Insights into redox metabolism of *Leishmania* parasite: Studies on key enzymes for novel drug candidates

A Thesis Submitted

By

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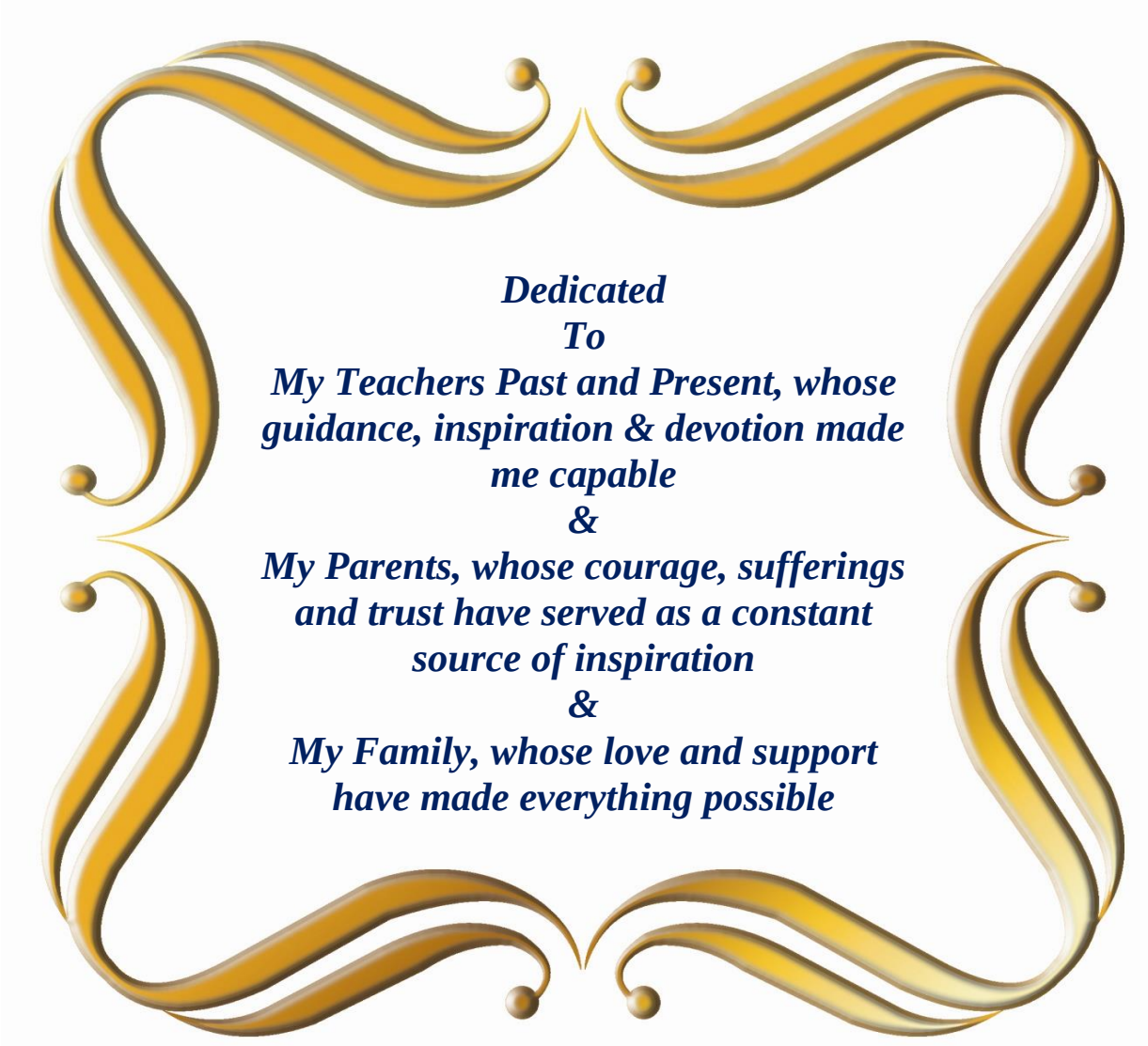
Dedicated
To
My Teachers Past and Present, whose guidance, inspiration & devotion made me capable
&
My Parents, whose courage, sufferings and trust have served as a constant source of inspiration
&
My Family, whose love and support have made everything possible

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

I hereby declare that the matter embodied in this thesis entitled "**Insights into redox metabolism of *Leishmania* parasite: Studies on key enzymes for novel drug candidates**" is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Dr. Vikash Kumar Dubey** (Supervisor) and **Dr. Rakhi Chaturvedi** (Co-supervisor).

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

Prakash Saudagar

August, 2013

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DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled "**Insights into redox metabolism of *Leishmania* parasite: Studies on key enzymes for novel drug candidates**" by **Mr. Prakash Saudagar (**Roll No: 09610619), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under our supervision at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for any kind of degree.

Dr. Vikash Kumar Dubey
(Supervisor)

Dr. Rakhi Chaturvedi
(Co-supervisor)

Acknowledgement

YOU OWE THE WORLD AND NOT THE OTHER WAY AROUND

- Jenny Holzer

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Abbreviations

ATP	:	Adenosine-5'-triphosphate
BLAST	:	Basic Local Alignment Search Tool
BET	:	Betulin
CL	:	Cutaneous leishmaniasis
CNT	:	Carbon Nanotube
DMEM	:	Dulbecco's Modified EagleMedium
DMSO	:	Dimethyl Sulfoxide
DNA	:	Deoxyribonucleic Acid
DTNB	:	5,5-dithiobis-(2- nitrobenzoic acid)
DTT	:	Dithiothreitol
EDTA	:	Ethylene Diamine tetra Acetic Acid
FBS	:	Fetal Bovine Serum
FESEM	:	Field Emission Scanning Electron Microscopy
FTIR	:	Fourier Transform Infrared Spectroscopy
f-CNT	:	Functionalized Carbon Nanotube
GSH	:	Glutathione
His	:	Histidine
MCL		Mucocutaneous leishmaniasis
MTT	:	3-(4,5-dimethyl thiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
RMSD	:	Root Mean Square Deviation
ROS	:	Reactive Oxygen Species
Spd	:	Spermidine
TCA	:	Trichloro Acetic Acid
TEM	:	Transmission Electron Microscopy
TryS	:	Trypanothione Synthetase
TryR	:	Trypanothione Reductase
VL	:	Visceral leishmaniasis

Contents

Chapter I

Introduction to Leishmaniasis: Literature review, current and future prospective*

1.1 Abstract

Intramacrophage obligate protozoan parasites of genus *Leishmania* are known to cause diverse set of infections collectively called leishmaniasis. The clinical manifestations of leishmaniasis are mainly governed by parasite, host factors and immune mediated inflammatory responses. Manifesting itself into three different clinical forms (cutaneous, mucocutaneous and visceral), leishmaniasis is a devastating disease impeding socioeconomic development and is estimated to affect over 150 million people worldwide. Leishmaniasis is classified as a major tropical disease by the WHO. Search for a successful vaccine against the parasite is still elusive. The main stream of treatment solely relies on chemotherapy. The current scenarios of chemotherapeutics against leishmaniasis pose a huge fall due to their high toxicity, high cost, tiresome to administer, and the growing incidence of resistance has seriously hampered their use. Thus, the search for new and improved anti-leishmanial drugs continues. Rationally designed, specific anti-leishmanial drugs can be identified against parasite target proteins by exploiting fundamental metabolic and/or biochemical divergence between host and parasite. Moreover, by exploring synthetic and natural products can result in identifying novel and potentially rich drug candidates against leishmaniasis.

*** Part of the review is submitted for publication**

1.2 Introduction

***1.2.1 Leishmaniasis*:** Leishmaniasis is a group of vector-born neglected tropical diseases caused by intramacrophage protozoan parasites of the genus *Leishmania*. Leishmaniasis has an extended history dating back to the extent that of first century AD. Leishmaniasis origins are unknown (*Momen and Cupolillo, 2000*). The first detailed clinical description of the disease was given by Alexender Russell in 1756 (*Hide et al., 2007*). In the Indian subcontinent the disease is called as kala-azar (*kālā* meaning black and *āzār* meaning fever). It is popularly known as "dum-dum fever" due to its high prevalence in place called Dum Dum, near Kolkata in India. The first discovery of the organism remained unclear. It is thought that in the year 1885, surgeon major Cunningham was possibly the first one to identify the parasite without being able to relate it to the disease (*Cunningham, 1885; Cox, 2002*). Peter Borovsky in his publication in the year 1898 gave first precise explanation of the parasite's relation to the host tissue and also appropriately referred it to protozoa. However, his findings were published in a Russian journal with low circulation so were not globally recognized during his lifetime (*Hoare, 1938*). Later, in the year 1903 William Boog Leishman and Charles Donovan both independently demonstrated the parasite in the splenic tissue from patients in India who died from "dum-dum fever" and named them as trypanosomatids, which were popularly known as Leishman-Donovan bodies (*Leishman, 1903; Donovan, 1903*). But it was Ronald Ross who anticipated that Leishman-Donovan bodies were the new parasite and named as *Leishmania donovani* (*Ross, 1903*). The transmission of leishmaniasis to humans by infected female sandflies was first described in the year 1942 by Swaminathan.

Ruled by parasite, host elements and immune mediate inflammatory reactions, the clinical manifestation of leishmaniasis comprises a broad spectrum of infections such as cutaneous, mucocutaneous and visceral form. Leishmaniasis is one of the most neglected tropical diseases in reference to insubstantial control tools and methods. Leishmaniasis is a poverty related disease, affecting indeed the poorest of the poor and also hampering the economic development of affected nations. The most lethal forms of infection are the visceral leishmaniasis (VL); causing absolute death, if untreated. It has been a major problem for endemic nations in the Indian subcontinents (India, Nepal and Bangladesh), and also Brazil

and Sudan for which high priority was given by their respective governments. *Leishmania* is transmitted by the bite of the female infected sandfly, which is the only vector of the disease. There are several factors that govern the spread of this disease such as human migrations, socio-economic factors, malnutrition, climatic change and environmental changes and also lack of awareness. Poverty intensifies the threat for leishmaniasis infection in several ways like poor living habitats (i.e. open sewage, spread of waste and poor sanitary conditions), which provide perfect condition for sandfly breeding. Malnutrition is another major factor that increases the risk of infection by *Leishmania*. Deficiency in vital elements leads to failure of the lymph node barrier and increased early visceralization of the parasite. Though, spread of infection in endemic and non-endemic areas are multi-factorial, in which lack of effective control steps for both *Leishmania* and sandfly, are main factors. The poor knowledge about the disease and lack of effective health policies are the major obstacles in complete eradication of leishmaniasis from all parts of the globe.

***1.2.2 Leishmaniasis Global Impact*:** Leishmaniasis is associated with poor socio-economic condition of the nations which can impair economic growth and development. Its rise can significantly hamper the nation's progress (*WHO, 2006*). Leishmaniasis is currently endemic in 88 nations ranging from deserts in western Asia to rain forests in Americas, covering intra-tropical temperate region of the globe with an estimated 350 million people at risk. Each year more than 90% of the cases reported are contributed mainly by 11 nations, India, Nepal, Bangladesh, Brazil, Sudan, Peru, Bolivia, Iran, Afghanistan, Syria, and Saudi Arabia (*Hide et al., 2007*). In recent times, the impact of leishmaniasis on public health has been re-evaluated and surprisingly, it was found that earlier assessments were very low compared to the actual global burden of the disease. According to the latest estimation, nearly 2 million fresh cases are reported annually, among which cutaneous leishmaniasis accounts for 1.5 million and visceral form claims the remaining 500,000 cases, with overall prevalence around 12 million people infected thought the globe currently (*Desjeux., 2004*). Most of the cases go unreported due to social stigma associated with the disease, moreover only 32 out of 88 nations that are affected by the disease report leishmaniasis cases. Hence, the actual global burden could be theatrically different (*WHO, 2006*).

