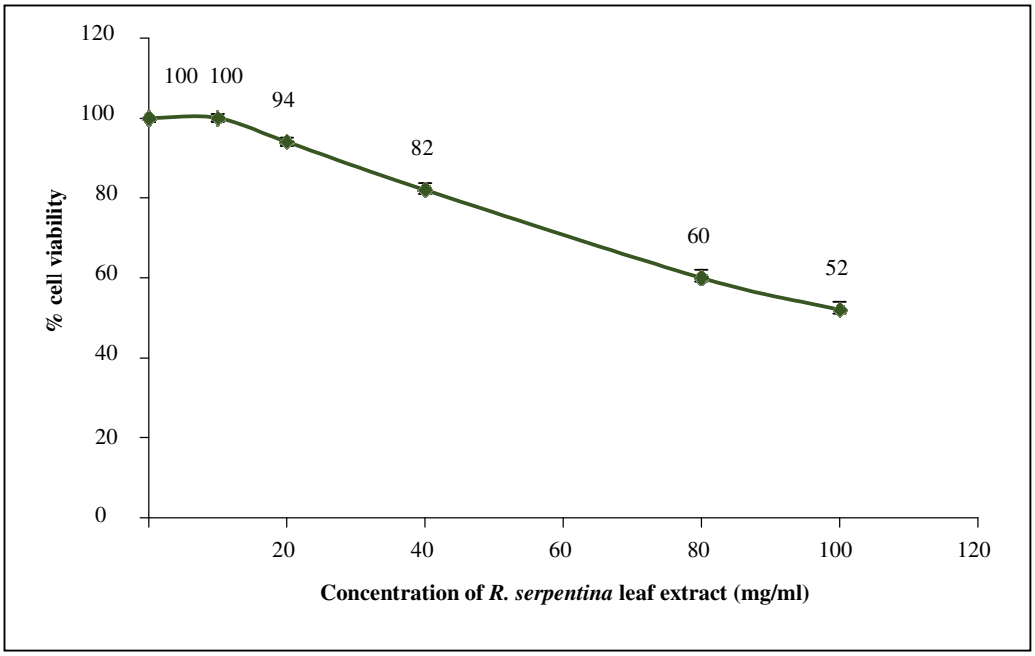

Figure: 5.14. Viability of hamster macrophages after treatment with different concentrations of *R. serpentina* leaf extract

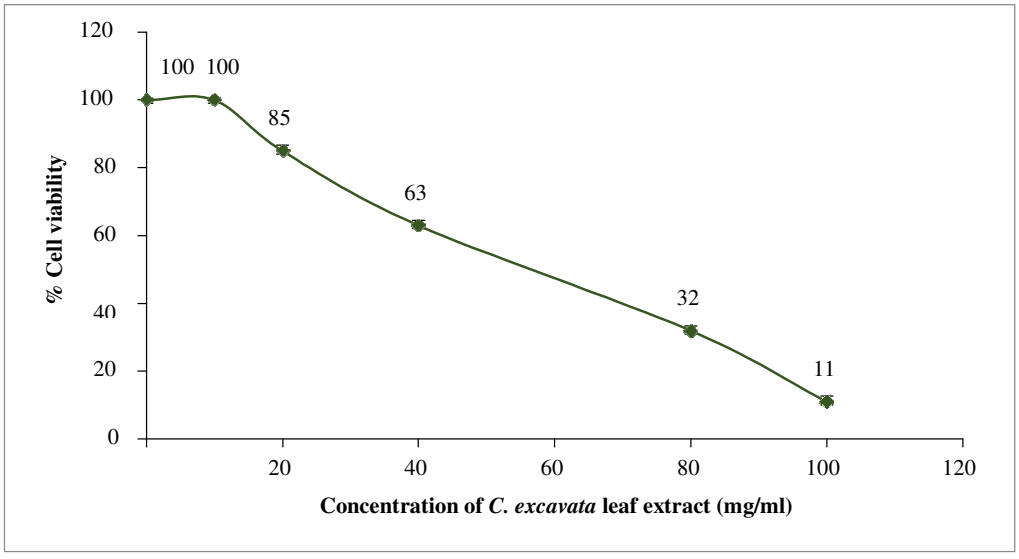

Figure: 5.15. Viability of hamster macrophages after treatment with different concentrations of *C. excavata* leaf extract

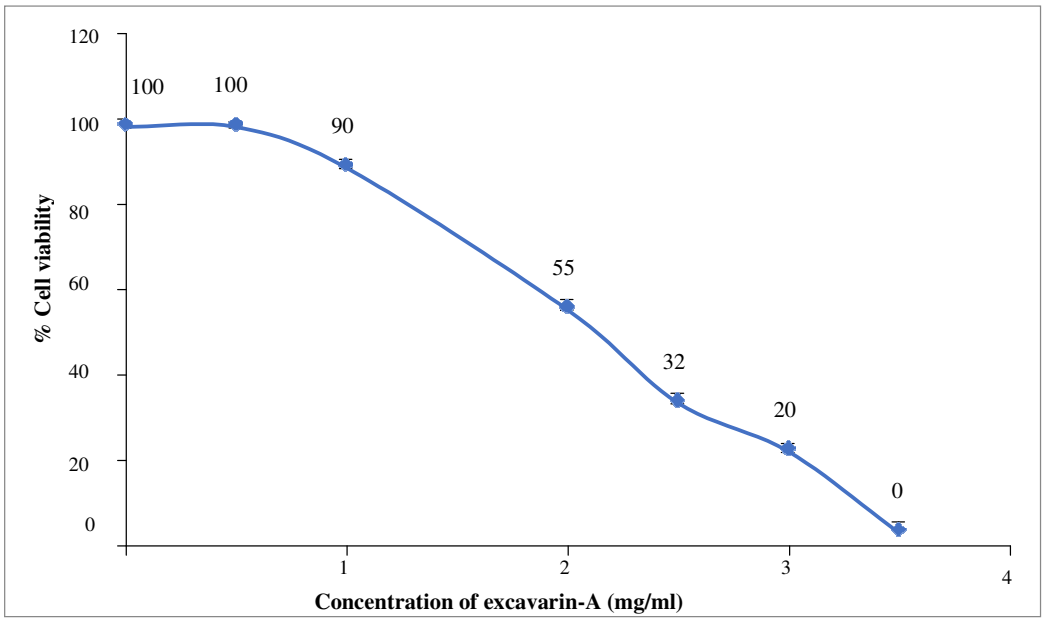

Figure: 5.16. Viability of hamster macrophages after treatment with different concentrations of excavarin-A