A new rising problem of concern against these diseases is their development as opportunistic infections in immunocompromised patients, such as those suffering from AIDS. This co-infection further complicates the treatment as both infections jointly destroy the immune system of the patient. In regions endemic for visceral leishmaniasis, many patients have asymptomatic infection. An associated HIV co-infection raises the risk of developing active visceral infection by between 100 to 2320 times (*WHO, 2006*). The 70% of adult visceral leishmaniasis cases in southern Europe are co-infected with HIV infection (*WHO, 2002*). Till now, as many as 35 nations across the globe have reported cases of VL/HIV co-infection (Figure 1.1), even though most of the reporting is concern to southern Europe countries.

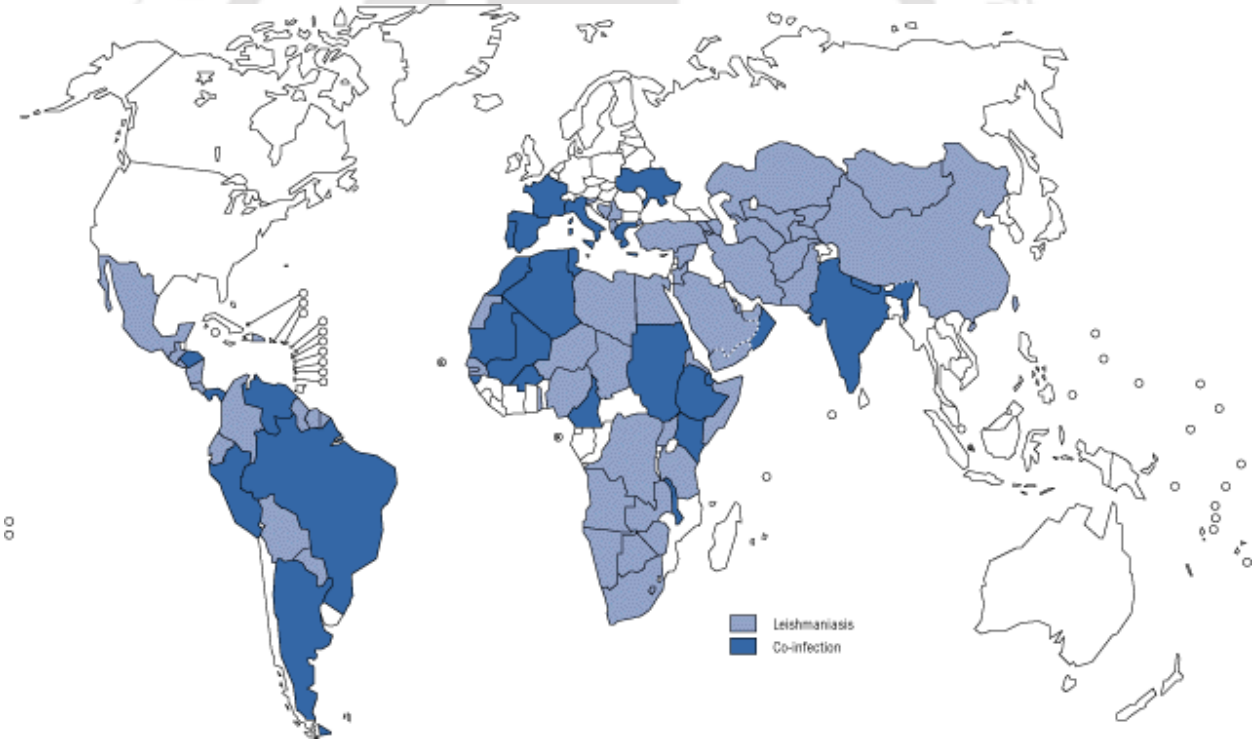


Figure 1.1: Geographical distribution of leishmaniasis (light blue) and co-infection with HIV (dark blue). As many as 35 countries throughout the world have reported cases of visceral leishmaniasis/HIV (VL/HIV) co-infection. Majority of the cases VL/HIV co-infection are reported from countries of southern Europe. As much as up to 70% of cases of VL in adults are associated with HIV infection. (Adopted from http://www.who.int/csr/resources/publications/CSR_ISR_2000_1leish/en/)

***1.2.3 Classification of Leishmania Parasite*:** The genus *Leishmania* are animal-like flagellated organisms which uses flagella for their locomotion, feeds by pinocytosis or phagocytosis, contain a large kinetoplast- a portion of the single mitochondrion containing a large amount of DNA. Based on these characteristics, members of the genus *Leishmania* are classified in the Domain Eukarya, Kingdom Protista, Sub kingdom Protozoa, Phylum Sarcomastigophora, Sub-phylum Mastigophora, Class Zoomastigophora, Order Kinetoplastida, and Family Trypanosomatidae. The genus *Leishmania* is divided in two subgenera based on the distribution of the midgut of the sandfly vectors. In the subgenus *Leishmania*, the promastigotes develop in the midgut and foregut of the insect, whereas in the subgenus *Viannia*, promastigotes are restricted to the hindgut. Species of the subgenus *Leishmania* belong to the *L. donovani* complex (*L. donovani, L. infantum chagasi, L. archibaldi); L. tropica complex (L. tropica, L. aethiopica, L. major) and L. mexicana complex (L. mexicana, L. amazonesis, L. pifanoi, L. garhami, L. venezuelensis*). Whereas, parasites of the subgenus Viannnia only occur in the new world. These are the species of *L. braziliensis* complex (*L. guyanesis, L. peruviana, L. panamensis and L. shawi*).

1.2.3.1 Systematic Position of Leishmania

Kingdom	:	Protista
Sub-Kingdom	:	Protozoa
Phylum	:	Sarcomastigophora
Sub-Phylum	:	Mastigophora
Class	:	Zoomastigophorea
Order	:	Kinetoplastida
Family	:	Trypanosomatidae
Genus	:	*Leishmania* (Ross, 1903)

***1.2.4 Life cycle of Leishmania and Morphology*:** *Leishmania* has a digenetic life cycle to survive two challenging environmental conditions as they shuttle between vector sandfly and the mammalian host. *Leishmania* parasites undergo morphological differentiation depending on the life cycle stage. The extracellular, flagellated and motile form of the parasite is called promastigote, which is present in sandfly. Whereas in mammalian host the parasite exists as intracellular, aflagellated and non-motile form called amastigote (Figure 1.2). The promastigote stage of the parasite are adapted for survive in gut of the sandfly and the amastigote stage of parasite in the phagolysosome of the macrophage cells of mammalian host (*Zilberstein and Shapira, 1994*). The digenic morphological forms and life cycle of *Leishmania* are shown in Figure 1.3. The only vectors for leishmaniasis are the certain species of sandflies (Figure 1.4) of the genus *Phlebotomus* in case of Old World *Leishmania* species or Lutzomyia for New World *Leishmania* species, which gets infected with *Leishmania* parasite largely form the natural reservoir hosts, such as dogs, rodents and canines. Sandfly ingests the amastigote parasites during its blood meal from the infected host. The amastigotes undergoes differentiation into procyclic promastigote forms which are long slender cells of ~15-20 μm/ 1.5-3.5 μm measurement and a 15-28 μm anterior flagellum. The flagellum helps in attachment and locomotion of the parasite in the gut wall of sandfly. The parasites further undergo transition and form metacyclic promastigotes which migrate to the esophagus and pharynx of the sandfly. When sandfly next takes the blood meal, it releases the parasites in the host, which are phagocytized by host macrophage cells. The parasite undergoes differentiation inside the parasitophorous vacuole which gets matured by the fusion of lysosomes, full of lysosomal enzymes. Astonishingly, the parasite cells survive within the acidic phagolysosome of macrophage cells, which are the prime defense cells against foreign invaders. Morphologically, amastigotes are smaller in size than promastigote with no flagella present. Amastigotes are ovoid in shapes measuring ~2-4 μm in diameter (*Herwaldt, 1999*). These amastigote cells thrive and proliferate inside the macrophage which gets ruptured and releases amastigote cells that are free to invade fresh macrophage cells. This cycle goes on which results in one of the clinical manifestations of leishmaniasis with different species showing different tropisms for macrophages in specific organs in the host.

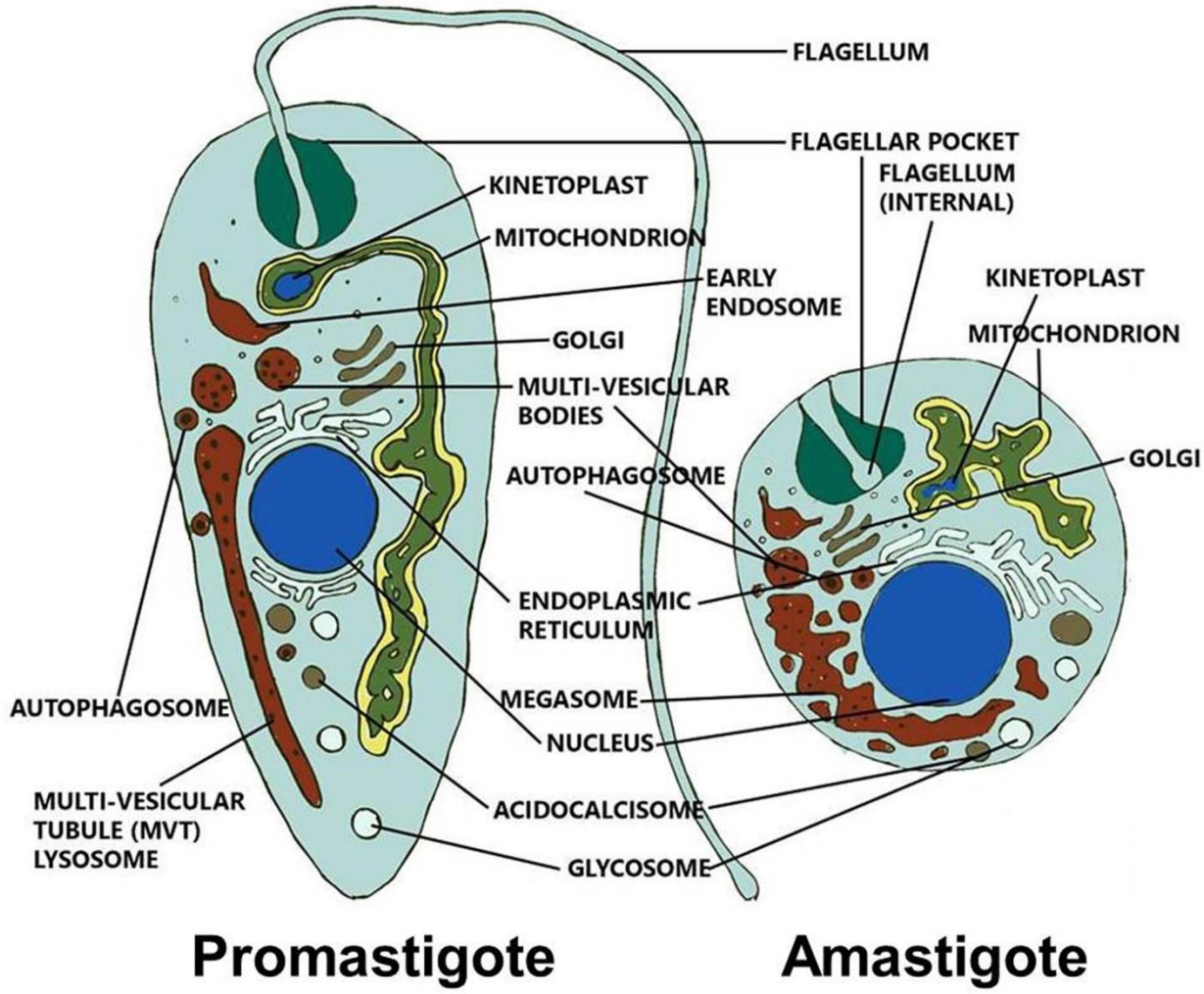


Figure 1.2: Diagrammatic comparison of two stages of *Leishmania* parasite. The sandfly stage of *Leishmania* parasite is promastigote form which is an elongated cell with anterior flagellum that helps in locomotion. The mammalian stage *Leishmania* parasite is the amastigote form which is ovoid in shape and smaller in size than promastigote with no flagella..