5.4. Discussion

The pathogen *Leishmania* sp.is capable of undergoing profound biochemical and morphological adaptations in order to complete its life cycle, to multiply and survive in different hosts. *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 defence mechanisms, its intracellular habitat offers almost complete protection. This makes the treatment for leishmaniasis complicated. The medication of first choice, the pentavalent antimonials, are toxic and administered exclusively by the parenteral route (Rocha et al., 2005; Joshi et al., 2006; Kumar et al., 2009). Second-line drugs include amphotericin B, pentamidine, miltefosine and paromomycin but limitations in terms of safety, resistance, stability and cost, low tolerability, long duration, and difficult administration, hinder its treatment. Hepatotoxicity, nephrotoxicity and cardiotoxicity, results in poor adherence to treatment, and compromises the effectiveness of therapy (Macêdo et al., 2021). Given this scenario, studies are needed that aim to identify an ideal antileishmanial agent which will have both direct and selective leishmanicidal effect.

To date many *in vitro* studies have been carried out to screen different referral plants or new ones, against *Leishmania* pathogen showing promising results (Yousefi et al.,2009; Maspi et al., 2010; Emami et al.,2012; Mahmoudvand et al.,2014; Kheirabadi et al.,2015; Bernuci et al.,2016; Vieira-Araújo et al., 2018; Badirzadeh et al., 2020). Different previous works showed 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 hepato protective (Gupta et al., 2010) activities of extracts of different parts of *R. serpentina*. Extracts of root, leaf and stem of *C. excavata* also showed different therapeutic activities previously (Sakong et al., 2011; Puongtip et al., 2011; Guntupalli et al., 2012). High content of secondary metabolites may offer good therapeutic value to both these plants

as we found in our work. In this study we screened the methanolic leaf extract of *R. serpentina* and *C. excavata* and excavarin-A for their antileishmanial activities.To the best of our knowledge, this is the first report of antileishmanial activity of *R. serpentina*.

We applied graded concentrations of methanolic leaf extract of *R. serpentina* and *C. excavata* along with a coumarin compound excavarin-A purified from *C. excavata* and a standard drug SAG against the pathogen *L. donovani*. Result showed that excavarin-A was most effective in inhibiting promastigotes and amastigotes of *L. donovani* and its activity was comparable to the standard drug included in this study. Coumarins belong to an active class of heterocyclic compound present in plant extracts, which are reported to have wide spectrum of therapeutic and biological activities like anticoagulant, antifungal, antioxidant, antitumor, antibacterial and anti-inflammatory (Rao et al.,2020). Excavarin-A was reported as a new coumarin compound by Kumar et al. (2012) with strong antifungal activity. We have also found, excavarin-A was to be a good antifungal, antileishmanial and antioxidant agent.

Many earlier reports evaluated leishmanicidal activity of plants or biomolecules on extracellular promastigotes as well as internalized amastigotes in animal macrophages, which showed that the active compounds acted differently against promastigotes and amastigotes. Compounds have direct contact with promastigotes, in contrast substances have to cross the cell membrane to reach the internalised amastigotes; moreover, amastigotes are adapted to survive in hostile intracellular environment. Thus, the promastigotes are more sensitive to active compounds (Ribeiro et al., 2014; Queiroz et al., 2014). The findings of Vieira-Araújo et al. (2018) also confirmed the fact. They isolated one molecule piperine from fruits of the plant *Piper nigrum* L. and tested for growth inhibition activities against both promastigotes and internalised amastigotes of *L. infantum*. They found lower IC_{50} values for promastigotes growth inhibition than that of amastigotes. Few more previous works concluded with similar findings (Monzote et al., 2010;

Bernuci et al., 2016) and ourresults are also in agreement with those observations. In each case, the concentration of substances needed for inhibition of amastigotes is higher than that of promastigotes.

Before recommending any natural product for therapeutic purposes, it is necessary to test whether the substance is cytotoxic for the animal cell in the limit of its effective concentration. Very frequently it is seen that several plant extracts give outstanding inhibitory effect against pathogens or has potentiality to cure diseased conditions, but it is cytotoxic to the living tissues which may cause immediate or long-term side effects. So, the samples were tested for viability of hamster macrophages in different doses. In a number of old and recent studies, cytotoxic activities of extracts of different plant or plant parts were tested on different cell lines and the inhibition of cell growth or cell proliferative activities were tested by MTT assay (Horiuchi et al., 1988; Hashemi et al., 2011; Rahman et al., 2014). In the present study, the crude extracts of *R. serpentina*, *C. excavata* and excavarin-A were checked for cytotoxicity by MTT assay. The macrophages were isolated from hamster peritoneal cavity, and tested with samples of plant leaf extracts in different concentrations. In a similar study, cytotoxicity test was performed on BLBC mice macrophage cell using MTT and the leaf extract of the plant *U. dioica* was found to be toxic at higher concentration (Badirzadeh et al., 2020). *C. excavata* leaf extract and excavarin-A showed good antileishmanial activity, however they were found to be cytotoxic within effective therapeutic ranges. But *R. serpentina* leaf extract was not found to be cytotoxic in cell viability test in its effective range of doses. At a higher concentration of 100mg/ml, which is much far from its therapeutic range, *R. serpentina* showed 52% viable cells when tested on host macrophages.

From the present work, it was evident that, crude leaf extract of *R. serpentina* had significant antileishmanial activity *in vitro*, and showed no cytotoxic effect in its therapeutic ranges of concentrations. Further study on animal model has been done with *R. serpentina* extract to explore its antileishmanial activity in

systematic environment. The *C. excavata* extract and excavarin-A were not considered for *in vivo* tests due to their cytotoxic activity observed in this study.

6.1. Literature review

In the humid and wet weather conditions of North Bengal, fungal infection is very common among local people. In general, fungal infections are the most common cause of many skin diseases in most of the developing countries. Opportunistic fungal infections, mainly resulting from the species of *Candida*, *Cryptococcus* and *Aspergillus* are life-threatening in immuno-compromised patients especially when affected with AIDS, cancer, or organ transplant. Resistance of many pathogens towards antifungal drugs is also a major factor that limits appropriate treatment. Therefore, it is necessary to search for more effective and less toxic novel antifungal agents from natural sources.

C. albicans is the most common causative agent of human infection among all *Candida* species, where infection ranges from mucosal surface to systemic organ (Miceli et al., 2011). Previous studies have shown that some plant extracts possess significant antifungal activities against different strains of *Candida* sp. but all are mainly screened *in vitro* (Soliman et al., 2017; Neto et al., 2017). The preliminary stage of clinical investigation for identification of biomolecules having antifungal activity is *in vitro* testing of natural materials which are mainly crude botanical extracts. However, this is not enough to invent new line of drugs because *in vitro* studies do not consider the individuals body system, organic metabolism or idiosyncrasies. Very few studies show antifungal activities of medicinal plant extracts on animal models. The latest advancements have focused primarily on mechanisms of action of natural compounds against opportunistic fungal pathogens at the genomic, molecular or proteomic levels (Martins et al., 2015).

6.1.1. *In vivo* evaluation of antifungal activity against candidiasis

In recent past, several studies on *in vivo* antifungal activities of medicinal plants evaluated on plant pathogens have been reported (Seepe et al., 2020; Dhar Purkayastha et al., 2018); but *in vivo* study on fungal pathogen of clinical importance still remains scarce. In this aspect, most of the *in-vivo* studies are concerned with candidiasis as this is the most frequently encountered fungal disease (Garber 2001). In a search for new antifungal treatments, the

mammalian animal models play a very important role for observing *in-vivo* efficacies. While mice are the most widely used models (Scorzoni et al. 2016), the ultimate choice of animal species along with the route of inoculation of the organism depends on the desired goal. In this review, the findings of few such studies are deliberated.

Zhang et al. (2005) studied the antifungal activity of eight steroid saponins isolated from n-butanol extract of *Tribulus terrestris* against six fluconazole resistant yeasts. *In vivo* testing of the purified saponin TTS-12, which showed maximum efficacy in preliminary tests was performed by the authors in a vaginal infection model, oestrogen-dependent rat vaginitis, developed with fluconazole-resistant *C. albicans*. The results showed remarkable therapeutic effect on candidal vaginitis upon vaginal administration of TTS-12. In another *in vivo* study, methanolic extract of *Syngonanthus nitens* scapes was tested in animal model (rat) infected by different *Candida* strains collected from patients with valvovaginal candidiasis. The treatment with cream containing the extract at different doses showed rapid clearance and eradication of vaginal fungal burden in experimental animals after 8 days of treatment (Araújo et al., 2013). Fabri et al. (2021) tested the antifungal activity of spilanthol, a bioactive alkylamide from the native Amazon plant species, *Acmella oleracea*. *In vivo* evaluation in an experimental vulvovaginal candidiasis model showed that infected rats treated with spilanthol recorded steady reduction of fungal burden when compared to the untreated animals.

The antifungal activity of aqueous extract of *Nigella sativa* seeds was studied on candidiasis in mice (Khan et al. 2003). Treatment of mice that were injected intravenously with *C. albicans* with the plant extract resulted in considerable inhibitory effect on the growth of the organism at the level of 5-fold decrease in cfu in kidneys, 8-fold in liver and 11-fold in spleen. In another study,antifungal activities of acetone extracts of leaves from *Combretum* species were tested against *C. albicans* and other pathogenic fungi associated with wound infections on immunocompromised Wistar rats (Masoko et al., 2010). The authors made wounds on the back of the rats and treatments were

administered topically using the extracts at 20% concentrations in aqueous cream. Their results showed that in the treated group, wound healing was evident as a rigid, dark, and thick crust formation after three days, but the lesion without treatment took longer time to heal. In a similar study, Sahgal et al. (2011) used crude methanolic seed extract of *Swietenia mahogany* against *C. albicans*. Here, experimental mice were infected with *C. albicans* suspension via injection at lateral tail vein and subsequently treated with 2.5 g/kg of seed extract by intraperitoneal injection after 24 hours. Their results showed significant reduction of the pathogen counts in both blood and kidney when compared to control group. In histopathological observations, the authors found that the treated kidney failed to exhibit the presence of either *C. albicans* or pseudomycelia. Similar study was performed by Jothy et al. (2012) with methanolic seed extract of the plant *Cassia fistula*. Mice were injected with *C. albicans* via intravenous route and treatment was performed with seed extract at 2.5 g/kg body weight administered intraperitoneally once daily for 3 days. Results revealed a six fold decrease in *C. albicans* burden in kidney and blood sample in the curative group mice when compared with those of the control group.

Dzoyem et al. (2014) studied the antifungal activity of methanol-dichloromethane extracts of twenty- one spices used in Cameroon. *In vivo* activity of *Olax subscorpioidea* extract which exhibited maximum activity during *in vitro* evaluation was tested in rat model of disseminated candidiasis caused by *Candida albicans*. The results revealed that oral administration to artificially infected rats at the dose of 2 g/kg of body weight recorded a significant reduction of cfu of *C. albicans* cells in the blood while a moderate decrease was observed in the kidney.