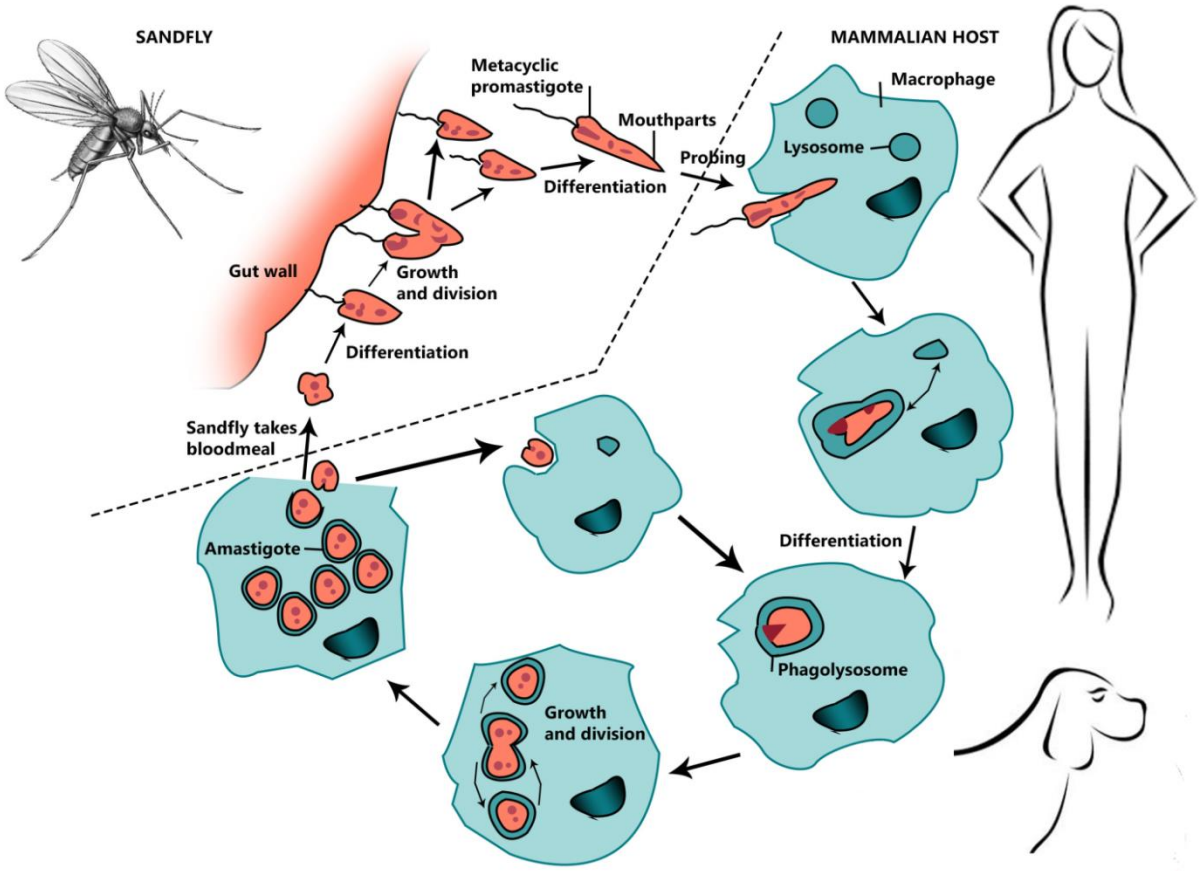


Figure 1.3: Life Cycle of Leishmania Parasite. The procyclic promastigote forms of *Leishmania* differentiate in sandfly into metacyclic promastigotes which are non-dividing and infective stage. These infective stage parasites are placed ready for injection at the opening of the midgut. At the time of blood feeding, the sandfly spit up metacyclic promastigotes, along with various salivary components. The macrophage cells that are present at the bite site phagocytize metacyclic promastigotes. After forming an intracellular habitation, metacyclic promastigotes transform into aflagellate amastigotes. Amastigotes undergo replication within host cells, which break when too many amastigotes are present, allowing re-infection of local macrophage cells. The transmission cycle is complete when infected macrophage cells are ingested up in a blood by a new sandfly, and amastigotes then transform into promastigotes in the sandfly midgut.

Table 1.1: Summary of the disease manifestation, geographical distribution of *Leishmania* sp. and their vectors.

Disease Manifestation	Geographic Distribution	Causative species	Vector
Visceral Leishmaniasis	Northeast India, Nepal Bangladesh, Burma	*L. donovani* (Asia)	*Phlebotomus argentipes*
Visceral Leishmaniasis	Mediterranean basin, Middle East, China, Central Asia	*L. infantum*	*P. perniciosus, P. ariasi*
Visceral Leishmaniasis	Sudan, Kenya, Horn of Africa	*L. donovani* (Africa)	*P. orientalis, P. martini*
Visceral Leishmaniasis	Central America, Northern South America, esp. Brazil	*L. chagasi*	*Lutzomyia longipalpis*
Cutaneous Leishmaniasis	Deserts in Middle East, Pakistan, North Africa	*L. major*	*P. papatasi*
Cutaneous Leishmaniasis	Sub-Saharan Savanna, Sudan	*L. major*	*P. duboscqi*
Cutaneous Leishmaniasis	Towns in Middle East, Mediterranean basin, Asia	*L. tropica*	*P. sergenti*
Cutaneous Leishmaniasis	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*
Cutaneous Leishmaniasis	Yucatan, Belize, Guatemala	*L. mexicana*	*L. Olmeca*
Cutaneous Leishmaniasis	Tropical forests of South America	*L. amazonensis*	*L. Flaviscutellata*
Mucocutaneous Leishmaniasis	Tropical forests of South and Central America	*L. braziliensis*	*Lutzomyia spp., L.Umbratilis*

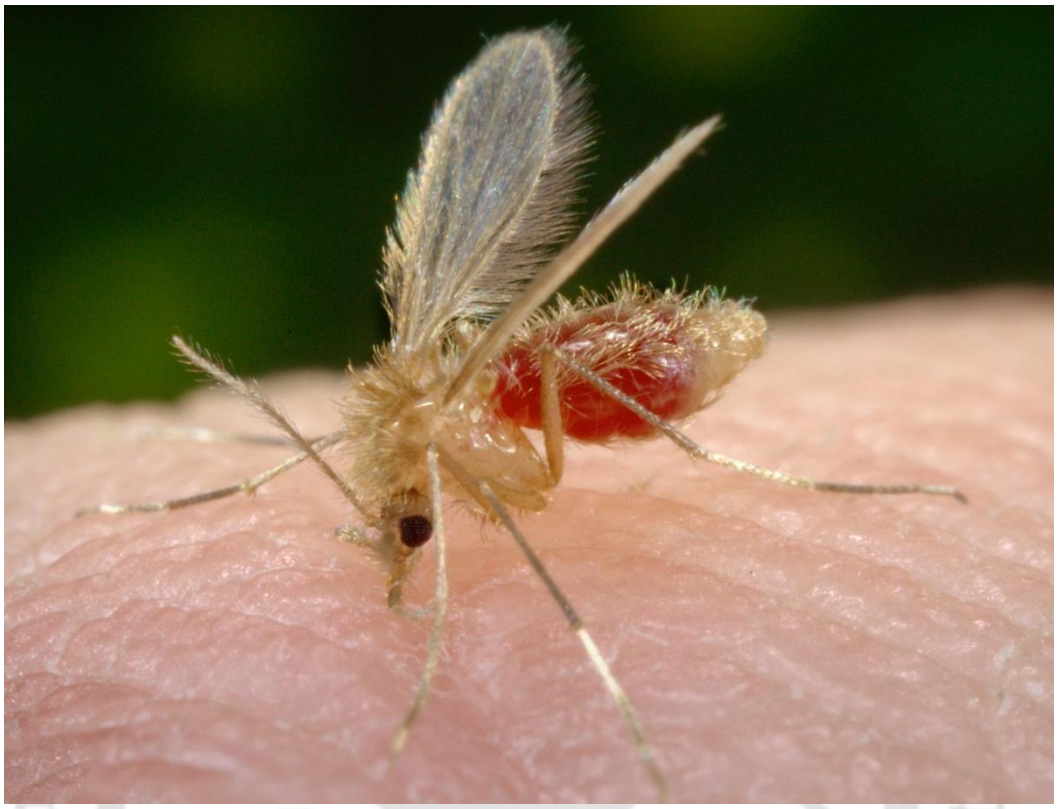


Figure 1.4: The insect vector of leishmaniasis, the phlebotomine sand-fly, is a 2-3 millimeter long insect. Over 30 species of the sand-fly are identified as vectors of leishmaniasis. The parasite is transmitted by female sandflies only, infecting itself with *Leishmania* parasites during blood meal from its infected mammalian host. (Taken from: http://phil.cdc.gov/phil/details.asp?pid=10275)

***1.2.5 Manifestations of Leishmaniasis*:** About 30 species of phlebotomine sandflies act as vectors in transmitting the parasite to the mammalian host. Several clinical manifestations are submerged under the term leishmaniasis- due to multifaceted pathology which mainly depends on complex association of three main companies intricate: vectors, parasites and hosts. Leishmaniasis has three different manifestations that range from ulcerative self-healing skin lesions called cutaneous leishmaniasis to life threatening disseminated visceral leishmaniasis. The other form of the disease is mucosal leishmaniasis in which the oral and nasal mucosal inflammation occurs. These clinical manifestations are caused by a total of about 21 different species of *Leishmania* (*Herwaldt, 1999; MacMorris-Adix, 2008*).

***1.2.5.1 Cutaneous Leishmaniasis*:** Among the three disease manifestations of leishmaniasis, cutaneous leishmaniasis (CL) is the most common and widespread infection of leishmaniasis. CL is characterized by development of red lesions at the site of the bite by infected sandfly (*Hide et al., 2007*). These lesions develop as papules, to nodules and ulcerate with central depression and raised borders (Figure 1.5a) ultimately to atrophic scars. Cutaneous infection can endure subclinical or develop clinical symptoms after an inconstant incubation time which averages several weeks to months. Cutaneous infection is generally self-curing without any specific treatment; however the residual scars often cause devastating social stigma. Elements of the natural history and pathogenicity of cutaneous infection, comprising the tendency for dormancy, fast self-healing, distribution, persistence, repetition, and re-infection, are poorly understood which include aspects related to the conduct of the vector, the parasite virulence factor, and the behavior of the host and innate and acquired immunity (*Herwaldt et al., 1992; Weigle and Saravia, 1996*).

***1.2.5.2 Mucocutaneous Leishmaniasis*:** The severe form of cutaneous infection is mucocutaneous leishmaniasis (MCL) (*Sangueza et al., 1993; James et al., 2006*). MCL mainly damages the mucous tissue of the mouth and nose. It is characterized by formation of lesions that frequently results in extensive destruction of the mucous membrane of the mouth, nose and face (*Hide et al., 2007*). In most of the cases, MCL is largely caused by parasites of the Viannia subgenus as a consequence of lymphatic or haematogenous distribution of parasite from the skin to the naso-oropharyngeal mucosa. In most of the patients MCL manifest after many years of cure of the cutaneous infection, however MCL can succeed while the cutaneous lesions are present over years after they cure. Characteristically, mucosal infection becomes apparent because of prolonged nasal symptoms following progressive naso-oropharyngeal damage (Figure 1.5b). Even with the clinically active infection, MCL is hard to diagnose due to amastigotes are scarce.

***1.2.5.3 Visceral Leishmaniasis*:** Visceral leishmaniasis (VL) is the most severe form of infections of *Leishmania* parasite with a death rate of 100% is attained if left untreated (Figure 1.5c). Appearance of freshly developed infection diverges from subclinical, to oligosymptomatic, to completely established visceral infection (kala azar). The visceral

infection is characterized by irregular sessions of fever, significant loss of weight, swelling of spleen (splenomegaly) and liver (hepatomegaly), anemia, weakness and night sweating are common symptoms that progress with time (*Davidson, 1998; Osman et al., 2000; Collinet al., 2004*). Visceral infection is known to especially affect children more (*Hide et al., 2007*). Patients suffering from VL are likely to develop diarrhea and develop retardation and can exhibit incomplete oligosymptomatic infections which generally decide instinctively over a flexible period of time, but can also evolve to kala azar (*Badaro et al., 1986; Gamaet al., 2004*). The blackening of the skin is one of the symptom from which the disease got its name kala azar in India (kala azar means black fever in Hindi). Post kala-azar dermal leishmaniasis (PKDL) is a dermal complication, caused as a sequel to VL (Figure 1.5d). A new emerging problem of concern with visceral infection is its development as an opportunistic infection in immunocompromised patients, such as AIDS. In VL/HIV co-infection patients the chances of treatment are less as both infections mutually destroy the immunity of the patient and eventually die (*Pintado et al., 2001; Murray, 2004; Fernandez-Guerrero et al., 2004*).