6.1.2. *In vivo* evaluation of antifungal activity against other superficial infections

Superficial fungal infections are found in the outermost layers of the skin, nails, hair and mucous membranes. There has been an increase in the incidence of these infections, mainly because of the increasing number of immunocompromised patients (Garber, 2001). The most common types of skin

diseases are caused by dermatophytes such as *Trichophyton* sp., *Microsporum* sp. and *Epidermophyton* spp. although non-dermatophyte yeasts such as *C. albicans* and other non-albicans *Candida* sp. along with the moulds, *Aspergillus* sp., *Fusarium* spp., *Acremonium* sp., *Scopulariopsis* sp. and *Scytalidium* sp. are also reported as causative agents in a growing number of cases (Tosti et al., 2000).

One of the past studies screened 50% ethanolic leaf extract of *Leptadenia reticulata* for antifungal activity against *Aspergillus flavus*. Here, wounds were made surgically in male albino rats on the posterior mid dorsal side of the animal and fresh spore suspension of *A. flavus* was introduced in the wounds by injection as a single dose. Treatment was done by application of cotton swab soaked in the plant extract on the wound at regular intervals of twice a day for 8 days which showed visible healing sign and the swab made from the healing wound region showed absence of the fungal hyphae (Sureshkumar, 2008).

Li et al. (2015) studied the antifungal activity of ellagic acid purified from the plant *Euphorbia humifusa*. Dermatophytosis was induced in guinea-pig infection model using suspension of *Trichophyton rubrum* as inoculum. Subsequently, the test compound was administered topically mixed with an ointment base once a day for 14 days. The treatment significantly enhanced the cure rate at all tested doses and microscopic examinations for the pathogen in the treated animals yielded negative result.

Garrido et al. (2015) tested efficacy of five Mexican traditional medicinal plants against human superficial mycoses causing fungus *in vivo*. Dermatophytosis was induced with *Trichophyton rubrum* in the sole of feet in mice to develop the tinea pedis model and subsequently from day 14 post-infection, the mice were treated intragastrically daily for seven days with 2.5 and 5 mg/kg of acetone extract of *Berberis hartwegiior* and *Zanthoxylum caribaeum* respectively. After 7 days of treatment with *B. hartwegii,* an improvement of100 with no signs of disease was observed. *Z. caribaeum treated* mice also showed significant improvement of disease condition.

In the present study, methanolic leaf extracts of *R. serpentina* and *M. oleifera* was screened for antifungal activities *in vivo* using rats as model animal after getting significant results in the *in vitro* experiments with the same leaf extracts against *C. albicans*.

6.2. Materials and methods

6.2.1. Preparation of plant extracts

Leaf extract of plants were prepared following the procedure described in previous section (Section 3.2.1).

6.2.2. Effect of plant extracts *in vivo*

For *in vivo* experiment, male albino rats of similar weight (80-100 gm) and age groups (10-12 months) were taken. Altogether 15 animals divided into 3 groups were infected with *C. albicans*. Of these five infected rats were untreated and marked as control group. Rest of the 10 animals were treated with plant extracts.

6.2.2.1. Infection with *C. albicans*

A wound of skin depth (round, 1 cm diameter) was made with sterilized surgical blade at mid posterior dorsal side of each animal. The test fungal strain *C. albicans* was cultured in PDA medium and a cell suspension of$1x10^5$ cells/ml was used as inoculum. The prepared solution was applied superficially on wounds on the skin of test animals with cotton swab at a dose of 1ml in each case. It took 2 days to develop skin infection at the site of application of fungal spore suspension (Sureshkumar, 2008). The animal ethics committee of the institute (IICB, Jadavpur, Kolkata) approved all animal experiments.

6.2.2.2. Treatment with plant extract

Ten infected animals were treated with diluted plant extracts superficially on the wounds. One milligram of crude extract was dissolved in minimum volume of physiological saline (0.9% NaCl) and applied over the wound of each infected animal. Five infected rats were treated with *R. serpentina* leaf extract and remaining five were treated by *M. oleifera* leaf extract. This process was repeated in regular intervals of twice a day for next 8 days. The whole

experiment was performed thrice. All the animals were kept in uniform diet and environment throughout the period of experiment.

6.2.3. Recovery of pathogen from test animals

After 8 days of treatment with plant extracts, fungal loads on the wounds were estimated by standard Lacto-Phenol Cotton Blue (LPCB) and Gram staining methods. For this, sample was recovered from the dry wound using a sterile swab from both treated and control groups and smeared on the microscopic slides. The slides were then stained and finally the stained samples were observed under the microscope (Olympus).

For LPCB staining, one or two drops of the stain was added to cover the smear. One edge of the drop of stain was touched with a cover slip edge, and lowered gently, avoiding air bubbles to cover the liquid. The preparation was thus ready for examination (Procop, 2017).

Although Gram stain is useful in staining bacteria, certain fungi such as *Candida* and *Cryptococcus* are observed to be Gram positive yeasts. For Gram staining fungal strain, the smear collected from the wound was spread on glass slide and fixed and dried by rapidly passing the slide through the flame of spirit lamp (keeping the smear uppermost). The fixed smears were then covered with crystal violet stain for 30-60 seconds. The stain was rapidly washed off with clean water, and the smear was covered with Lugol's iodine for 30-60 seconds to form crystal violet-iodine complex. Next, iodine was washed off with clean water and decolorized rapidly (within few seconds) with acetone-alcohol, and again washed immediately with clean water. At last, the smear was counterstained with Safranin solution for 30 seconds. The stain was then washed off with clean water and examined under microscope (Procop, 2017).

6.2.4. Analysis of blood sample of test animals

After the period of treatment, on 9^{th} day of experiment, blood samples were collected from both control and treated rats. The blood samples were collected on glass vial containing EDTA (anticoagulant) and the total count (TC) of the leukocytes was determined. For finding the total leukocyte count,

a sample of blood was diluted with Turk's fluid (1.5 ml glacial acetic acid, 1.5 ml of 1% gentian violet solution and 100 ml distilled water) which destroyed the RBC and stained the nuclei of the leukocytes to make them visible. Initially 1 ml of Turk's fluid was taken in a watch glass, the counting chamber was placed on a microscope stage containing 16 WBC squares. A WBC pipette was filled with the blood sample up to the mark 0.5 and Turk's fluid was sucked to the mark of 11, and the blood and fluid were mixed thoroughly. That gave the dilution of ratio 1:20. In another pipette, blood was drawn up to mark of 1 followed by Turk's fluid up to mark of 11. After discarding first two drops of fluid from the pipette, the chambers on its both sides were charged. The cells were allowed to settle for the next 3 to 4 minutes and the chambers were transferred under the microscope, first under low magnification and then switched to high magnification. The leukocytes were seen as round shaped cells with clear unstained cytoplasm and deep blue-violet nuclei. WBCs were counted in six squares marked in the counting chamber, where cells were in four groups of sixteen squares, i.e., in a total of 64 squares. In a counting chamber, the volume of a square is 1/160 mm^3, therefore the volume of 64 squares is 4/10 mm^3. Thus, the total volume of diluted blood, in which WBCs were counted, was 4/10 mm^3. By multiplying the number of WBC count by 10/4, the WBC count in 1 mm^3 diluted blood was found. The blood was diluted 20 times, so 1 mm^3 of undiluted blood from the first pipette contained A x 10/4 x 20=A x 50 WBCs (A=WBC count). In case of second pipette, it was A x 10/4 x 10=A x 25. Two counts were taken for comparison and confirming the accuracy (Procop, 2017).

6.2.5. Data analysis

Total count of WBC (mean $\pm$ SD) were analysed by student "t" test.

6.3. Results

The wounds caused by the infection of *C. albicans* were treated with leaf extracts of *R. serpentina* and *M. oleifera*; this caused healing of infection which was reflected by the reduction of fungal load in the samples collected from treated rats. The observation was confirmed by LPCB and Gram staining

methods. On the other hand, in infected but untreated rats, a significant presence of *C. albicans* was observed in samples collected from the wound after 8 days (Table 6.1).

The process of recovery from infection is faster in *R. serpentina* treated animals in comparison to *M. oleifera* treated group. On the fourth day of treatment with *R. serpentina* leaf extract, the scar on skin of the experimental animal totally disappeared; but in case of *M. oleifera,* few signs of infection were still present (Fig: 6.1). On the eighth day of treatment, the body fur was regained at the site of the wound in *R. serpentina* treated group, but very little skin hair was observed in case of *M. oleifera* treated animals.

In both *R. serpentina* and *M. oleifera* treated rats, there was a significant increase in mean WBC count in comparison to untreated group indicating that both the plant leaf extracts have a protective role in reducing fungal burden in the animals. (Table: 6.2).

Table: 6.1. Treatment of skin lesion induced by *C. albicans* using plant extracts.

Treatment	Occurrence of *C. albicans* at different days during treatment			Nature of wound at different days during treatment		
	0 d	**4 d**	**8 d**	**0 d**	**4 d**	**8 d**
untreated control	+++	+++	+++	Severe	Severe	Severe
Infected and treated with *R.serpentina* leaf extract	+++	+	-	Severe	Superficial	Absent
Infected and treated with *M. oleifera* leaf extract	+++	++	+	Severe	Moderate	Superficial