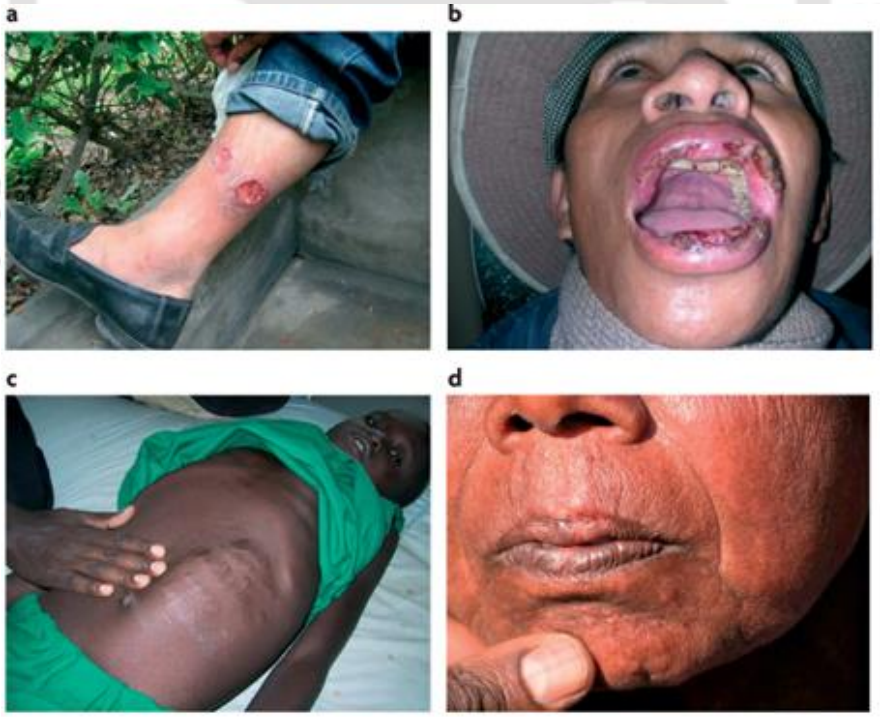


Figure 1.5: Clinical Manifestations of Leishmaniasis: (A) A patient from Peru with cutaneous leishmaniasis. **(B)** A patient from Bolivia with mucosal leishmaniasis. **(C)** A patient from Uganda with visceral leishmaniasis. **(D)** A patient from India with nodular post-kala-azar dermal leishmaniasis (PKDL). (Adopted with permission from *Nature Reviews Microbiology* 2007, 5: S7-S16)

***1.2.6 Current Available Drugs and their Status*:** Till date the treatment of leishmaniasis infection is primarily relayed on chemotherapy, due to unavailability of vaccine candidates either preventative or prophylactic. The present available drugs in the market for leishmaniasis include pentavalent antimonials, amphotericin B and its liposomal formulations; miltefosine, paromomycin and pentamidine (Figure 1.6). Once the patient is confirmed positive for leishmaniasis, generic pentavalent antimonials has been the first choice of chemotherapy in leishmaniasis endemic nations particularly in Indian subcontinent (*Croft et al., 2006*). In addition, the substitutes for generic antimonials in treating the disease were sodium stibogluconate and meglumine antimoniate. Pentavalent antimonials, are actuality used as the first line of treatment for leishmaniasis for more than five decades and still they are the primary drugs of choice in areas where drug resistance is not recorded (*Singh et al., 2006*). The increasing frequency of resistance has upraised serious concern for its use in leishmaniasis endemic regions (*Maltezou, 2010*). The pentavalent antimoniate (SbV) is thought to be a pro-drug, which is further changed to active trivalent antimonite (SbIII) form of the drug which is highly effective on the parasite compaired to SbV (*Ephros et al., 1999*). The conversion of SbVto SbIIItakes place either in parasite or in macrophages is still a question (*Shaked-Mishan et al., 2001; Frézard et al., 2009*). The loss of reductase activity facilitated by parasite may possibly lead to drug resistance, which may further reinforce the evident that the *Leishmania donovani* amastigote resistance to SbV has lost their reductase activity. This possibility is supported by the recent finding of a parasite thiol dependent reductase 1 (TDR 1) enzyme involved in catalyzing the reduction of SbV to SbIII using glutathione as a reductant (*Torres et al., 2010*). Moreover, a new characterized antimoniate reductase enzyme in *Leishmania* species, arsenate reductase 2 (ACR2) increases the sensitivity of *Leishmania* to SbV (*Santos et al., 2003*). The evident that the reduction of SbV to SbIII takes place in macrophage rather than parasite is also reported (*Sereno et al., 1998*). The previous studies with organisms like fungus and bacteria shown that the metal reduction is carried out by host specific enzymes, further suggesting the conversion is host specific. The main reason of acquired resistance is due to extensive misuse of antimonial drugs in endemic regions where they are made widely available, causing the loss of drug activation by the parasite. In an in vitro study the disability of the SbV resistant strain to reduce SbV to SbIII was reported (*Bolhassani et al., 2011*). Further, in a study with SbIII

resistant leishmanial strain the parasite showed low accumulate of metals possibly due to decreased uptake or high efflux (*Brochu et al., 2003*). Moreover, in clinical isolates of resistance parasites many genes have been identified suggesting the various factors responsible for drug resistance (*Decuypere et al., 2012; Choudhury et al., 2008; Carter et al., 2006; Decuypere et al., 2005; Singh et al., 2003*).

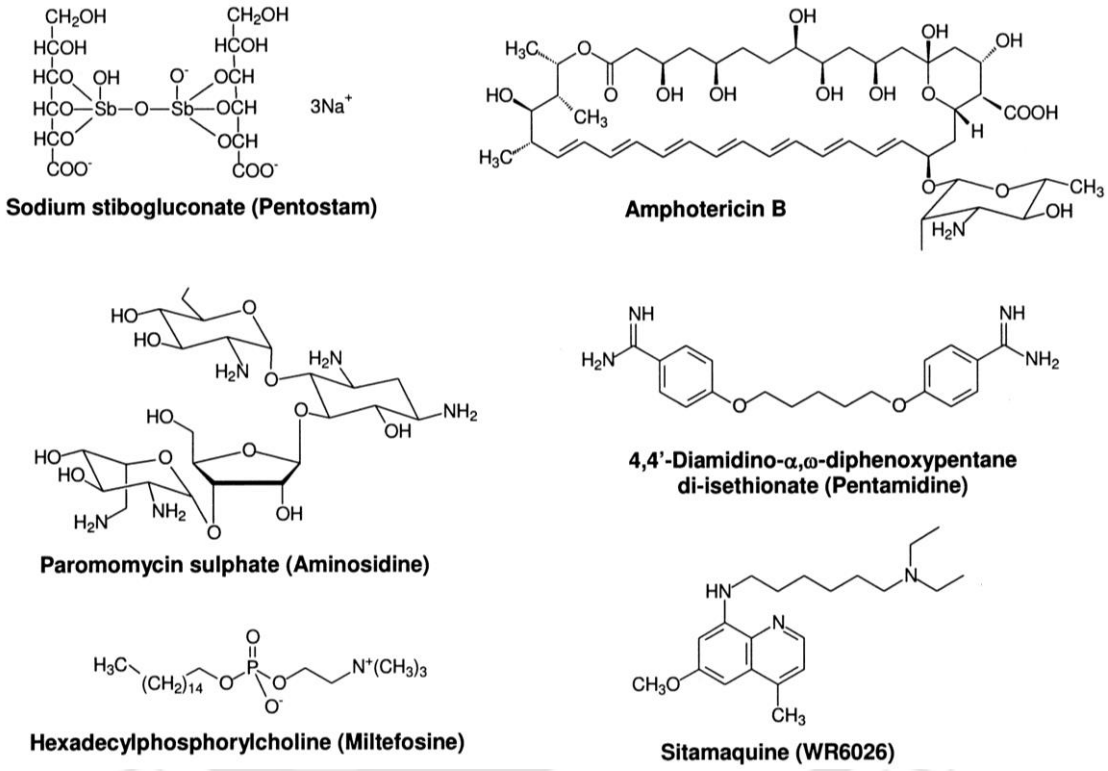


Figure 1.6: Current drugs that are used for treatment of leishmaniasis. Among the commercially available drugs, such as pentavalent antimonials, amphotericin B, paromomycin, pentamidine and sitamaquine, pentavalent antimonials were first drugs used against leishmaniasis. The latest and most successful drug is miltefosine. These available drugs suffer severe drawbacks in terms of cost, efficacy, administration mode and toxicity.

The other alternates for generic pentavalent antimonials, which are being used as second line of defense in curing leishmaniasis, are amphotericin B (AmB) and its liposomal formulations, miltefosine, paromomycin and pentamidine. AmB, one of the most successful drugs of leishmaniasis, is a polyene antifungal drug generally used to cure systemic fungal infections. AmB is the choice of drug for leishmaniasis especially in the endemic regions of Bihar state in India where antimonials resistance is high (*Bern et al., 2006*). AmB is known

to show high affinity for ergosterol, one of the major components found in cell membranes of *Leishmania* parasite and fungus. Besides its high efficiency, AmB is known to show side effects and toxicity (*Laniado-Laborin and Cabralis, 2009*). The toxicity and side effects of AmB is masked in its three clinical formulations liposomal AmB (L-AmB: Ambiosome), AmB lipid complex (ABL: Abelcit) and AmB colloidal dispersion (ABCD: Amphocil) wherein the deoxycholate have been substituted by other lipids. These AmB lipid formulations not only retained their drug property but also have increased efficacy by more than 95%. However, high cost limits its use as victims of leishmaniasis are from poor background. The anti-leishmanial property of AmB and its lipid formulation is mediated through both, cholesterol of macrophage and sterol of *Leishmania* as cholesterol is complexed with AmB and distinctly prevents the parasite binding to host macrophage (*Paila et al., 2010*). Further, AmB at higher concentration (<0.1 M) act on the sterol present in leishmanial cell membrane forming aqueous pores which results in osmotic cell lysis (*Ramos et al., 1996*). Despite of high efficacy shown by AmB, it is allied with toxicity and emerging drug resistance. The nephrotoxicity caused by AmB is mediated through increased salt and $Ca2^{+}$ concentration in kidney tubular cells that lead to apoptotic death of the cell. The absence of ergosterol (main target of AmB) was observed in an in vitro studies conducted with drug resistant *Leishmania* (*Pourshafie et al., 2004*). In an early investigation, it was demonstrated that success of AmB in curing the infection significantly depends on the immunity state of the patient and successive relapse may boost emergence of drug resistance parasites (*Di Giorgio et al., 1999; Lachaud et al., 2009*).

The only available oral drug against VL is miltefosine. It is an alkylphosphocholine moiety, developed mainly for cancer treatment (*Croft and Coombs, 2003*). Miltefosine was proved to be very effective drug for VL which was evident from successful phase I/II/III trails and its propensity was finally judged after successful phase VI trail, which effectively treated the VL in endemic regions (*Sundar and Chatterjee, 2006; Bhattacharya et al., 2007*). The miltefosine and ambisome combination was tested, which gave high efficacy with good tolerance levels, but high toxicity and side effect was the limiting factor (*Sundar et al., 2010*). The combination of miltefosine and ambisome has also been evaluated, and has been found effective with good tolerability but unfortunately side effects raise questions against extreme efficacy of this combination. The main adherence of the drug is compromised by its

long terminal residence, time and teratogenicity. Miltefosine has a median long half-life of approximately 152 hours, which could encourage development of clinical resistance. Further, its teratogenicity and abortifacient nature limits its use in pregnancy. The exact mechanism of anti-leishmanial activity mediated by miltefosine is still not known, but it is known to elicit apoptosis in *Leishmania* parasite (*Paris et al., 2004*). Miltefosine is also known to reduce the membrane lipid of promastigote cells and increase phosphatidylethanolamine levels signifying a fractional inhibition of phosphatidylethanolamine-N-methyltransferase that leads to reduced parasite multiplication (*Losieau and Bories, 2006*). Paromomycin is an aminoglycosidic antibiotic which is known to have both antibacterial and anti-leishmanial activity. Paromomycin can cure CL very effectively and is also used for VL. The use of paramomycin is restricted in endemic zones due to its restricted availability (*Thakur et al., 2000; Thaku, 2003*). The mode of action of paromomycin is still unknown. In recent times, it was reported that paromomycin has an affinity to glycocalyx of *Leishmania*, proposing primary target could be mitochondria (*Jhingran et al., 2009*). Because of its restricted use no drug resistance is reported yet, whereas resistance with in vitro leishmanial strains is reported. But due to aminoglycosidic nature of paromomycin, rapid emergence of resistance cannot be ruled out.

Sitamaquine is the only drug that was particularly developed for treatment of VL. Chemically it is 8-aminoquinoline which is a collaborated product develop by GlaxoSmithKline and Wlater Reed Army Institute (*Sundar and Chatterjee, 2006*). Sitamaquine has an advantage of oral administration. Sitamaquine efficacy against VL with good tolerance was demonstrated successfully in phase II trial conducted in India (*Jha et al., 2005*). Despite of its good efficacy, it suffered from side effects such as dyspepsia, nephritic syndrome, vomiting, cyanosis and glomerulonephritis. The results of the Kenyan phase II trial were not the same as Indian trial (*Wasunna et al., 2005*). The side effects observed in Kenyan trial were kidney failure, headache and abdominal pain. In an animal studies conducted on CL with BALB/c mice, sitamaquine failed to reduce the parasite burden and lesions continued to progress. Thus, due to improper activity and efficacy further clinical trials have been stopped (*Garnier et al., 2006*). Another second line drug used in treating leishmaniasis is pentamidine which is an aromatic diamine. Pentamidine salts, methansulphonate and isothionate are used for treating VL. In India it was used for treating

SbV unresponsive patients, but due to its high risk of resistance with decreasing activity has stopped its use in India. Like most of the leishmaniasis drugs its mode of action is not known still. Few studies claim that it makes its entry into parasite by polyamine and arginine transporters (*Basselin et al., 2000; Kandpal and Tekwani, 1997*). The high toxicity by pentamadine causes nephrotoxicity, hypoglycemia and hypotension.

In spite of the substantial development that has been done during past decade in chemotherapies for leishmaniasis (*Castillo et al., 2010*), there are limitations with all drugs like safety, feasibility, efficacy, price, toxicity, side effects and possibility of mounting resistance. The major concern of current therapeutics is steady rise in resistance to antimonials in particular and others in common, mainly in Indian subcontinents. In recent period, clinical trials of combination treatments are in use. Combination of two or more therapeutics might decrease treatment period and drug doses and therefore drug toxicity but chances of resistance development against present available drug regimen cannot be ruled out. The primary combination medicines presently under consideration are liposomal amphotericin B (LAmB) and miltefosine, antimonials and LAmB, paromomycin and LAmB and antimonials and paromomycin in India and also being assessed in various countries. There is no vaccine candidate available, and present latest growth on leishmanial research does not ensure the availability of future candidate leishmanial antigens/immunogens. Therefore, identification of new drug targets and development of novel leishmanicidal drugs must be the main concern area of research.

***1.2.7 New Potential Drug Targets for Anti-leishmanial Drug Development*:** Leishmaniasis being the second largest parasitic killer (next to malaria) is been treated using non-leishmanial drugs, whose molecular mode of action is poorly understood. Recent research on leishmaniasis have mainly focused on clinical trials and detection studies due to funding encouraged by global agencies such as WHO to disease endemic regions like Indian subcontinent. It is clearly evident from recent leishmanial research publications that the primary focus is being given only on trials/combination treatment of existing non-leishmanial drugs, diagnostic methods and tools, but very little emphasis is made on other aspects by researchers. Leishmanial research majorly lacks disease control strategies with no emphasis made on identifying new drugs and drug targets and vaccine candidates. By exploring

fundamental metabolic and/or biochemical divergence between host and the parasite new potential drug targets can be identified. The selected target enzyme must be essential for parasite survival with no structural or functional similarity with host enzymes to maintain the specificity. Moreover, approaches to target multiple enzymes of a metabolic pathway simultaneously may possibly exhibit more efficacy and efficiency.

***1.2.7.1 Enzymes of Polyamine Biosynthesis*:** Polyamine like spermidine, spermine and putrescine play significant role in growth and differentiation of *Leishmania* cells from promastigote to amastigote form (*Tavares et al., 2005*). Polyamines are also known to down regulate lipid peroxidation synthesis and create favorable atmosphere for survival (*Vannier-Santos et al., 2008*). The enzymes of polyamine biosynthesis, such as ornithine decarboxylase, spermidine synthase, s-adenosylmethionine decarboxylase and arginase are overexpressed by *Leishmania.* However, polyamine levels remains unaffected or slightly changes at the time of their growth and metabolism which suggest presence of regulatory mechanisms (*Roberts et al., 2007*). Any of these polyamine regulatory mechanisms can provide a great opportunity for future drug target. In *Leishmania*, conversion of arginine to L-ornithine is catalyzed by arginase enzyme. Further decarboxylation of L-ornithine by enzyme ornithine decarboxylase produces putrescine, which sequentially gets converted to spermine and spermidine, the essential substances for cell progression and proliferation of parasite plus Th2 type response which is helpful in parasite survival in host (*Baiocco et al., 2009a*). However, trypanosomasis polyamine inhibitor alpha-difluoromethylornithine failed to act on leishmaniasis thereby proving the differences amongst the closely linked parasites. Hence, extra efforts are required in identification of specific inhibitors of polyamines of *Leishmania* (*Reguera et al., 2005*). Other attractive targets are the transporters of polyamine which maintains the polyamine levels in parasite cell. Therefore, identification of inhibitors of polyamine biosynthesis and transportation may emerge as effective anti-leishmanial therapies.

***1.2.7.2 Enzymes of Sterol Biosynthesis*:** Stigmasterol and ergosteral are the main endogenous sterols found in *Leishmania* species, where as in mammalian host it is cholesterol, hence enzymes of sterol biosynthesis are attractive drug targets. The two major

functions of ergosterol are, it forms the major component of cell membrane and may show hormonal role. The strong anti-leishmanial activity of AmB is known to mediate through interaction with ergosterol present in cell membrane of *Leishmania*. However, emergence of resistance to AmB, directs researchers to identify a substitute molecule of AmB. Azasterols shows anti-leishmanial activity by inhibition of 24-methyltransferase, one of the key enzymes of ergosterol biosynthesis (*Lorente et al., 2005*). The other sterols were also found to active against *Leishmania*. But still, the parasite has potential to utilize host sterols to survive the altered sterol of the cell, which is an important consideration to be noted while developing a drug. The more suitable way would be developing combine therapy with inhibitors of sterol biosynthesis and that of other metabolic pathway. Beside combinational therapy, identification of a new potential site in sterol metabolic pathway of *Leishmania* can also emerge as an attractive drug targets (*Andrade-Netoa et al., 2011*).

***1.2.7.3 Enzymes of Glycosomal Pathway*:** All trypanosomatids including *Leishmania* utilize carbon sources of the host to overcome their energy requirements. The parasite utilizes glucose from the blood of the mammalian host and other remaining vital molecules such as amino acids and lipids from macrophage phagosomes. In *Leishmania*, peroxisomes like organelle are present in parasite glycosomes, which play vital role in various metabolic processes which include glycolysis, purine salvage pathways, oxidation of fatty acid, lipid biosynthesis etc. Superoxide radicals are produced as a byproduct in huge amount by these metabolic processes. To guard the glycosomal enzymes from damage due to superoxide radical, *Leishmania* parasite have evolved with Fe-superoxide dismutase (FeSOD's). The differentially expression FeSOD's, Lcfesodb1 and Lcfesodb2 in *Leishmania chagasi* amastigotes and promastigotes stage, respectively, are well characterized. These enzymes play vital role in survival of the parasite by scavenging superoxide radicals inside glycosomes (*Plewes et al., 2003*). The absence of FeSOD in mammalian host attracts researchers to exploit them as potential drug target. In addition, only initial few steps of glycolysis pathway take place inside glycosome, and the rest in cytosol. Thus the other cytosolic enzymes of glycolysis pathway of the parasite can also be targeted because of their exclusive nature of glycolytic pathway and evolutionary distance between *Leishmania* and

mammalian host, thus intermediate enzymes of glycolysis pathway can be possible drug targets.

***1.2.7.4 Mitogen Activated Protein Kinases (MAP kinase)*:** The significant role of MAP kinase in various aspects of immune reactions from beginning of innate immunity to triggering of adaptive immunity is well known. Moreover, they also play important role in cell proliferation, differentiation and cell death processes. MAP kinases are important signaling proteins that regulate different cellular processes (*Weise et al., 2004*). In *L. Mexicana*, 15 MAP kinases have been recognized (*Weise, 2007; Weise et al., 2004*). The importance of MAP kinase in transformation and cell proliferation were shown in *Leishmania* mutant missing MAP kinase gene. The mutant amastigote cells devoid to MAPkinase gene lost their cell division property and also eradication of parasite in infected macrophage cells, which highlight the importance of MAP kinase for amastigote survival (*Weise, 2001*). MAP kinases are equally important to *Leishmania* promastigotes stage, which is evident from in vitro study where elevated expression of MAP kinase resulted in stage defined phosphotransferase activity and deposition in axenic amastigotes but not in promastigotes (*Morales et al., 2007*). Identification and targeting MAP kinase unique to *Leishmania* have high possibility of potential drug target. Therefore, efforts are needed to identify unique MAP kinase of *Leishmania* for targeting. The MAP kinase identified shell not only provides a potential target but can be utilized for developing therapeutic immunemodulation.

***1.2.7.5 Leishmania Topoisomerases*:** The torsional stress in DNA is released by introducing DNA breaks either on single strand (Type l Topoisomerases) or on both the strands (Type ll Topoisomerases) by ubiquitous enzymes called DNA topoisomerases. The topoisomerases are most important chemotherapeutic targets in diseases like cancer and bacterial infections (*Schneider et al., 1991; Heisig, 2001*). In comparison, protozoan parasites are not different, they too need topoisomerases particularly topoisomerase II because of the presence of multiple mini and micro circular DNA in kinetoplastids mitochondria. In an investigation it was shown that elevated expression of topoisomerase II in *Leishmania donovani* enhances activity of arsenite resistance in parasite (*Singh et al 2005*). The prime site of action for

topoisomerase II is mitochondria due to topological problems connected with mitochondrial DNA. The known drugs such as fluoroquinolones, novobiocin and etoposide can be used for targeting leishmanial topoisomerase II enzyme (*Rosypal et al., 2010*). More focus is needed in the direction of topoisomerase targeted drug interaction and development to fight the drug resistance problem.

***1.2.7.6 Metacaspases*:** In *Leishmania*, presences of metacaspases which are distinctly related to caspases are thought to be responsible for caspase like activity. The metacaspases in *Leishmania* are poorly understood. The apoptosis process in *Leishmania* is thought to have a possible role of metacaspases. The two metacaspases of *Leishmania donovani*, LdMCA1 and LdMCA2 are reported to have 98% homology with each other and said to contain proline rich C-terminal domain and are equally expressed in both stages of the parasite (*Lee et al., 2007*). The essential role of metacaspases in segregation of the kinetoplast and nucleus is studied in *Leishmania major* (*Denise et al., 2006*). By treatment with H_2O_2, *Leishmania* parasites metacaspases elicites apoptosis. Further parasites that overexpress metacaspases are found to show high sensitivity to H_2O_2 induced apoptotic death (*Gonza'lez et al., 2007*). Targeting metacaspase biosynthesis process by potential inhibitors and prove their essentiality in parasite survival may help emerge these enzymes as potential targets for leishmaniasis. Moreover, metacaspases themselves can also be targeted as they are essential for parasite survival and chromosomal segregation (*Meslin et al., 2011*). Nevertheless, more research is necessary to better understand the entire functions of metacaspases in *Leishmania*.