+++ High occurrence, ++Moderate occurrence, +Low occurrence, -Absence

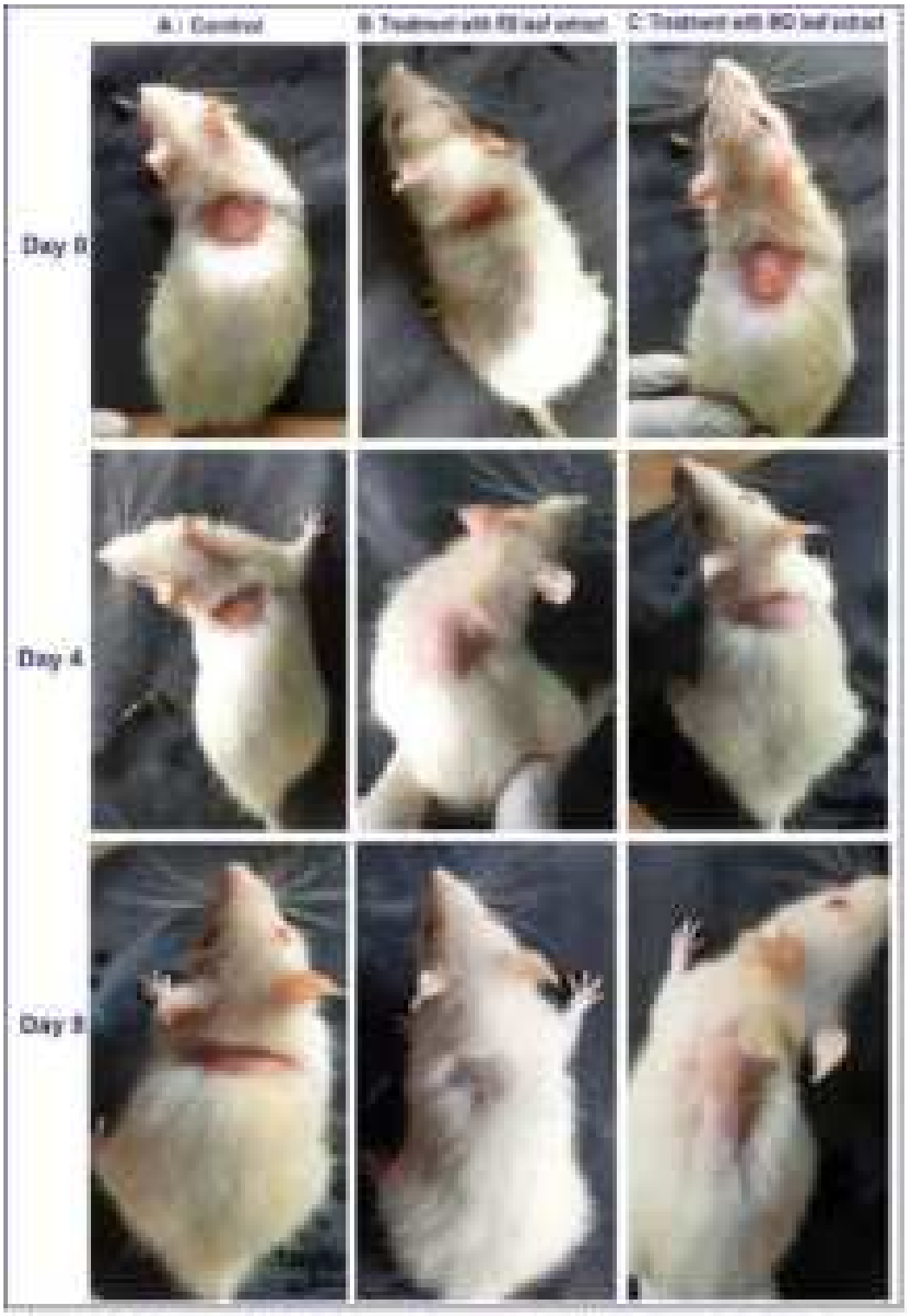

Figure: 6.1. Effect of plant extracts on superficial infections by *C.albicans* on experimental animals: A. Control group experimentally infected with C.albicans. B. Infected animals treated with leaf extracts of *R. serpentina* (RS) and C. Infected animals treated with leaf extracts of *M. oleifera* (MO)

Table: 6.2. Total leukocyte count in blood samples of experimental rats infected with *C. albicans* and treated with plant extracts.

Sample	White blood cell count[#] Cells/mm^3
Infected but untreated rat	7634±141
Infected rats treated with *R. serpentina* leaf extract	8735±130
Infected rats treated with *M. oleifera* leaf extract	8642±122

[#] (average no. in 5 rats± SD)

6.4. Discussion

As eukaryotic pathogens, human fungal diseases differ from other infections fundamentally. Fungi share many similarities with their host cells, this impairs the activity of antifungal compounds. A single fungal pathogen can infect a wide range of cell types and multiple tissues in the same patient especially if the host is immune compromised in any way (Rodrigues and Nosanchuk, 2020).The Global Action Fund for Fungal Infections has highlighted the devastating impact of focal fungal diseases in patients in advanced stage of AIDS(GAFFI,2018). In addition, patients on anticancer therapies and other immunosuppressive medications are affected, which have contributed to the excess number of deaths due to fungal diseases worldwide. The Center for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have emphasized on this fact in their reports (CDC, 2017; WHO, 2020b). Despite this fact, fungal diseases have been continually neglected over the years and persistence of its neglected status is caused by failures in science, market, and public health. The financial support for fungal disease research is incredibly low, drug development field is progressing at a very slow pace. Classical antifungal drugs Amphotericin ß discovered in 1955, still remain the 1st line medication for fungal infection. The affordable conventional formulations include significant side effects. On the other hand, most effective and least toxic formulation like liposomal Amß, is available at a very high cost.

As the deadliest fungal infections affect mainly the neglected and poorest populations of the world, it faces market failure in the form of lack of interest from the pharmaceutical sector in the development of medicines, vaccines, and diagnostic tests for human fungal diseases or for drug commercialization (Rodrigues and Nosanchuk, 2020). In this scenario, it is the need of the hour to unturn the rubble from natural resources in search of new therapeutic formulation which is effective, low cost and devoid of any side effect.

In the last two decades, phytotherapeutic agents derived from natural resources with antimicrobial and antifungal potentiality, has grabbed the attention of scientific researchers (Martins et al., 2014). Various *in vitro* studies have been conducted to screen the antifungal activities of different medicinal plants. But plant-derived bioactive molecules show synergic, antagonistic and polyvalent relationship when they enter into human body. Their mode of action, structural and functional conformations are also modulated due to organic metabolism or in complex environment of the host body (Martins et al., 2015). Thus, *in vitro* screening in laboratory set up is not enough to establish the activity of any plant extract or any biomolecule derived from medicinal plants; and so, *in vivo* screening and clinical trials are needed (Sealbert et al., 2000; Alviano et al., 2009). Some studies are reported in past showing antifungal activities of medicinal plants *in vivo* (Masoko et al., 2010; Araújo et al., 2013; Sahgal et al., 2011; Jothy et al., 2012; Garrido et al., 2015). The present study intended to further extend the positive findings of *in vitro* screening in animal model.

A very simple *in vivo* experiment was designed where skin infection was developed in experimental animals with *C. albicans,* and then effectiveness of the plant extracts was observed by superficial application. Although the study was very basic in nature, it showed visible results. Superficial infections on experimental animals (male albino rats) were treated with diluted methanolic leaf extract of *R. serpentina* and *M. oleifera*, twice a day for the next 8 days, and the control group remained untreated. Standard LPCB and Gram staining showed significant recovery which were also visible prominently with the

naked eye. *R. serpentina* showed better healing in comparison to *M. oleifera,* but both the plant extracts reduced skin infections when compared to the untreated group. On the fourth day of treatment, the scar completely faded out in *R. serpentina* treated animals, and on 8th day the skin hairs reappeared. In case of *M. oleifera* treated group of animals, on the 8th day of treatment, the scar disappeared totally but very little amount of skin hair was observed at the site of wound. Haematological analysis of blood samples from animals of both treated and untreated groups showed significant results.

Total count of WBC had increased in both cases of treated animals. This finding was in agreement with previous work described by Suresh Kumar (2008). As a part of host's innate immune system, neutrophil, leukocytes or polymorphonuclear leukocytes (PMNLs) are known to function as major phagocytic cells, which cause elimination of the fungi during fungal infection (Demirezen et al., 2015). The elevation in total count of WBC which was found by haematological analysis of blood samples of treated animals, suggests that the leaf extracts of both the plants have protective roles in improving host defence to counter fungal attack. From the findings of the present work, it is evident that methanolic leaf extracts of *R. serpentina* and *M. oleifera* possess antifungal activities against *C. albicans*. The active principles responsible for the antifungal activities, and their mode of action may be explored through further research.