***1.2.7.7 Dihydrofolate Reductase (DHFR)*:** DHFR is an important enzyme involved in folate metabolism, associated with synthesis of thymidine (*Blaney et al., 1984; Booth et al., 1987*). DHFR carries out NADPH dependent reduction of dihydrofolate to tetrahydrofolate. Hence, inhibition of DHFR averts synthesis of thymidine and consequently DNA synthesis. Luckily, the crystal structures of DHFR from *Leishmania major* and *Trypanosoma cruzi* are available, which can be exploited to study molecular library datasets and also to design inhibitors of DHFR (*Liang et al., 1998; Senkovich et al., 2009*). An approach to identify unique leishmanial DHFR inhibitors by means of molecular database search has also been made to explore the Cambridge structural database. However, DHFR as drug target needs more

research (*Zuccotto et al., 1998; Kuntz, 1992*). The conversion of dihydrofolate from methyhylenetetrahydrofolate (M-THF) and thymidine is catalyzed by dihydrofolate reductase-thymidylate (DHFR-TS) enzyme, which is shown to be associated with *Leishmania* survival and leishmanial cells missing this enzyme did not stay alive in animals (*Veras et al., 1999*). In spite of these advantages many resistance processes to DHFR have been shown including elevated expression of the enzyme DHFR-TS and enzyme ptrI (*Wang et al., 1997; Bello et al., 1994*). The enzyme, PTR1, is involved in conversation of biopterin to dihydrobiopterin and tetrahydrobiopterin but can also undertake the convert dihydrofolate to tetrahydrofolate. Therefore, a combined target strategy of both DHFR and PTR1 may be more operative drug target.

***1.2.7.8 Enzymes of Thiol Metabolic Pathway*:** In mammalian host *Leishmania* parasite thrives and proliferates in lethal lysosome's environment of macrophage cells. How parasite is able to survive and escape from reactive nitrogen and reactive oxygen species that is produced in mature parasitophorous vacuole of macrophage is still remains elusive. From the recent discovery of the redox metabolism in trypanosomatids (Figure 1.7), it is clear that parasite has evolved with unique redox metabolism to fight respiratory burst produced by the host macrophage cells. Antioxidant defense mechanism of *Leishmania* parasite relies on cascades of three enzymes namely trypanothione synthetase (TryS), trypanothione-recycling flavoenzyme trypanothione reductase (TryR) and tryparedoxin-recycling enzyme tryparedoxin peroxidase (Px). A dithiol, trypanothione [$T(SH)_2$] is the central reductant metabolite of parasite redox metabolism is synthesized by TryS and $T(SH)_2$ is kept in reduced state by TryR enzyme whose functionally analogous enzyme found in host is GSH/glutathione reductase (*Krauth-Siegel and Inhoff, 2003; Krauth-Siegel et al., 2003*). Why parasite has evolved with $T(SH)_2$ still remains an enigma. The $T(SH)_2$ transfers its reducing equivalent to thioredoxin and tryparedoxin, which in turn reduces peroxidase for detoxification of toxic reactive species generated during synthesis of hydroperoxide and deoxyribonucleotide. By keeping low molecular mass thiols ovathiol, glutathiol trypanothione and monoglutathionyl spermidine (Figure 1.8) in their reduced state, the parasite facilitate the cellular metabolic pathways (*Cunningham et al., 2001*).

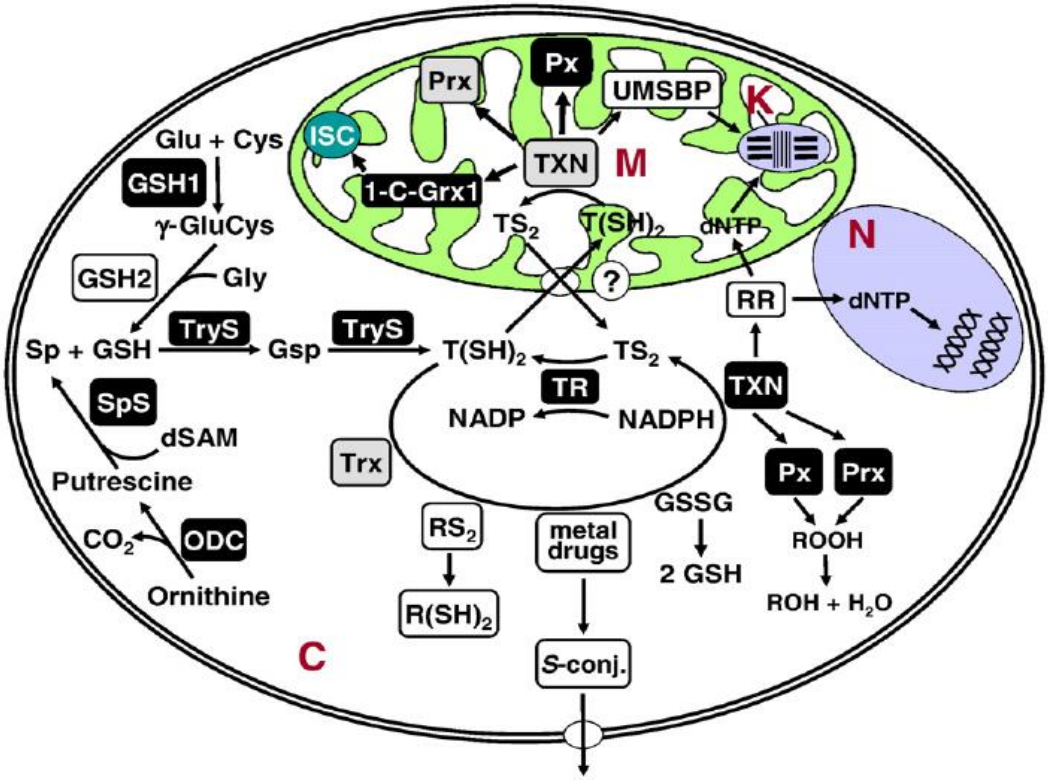


Figure 1.7: **Redox metabolism of *Leishmania* parasite:** Glutathione and spermidine are used by trypanothione synthetase (TryS) for trypanothione synthesis. Trypanothione reductase (TR) reduces trypanothione [$T(SH)_2$] which helps in removal of ROS and DNA synthesis. In the cytosol, $T(SH)_2$ reduces disulfides in proteins (RS_2) such as thioredoxin (Trx), tryparedoxin (TXN) and glutathione disulfide (GSSG) as well as dehydroascorbate (not shown). Trypanothione disulfide generated in these reactions is reduced back to the active dithiol by the NADPH-dependent trypanothione reductase (TR). $T(SH)_2$ can be conjugated to metal-containing drugs (S-conj) that are sequestered inside the cell or extruded, probably by specific transporter. 2-Cys-peroxiredoxins (Prx) and glutathione-peroxidase-type enzymes (Px) act as tryparedoxin peroxidases which detoxify hydroperoxides (ROOH) by converting them into the respective alcohol (ROH). TXN delivers also electrons to ribonucleotide reductase (RR) which synthesizes the deoxyribonucleotides (dNTP) required for DNA synthesis**.** (Figure and part of figure caption adopted with permission from Biochimica et Biophysica Acta. *1780*, 1236–1248)

$T(SH)_2$ is capable of reducing Fe and NO reactive species into a stable complex, dinitrosyl iron with an efficiency of 600 times faster than the GSH reductase mechanism of the mammalian host. This high reducing efficiency of $T(SH)_2$ protects *Leishmania* parasite from possibly hazardous nitric oxide species. The redox metabolic divergence between *Leishmania* and the mammalian host and the sensitivity of parasite towards oxidative stress attract enzymes of trypanothione based redox metabolism of parasite as potential drug targets for anti-leishmanial drug development (*Ascenzi et al., 2003*). Hence, specific inhibitors against the redox enzymes of the parasite may be an ideal drug that will kill parasite without cross firing the host components.

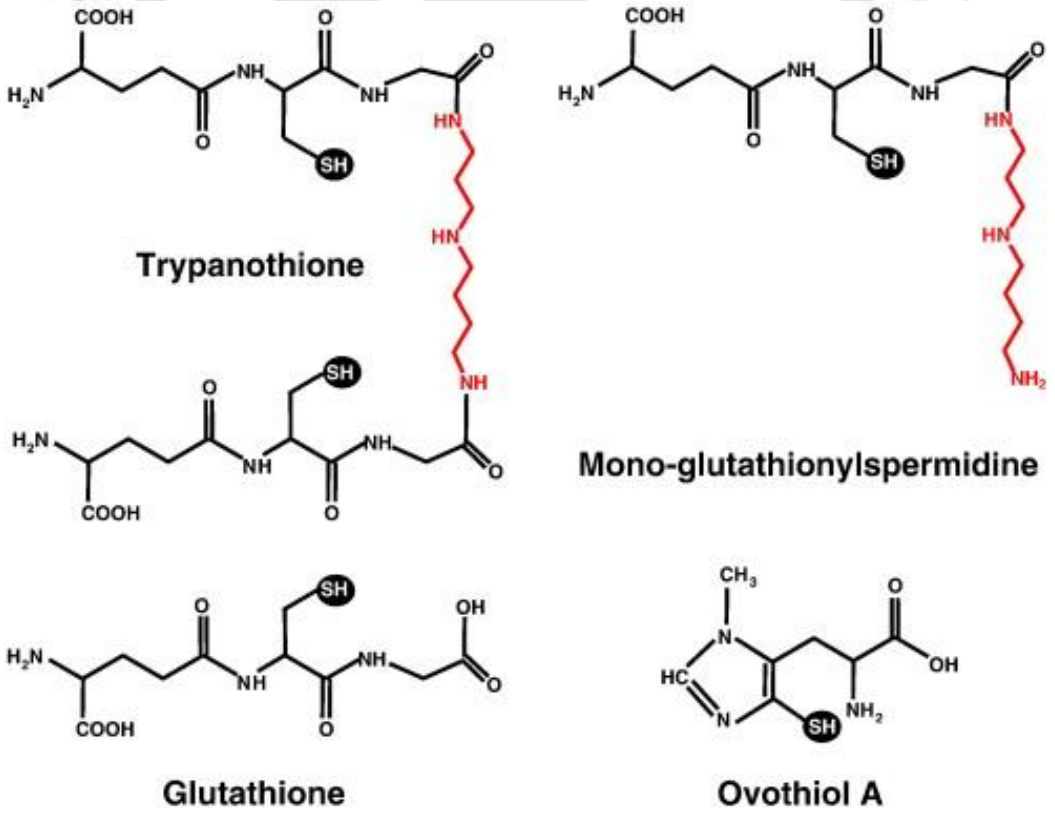


Figure 1.8: Low molecular mass thiols occurring in trypanosomatids. The polyamine moiety in the trypanothione [bis(glutathionyl)spermidine] and mono-glutathionylspermidine molecules are depicted in red. Sulfhydryl groups are highlighted by a black background. (Figure and part of figure caption adopted with permission from Biochimica et Biophysica Acta. *1780*, 1236–1248)

***1.2.7.8.1 Trypanothione Synthetase (TryS)*:** TryS (EC 6.3.1.9), an enzyme responsible for biological synthesis of trypanothione [$T(SH)_2$] in kinetoplastid group of parasitic protozoans such as *Leishmania*. As $T(SH)_2$ being the principal thiol in kinetoplastids group of parasites, TryS plays a pivotal role in maintaining the cellular redox balance by synthesizing $T(SH)_2$. The TryS carries out the biosynthesis of $T(SH)_2$ by covalently attaching two glutathione (GSH) onto spermidine. Reduced trypanothione is known to involve in carrying out many important cellular functions, such as detoxification of H_2O_2, detoxification of metals and drugs, maintaining the redox balance by protein disulfide reduction and indirectly involve in synthesis of deoxyribonucleotide (Figure 1.9). Hence, inhibition of biosynthesis of $T(SH)_2$ can lead to the immense stress on biochemical functions of the parasite and finally leading to death of the parasite. The enzyme, TryS, is an attractive drug target, as it is essential for the survival of the parasite and its absence in host system attracts search for new drugs targeting TryS. In *T. brucei*, the essentiality of TryS for survival of the parasite was shown by gene knockout studies, which genetically validated TryS as a drug target (*Comini et al., 2004; Ariyanayagam et al., 2005*). There was a gradual decrease upto 90% in the levels of $T(SH)_2$ in knockouts of *T. brucei* for 24 h. With the decline in the levels of $T(SH)_2$ upto 10 %, the parasite fails to fight against oxidative stress and sensitized to even a small extracellular stress. Recently it was also shown that the antioxidant enzymes of the parasite including TryS were associated with the virulence in *T. cruzi* (*Piacenza et al., 2009*), thus highlighting the importance of TryS as a potent drug target. There was a struggle for the TryS inhibitors, in search for TryS inhibitors it was found that most of the trypanothione reductase (TryR) inhibitors are known to inhibit TryS (*Torrie et al., 2009*) and also screening molecular libraries helped identify few of the inhibitors (*Saudagar and Dubey, 2011*). Very recently, design and synthesis of a good number of *Trypanosoma brucei* TryS inhibitors have been reported and known to show promising results for drug development (*Spinks et al., 2012*). These compounds having been tested on parasite survival showed prominent results conforming on-target activity of the inhibitors within the parasite. In a process of drug discovery against kinetoplastids group of parasites TryS have proven to be very vital target and showed promising results.

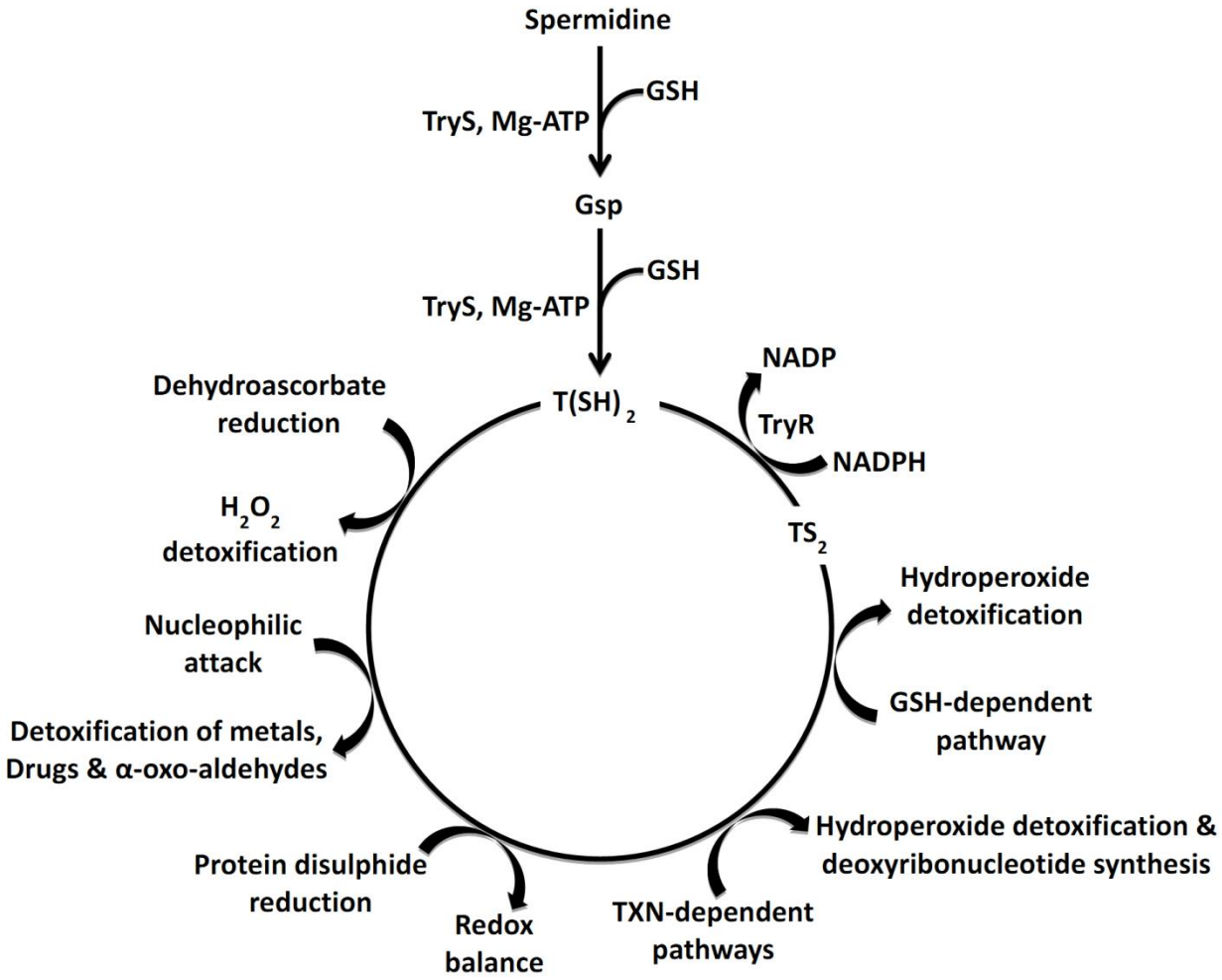


Figure 1.9: Schematic diagram representing the various metabolic functions carried out by trypanothione [$T(SH)_2$]

The importance of TryS in parasite survival and also virulence is shown to be dependent on the levels of $T(SH)_2$. In a recent report the kinetic construct of *T. cruzi* $T(SH)_2$ metabolism was studied by mimicking physiological conditions of enzymes and metabolite concentrations as that in the parasite to identify the enzymes involved in controlling $T(SH)_2$ synthesis and concentration (*Olin-Sandoval et al., 2012*). It was found that the flux of $T(SH)_2$ is mainly maintained by γ- glutamylcysteine synthetase (γ –ECS) and TryS and partly by spermidine uptake. Trypanothione reductase (TR) is reported to show negligible $T(SH)_2$ flux control. The model predicted that by inhibition of TryS and γ –ECS by 63 and 58 %, respectively, the flux of $T(SH)_2$ can be decreased by 50 %. In this model, the importance of

TryS in maintaining $T(SH)_2$ concentrations is highlighted. Experiments in the living system is required for further clarification of role played by TryS in maintaining $T(SH)_2$ flux in the cell. TryS along with γ–ECS can emerge as a promising multi-target strategy in treating diseases caused by kinetoplastids group of parasites. The important role played by TryS in maintaining $T(SH)_2$ fluxes in relevance to monitoring other enzymes is still being studied. Taking the advantage of TryS being absent in the host and by exploring the biochemical pathways that regulate the synthesis of $T(SH)_2$, TryS can emerge as a major drug target in treating leishmaniasis.

***1.2.8 Development of New Anti-leishmanial Drugs*:** Anti-leishmanial drug development have taken new impetus in past decades mainly because of the growth in parasite biology, understanding parasite metabolism, parasite genome sequence, new assay techniques, new bioinformatics tools and moreover a big number of collaborations and networks and agencies to encourage the discovery of new anti-leishmanial agents. At present, the progresses of both natural and synthetic molecules have significant prominence in the hunt of new therapeutic substitutions.

***1.2.8.1 Anti-leishmanial Synthetic Compounds*:** In the recent year's development of new drug like molecules have significantly evolved mainly due to advances in technology of structural and molecular biology and also computational chemistry. The introducing structural changes in native molecule (called lead compound) to acquire new derivatives has been the most successful methodology for the design of new drugs based on well-known and validated target molecules in the parasite (*Liñares et al., 2006*). Studying relation between the lead molecule and the validated target have provided the knowledge of physicochemical and structural properties drugs and targets and as well as helped in identifying active pharmacophore group for drug development (*Liñares et al., 2006*). The hybrids with pharmacophore group may be achieved with the aim to enhance the efficacy and decrease the toxicity of the compound. This method along with bioinformatics tools has potentials to virtually screen the potential compounds.

Subsequently, the design of selective inhibitors has increased the possible means for monitoring the parasites growth without injuring the host cells (*Santos et al., 2008*). The

most promising specific targets are redox enzymes of the parasite such as TryS and TryR (*Saudagar and Dubey, 2011; Krauth-Siegel et al., 2003*) kinetoplast (*Motta, 2008*), cysteine proteases (*McKerrow et al., 1999*), topoisomerases (*Das et al., 2008*), fatty acid and sterol pathways (*Roberts et al., 2003*), mitochondria (*Sen and Majumder, 2008*). Anti-leishmanial property of many synthetic products has been demonstrated. The anti-leishmanial property azasterols is mediated through inhibition of 24-methyltransferase and shows activity against both promastigote and amastigote stages of *Leishmania* parasite (*Magaraci et al., 2003*). The in vitro anti-leishmanial activity of new alkyl-lysophospholipid derivatives edelfosine and ilmofosinetested against *Leishmania donovani* promastigotes and amastigotes demonstrated high activity (*Azzouz et al., 2005*). Certain III NAD-dependent deacetylase is known to get inhibited by nicotinamide, which showed in vitro anti-leishmanial activity on *Leishmania infantum* promastigotes and amastigotes (*Sereno et al., 2005*). Similarly other examples are given Table 1.2. Moreover, efforts are being made to identify inhibitors of *Leishmania* parasite by screening molecular library. The screening chemical library is an advantageous in identifying the lead compounds but is laborious time consuming at the same time. For a known validated target, the inhibitor screening process can be made faster using computational screening of molecular libraries against the target protein (*Venkatesan et al., 2011a; Venkatesan et al., 2011b*). The advantage of synthetic drugs is that they can be synthesized according to the market need and more over cost can be subsidized significantly for endemic regions and low intellectual property complications (*Kingston and Newman, 2005*). However, synthetic drugs may show high toxicity and with very low number have been clinically studied.

***1.2.8.2 Potential Anti-leishmanial Natural Products*:** Many people in endemic regions mostly depend on traditional treatment methods which mainly involves the use of medicinal plants (*Fournet and Muñoz, 2002; Chan et al., 2001*). The natural products represent wide source of chemical structures with extensive diversity and accessibility in nature. The drug discovery research program promoted by Tropical Diseases Program of the World Health Organization (TDR/WHO) has considered an urgency of pharmacological research of plants (*Chan et al., 2001*). In the past few years' extensive research of natural products against *Leishmania* parasite has been done. Few studies have discovered the exploration of new

molecules from marine sources or microorganisms such as a glycoprotein extracted from the sea sponge *Pachymatisma johnstonii*, have high in vitro activity against *Leishmania* species (*Le Pape et al., 2000*) and a fungal metabolite aphidicolin extracted from *Nigrospora sphaerica* have inhibited both *Leishmania donovani* promastigotes and amastigotes growth (*Kayser et al., 2001*). Nevertheless, plants have been the major sources of natural products. The anti-leishmanial property of plant extracts from diverse geographic zones has been reported. The oil extracted from a Brazilian medicinal plan *Croton cajucara* induces nitric oxide production and effectively inhibits *Leishmania amazonensis* growth (*Rosa et al., 2003*). The essential oils of few plants contain nerolidol which is reported to have anti-leishmanial property and effectively inhibit the growth of *Leishmania* species, further their mode of action is predicted to interfere with the sterol synthesis pathway of the parasite (*Arruda et al., 2005*). Many studies have reported the identification of potential molecules purified from natural source, which exhibited anti-leishmanial activity. Table 1.3 lists few of the most capable compounds produced from natural sources. Due to high toxicity shown against host cells, most of the compounds fail; these compounds need to be hybridized with different functionalization to optimize their anti-leishmanial activities with lower toxicity.