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.

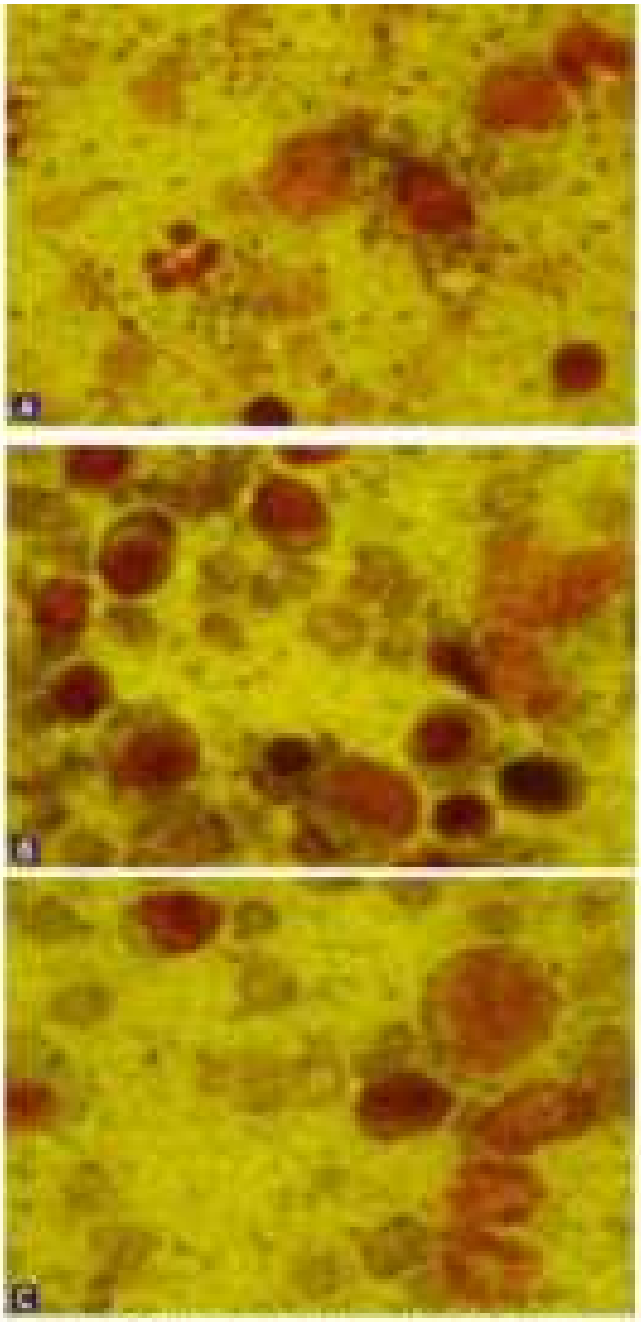

Figure: 7.1. Giemsa stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in liver. Black spots within the macrophage indicate amastigotes (arrows). [A] Untreated Leishmania infected macrophage. [B] Leishmania infected macrophages treated with 50 mg/kg body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

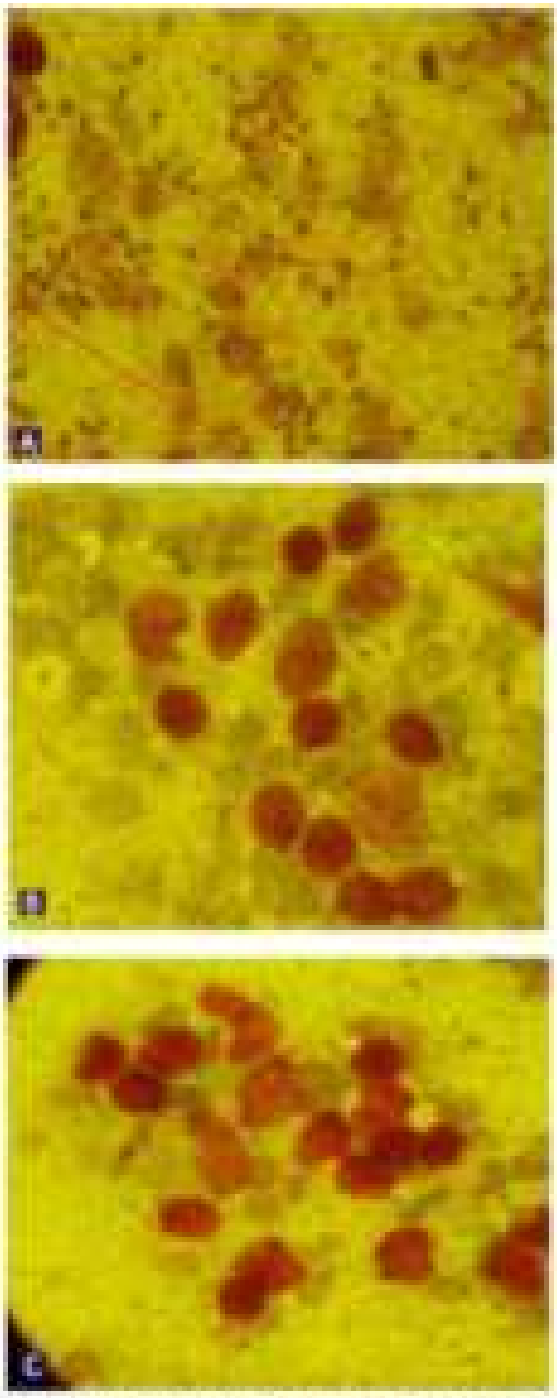

Figure: 7.2. GIEMSA-stained micrographs showing effect of *R. serpentina* leaf extracts on intracellular amastigotes in spleen. Black spots within the macrophage indicate amastigotes (arrow). [A] Untreated Leishmania infected macrophage. [B] Leishmania infected macrophages treated with 50 mg/kg body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

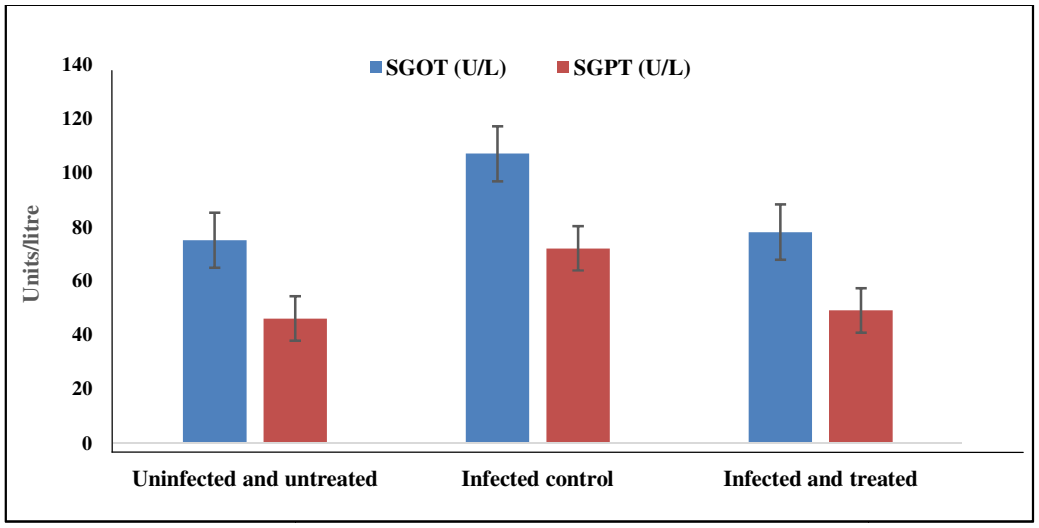

Figure: 7.3. Effect of *R. serpentina* on serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels (units/litre) in *Leishmania* infected hamsters.

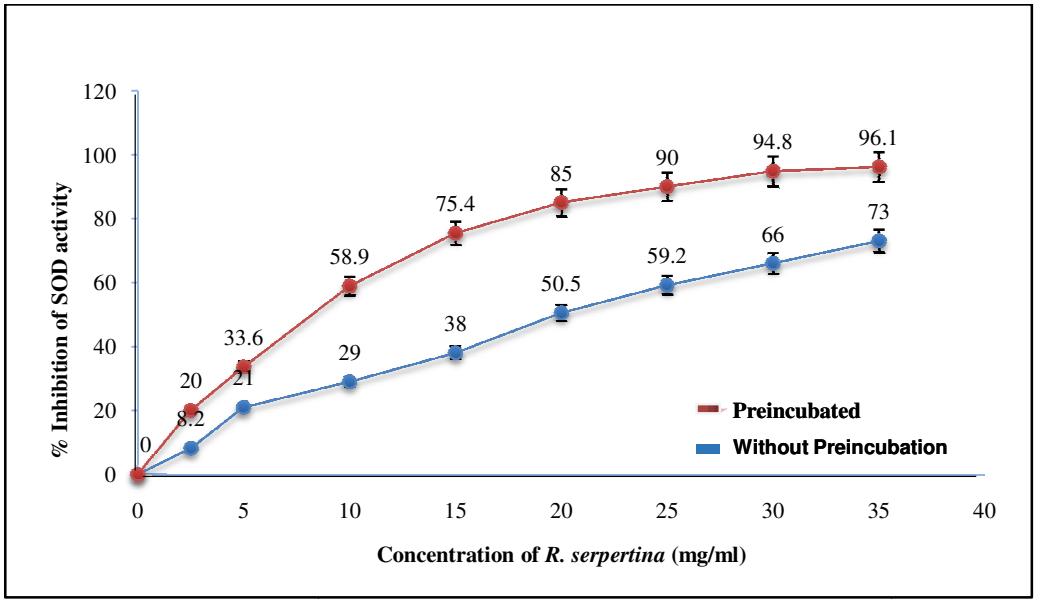

Figure: 7.4. Leishmanial SOD activity when enzyme (promastigote lysate) and *R. serpentina* leaf extracts were added to the reaction mixture either simultaneously (▬) or after preincubation for 30 minutes (▬)

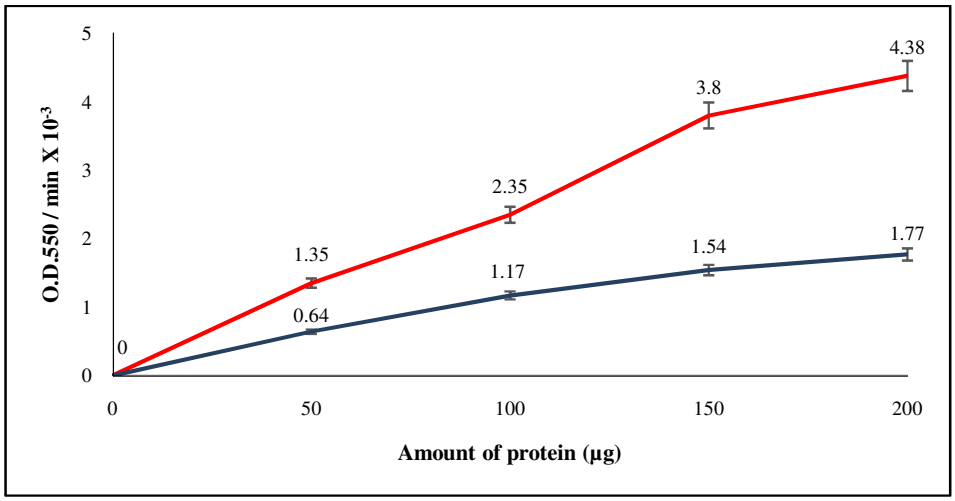

Figure: 7.5. Rate of superoxide radical release before (▬) and after (▬) treatment of *Leishmania* pathogen by *R. serpentina*. Formation of blue formazan derived from reduced nitroblue tetrazolium in the presence of superoxide radical was measured spectrophotometrically.