Table 1.2: Anti-leishmanial activities of synthetic compounds and their mode of action

Compound	Mode of action	Anti-leishmanial activity	Reference
Azasterols	Inhibitors of 24-methyltransferase	Showed activity against promastigotes of *L. donovani* and axenic amastigotes of *L. amazonensis*	Magaraci et al., 2003
Nicotinamide	Inhibitor of certain III NAD-dependent deacetylase	Caused in vitro inhibition of *L. infantum* promastigotes and amastigotes	Sereno et al., 2005
3-substituted quinolines	Potential activators of macrophages	Anti-leishmanial in vitro effects against *L. chagasi* promastigotes and amastigotes was observed	Tempone et al., 2005
Triazole SCH 56592	Inhibitor of ergosterol synthesis pathway	Exhibited in vitro and in vivo activity against *L. amazonensis* and *L. donovani*	Al-Abdely et al., 1999
9, 9-dimethylxanthene tricyclics	Inhibitors of trypanothione reductase	Caused in vitro inhibition of amastigotes of *L. donovani*	Chibale et al., 2000
Edelfosine and Ilmofosine	New alkyl-lysophospholipid derivatives	Demonstrated high in vitro activity against *L. donovani* promastigotes and amastigotes	Azzouz et al., 2005
N-acetyl-l-cysteine	Precursor of glutathione	Showed in vivo activity against *L. amazonensis* in BALB/c mice	Chagas et al., 2008
Perifosine	New alkyl phospholipid derivatives	Displayed significant activity against promastigotes of *L. braziliensis, L. amazonensis, L. major* and *L. infantum*	Cabrera-Serra et al., 2007

Table 1.3: Anti-leishmanial activities of natural compounds and their source

Compound	Source	Anti-leishmanial activity	Reference
Canthin-6-one alkaloids	*Zanthoxylum chiloperone*	Demonstrated in vivo activity in BALB/c mice infected with *L. amazonensis*	Ferreira et al., 2002
Licochalcone A	*Chinese licorice*	Exhibited activity in vitro and in vivo against *L. major* and *L. donovani*	Chen et al., 2003
Trichothecenes	*Holarrhena floribunda*	Exhibited anti-leishmanial activity against promastigotes and amastigotes of *L. donovani*	Loukaci et al., 2000
2', 6'- dihydroxy-4'methoxychalcone	*Piper aduncum*	Exhibited in vitro activity against promastigotes and amastigotes of *L. amazonensis*.	Caio et al., 1999
Maesabalide III	*Maesa balansae*	Caused in vitro and in vivo activity against *L. donovani*	Maes et al., 2004
Coronaridine	*Peschiera australis*	Showed in vitro activity against promastigotes and amastigotes of *L. amazonensis*	Delorenzi et al., 2004
Parthenolide	*Tanacetum parthenium*	Displayed activity against promastigotes and amastigotes of *L. amazonensis*	Tiuman et al., 2005
Plumbagin	*Pera benensis*	Demonstrated in vivo activity in BALB/c mice infected with *L. amazonensis* and *L. venezuelensis*	Fournet et al., 1992

***1.2.9 Significance of current research*:** Several parasitic specific protein/metabolic pathways such as polyamine biosynthesis, sterol biosynthesis, glycosomal process, MAP kinases, topoisomerases, metacaspases, and parasite thiol metabolism have been identified as targets for anti-leishmanial drug discovery. Extensive research is needed to identify anti-leishmanial agents that can inhibit parasite specific enzymes of unique metabolic pathways of the *Leishmania* parasite without cross firing the mammalian host cells. Such parasite specific inhibitors have a huge scope to emerge as potential drug for leishmaniasis. Natural compounds have always been the main source of molecule in drug discovery process due to their structural and metabolic diversity. Several plant extracts have been reported to contain anti-leishmanial activity, but most of their mode of action is not known. The anti-leishmanial property of most of the plants has been validated by generic means very few have been validated by chemical means. The search for inhibitors against known validated targets can be made faster with the help of bioinformatics tools. Computational screening of available molecular libraries against known targets will help in sorting out the high affinity molecules that are most likely to be the inhibitors of the target studies. Finally the screened molecules can be validated by chemical means to identify the potential inhibitor of the target. Screening of molecular library and docking studies against known target, TryS, was one of the main initial tasks that I have undertaken during my Ph.D. research. The research in this thesis is primarily focused on the validated specific target enzymes of parasite for identification of novel inhibitors that can emerge as potential drug candidates for leishmaniasis. Our efforts for the identification of new inhibitors, essential for the control of leishmaniasis relay mainly on screening of potentially effective compounds against parasite specific target and parasite growth. We have explored the metabolically diverse redox enzymes of the parasite for specific targeting. Trypanothione synthetase (TryS), one of the key enzymes of parasite's redox metabolism was studied. We initiated our study by *in silico* homology modeling of TryS of *L. donovani* which was used for virtual screening of natural product data set to identify the potent inhibitors of TryS. The top hits identified were chemically evaluated. We have cloned and expressed *L. donovani* TryS (*Ld*TryS) in bacterial expression system and enzymatically active protein was purified. Further, the biochemical properties of *Ld*TryS enzyme were studied. The *Ld*TryS inhibition studies were carried out with commercially available top hits identified in *in silico* screening. We have identified four compounds

tomatine, conessine, uvaol and betulin that inhibited *Ld*TryS and also were effective anti-leishmanial agents. Betulin was further explored to understand molecular mechanism of parasite death. It was identified that betulin induced ROS generation, triggers the apoptotic cascade in *L. donovani* promastigote cells. Further, we found that betulin significantly decreases parasite burden inside the macrophage cells in vitro. Functionalized carbon nanotubes attached betulin (f-CNT-BET) was formulated, which was more effective in targeted delivery of drug to *L. donovani* amastigote parasite inside the macrophage cells in vitro. Further, in our regular screening process for inhibitors against redox enzyme of the parasite, we have identified oxabicyclo derivatives as a new class of anti-leishmanial agent that inhibited TryS and TryR redox enzymes of *Leishmania*. We have explored the molecular mechanism of parasite death caused by the most potent oxabicyclo derivative (PS-203). We found drastic decrease in parasite $T(SH)_2$ due to the inhibitory effect caused by PS-203 that led to accumulation of ROS in parasite and caused apoptotic death. Further, the effect of oxabicyclo derivative was tested on *L. donovani* amastigote parasite in in vitro and in vivo studies. This complete work presented in the thesis is divided into four main specific objectives:

i. Molecular modelling of trypanothione synthetase (TryS) of *L. donovani* and virtual screening of natural product dataset for inhibitors of trypanothione synthetase.

ii. Cloning, expression, characterization and inhibition studies on trypanothione synthetase, a drug target enzyme, from *Leishmania donovani*.

iii. Anti-leishmanial Property of Betulin: Depicting the molecular mechanism of parasite death and its targeted delivery.

iv. Molecular mechanism underlying antileishmanial effect of oxabicyclo derivatives: Inhibition of key redox enzymes of the parasite.

Chapter II

Molecular modelling of trypanothione synthetase (TryS) of *Leishmania donovani* and virtual screening of natural product dataset for inhibitors of trypanothione synthetase.*

2.1 Abstract

Trypanothione synthetase (TryS) is a validated target against leishmaniasis. In search for new drugs, the current study aimed to identify potential natural product inhibitors of trypanothione synthetase which can be further developed as anti-leishmanial drug. This chapter reports modelled structure of trypanothione synthetase (TryS) of *Leishmania donovani*. The quality of model is validated by PROCHECK validation package. Further, *in silico* virtual screening of a natural product data set of 800 diverse chemical entities was performed. The modeled TryS structure was used in the virtual screening process and docking studies to identify potential lead compounds. The best top hit compounds were sorted based upon their binding energy. The top hits were structurally similar; implying that compounds with such chemical entities can be potential inhibitors of the enzyme. The interactions were also conserved; either they bind residues surrounding the ATP binding cleft which act as anchoring residues or to the residues surrounding the substrate binding.

*** Part of the work is published in *Journal of Proteins and Proteomics*. 2, 41–48**

2.2 Introduction

Leishmania is a genus of trypanosomatid protozoan parasites causing widespread diseases collectively called leishmaniasis. The most fatal form of the disease is visceral leishmaniasis caused by *Leishmania donovani*. Glutathione in conjugation with glutathione reductase and glutathione peroxidase maintains the redox-homeostasis in mammals. In case of trypanosomatids similar role is played by trypanothione/trypanothione reductase system (*Colotti and Illari, 2011*). Trypanothione is a dithiol conjugate and the enzyme trypanothione reductase helps to maintain the thiol in reduced form (*Fairlamb et al., 1985*). The lack of functional alternatives to the thiol metabolism and the parasite's sensitiveness to oxidative stress combined with the absence of such system in humans makes the enzymes belonging to this pathway attractive target for drug design (*Oza et al., 2005*). Enzymes, such as trypanothione reductase, tryparedoxin and trypanothione synthetase (TryS) have been validated as drug targets for trypanosomiasis (*Colotti and Illari, 2011*). Trypanothione synthetase is one of the key enzymes of the pathway which is involved in the synthesis of trypanothione and also maintains the levels of polyamines which are critical for cellular proliferation and differentiation (*Wilkinson et al, 2003; Comini et al., 2004*). In case of *Leishmania*, TryS is bi-functional possessing synthetase and amidase activities. The enzyme catalyses the biosynthesis of trypanothione in an ATP dependent reaction and also hydrolyses dithiol (*Oza et al., 2008*). The crystallographic structure of TryS from *Leishmania major* is available in three different forms and it has revealed the presence of two major domains viz the N-terminal and the C-terminal domains. The C-terminal domain is a characteristic papain-like cysteine protease domain; the domain catalyzes biosynthesis of $T(SH)_2$ by step wise addition of two molecules of glutathione to one molecule of spermidine by hydrolysis of an ATP which binds to the ATP-common grasp fold present at the C-terminus. The N-terminal domain catalyzes hydrolysis of trypanothione to glutathione and glutathione-spermidine conjugate and then further into glutathione and spermidine (*Fyfe et al., 2008*).

TryS has been validated genetically and biochemically as a drug target in *Leishmania* and other trypanosomatids. In case of *Trypanosoma brucei*, techniques such as RNA interference and gene knockout studies have provided evidence that it is essential for survival of both bloodstream and procyclic forms of the parasite (*Torrie et al., 2009*). TryS can be a

potential target since it is important for survival of the parasites and inhibition of the enzyme will lead to alteration in polyamine levels as well as synthesis of thiols resulting in depletion of substrate for trypanothione reductase and disturbs redox homeostasis of the parasite. In the present study, *in silico* techniques was used for identification of inhibitors to TryS from *Leishmania* parasite. The homology modeling of TryS from *Leishmania donovani* is performed as crystal structure of *Leishmania donovani* TryS is still not yet solved. *Leishmania major* TryS as template for modeling *Leishmania donovani* TryS. The model generated was validated using procheck validation server and further used for virtual screening of natural product dataset for identifying inhibitors of TryS.

2.3 Materials and methods

***2.3.1 Sequence alignment*:** The complete protein sequence of TryS (UniProtDI: Q8IFU8) from *Leishmania donovani* (*Ld*TryS) was taken from UniProt database (http://www.uniprot.org). The closely related sequences present the in databases was evaluated by BLAST program (*Altschul et al., 1997*) (http://www.ncbi.nim.nih.gov/blast) against the PDB. The BLASTP was performed to search the structural similarity of these sequences with the protein data bank. The alignment was done among target protein sequences TryS (UniProtDI: Q8IFU8) with the template PDB ID: 2VOB using ClustalW (*Thompson et al., 1994*) (http://www.ebi.ac.uk/Tools/clustalw/).

***2.3.2 Homology modeling*:** *Leishmania major* trypanothione synthetase PDB code: 2VOB (*Fyfe et al., 2008*) taken from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) served as template for the homology modeling process based on its sequence similarity to *Leishmania donovani* TryS. MODELLER (*Marti-Renom et al., 2000*) is a computer program that generates 3-D model structures of proteins and their assemblies based on restraints on the spatial structure of the amino acid sequence(s) and ligands to be modelled. Several models were generated using MODELLER and were sorted based on their DOPE (Discrete Optimized Potential Energy) scores. Based on the lowest DOPE score the best models were selected in order to check the stereo chemical quality of the given generated model and the model reliability was checked using PROCHECK (*Laskowski et al., 1993*).

***2.3.3 Evaluation and validation of protein model*:** The *Ld*TryS models quality was evaluated with respect to stereo chemical geometry and energy. The ProSA (http://prosa.services.came.sbg.ac.at/prosa.php) (*Wiederstein and Sippl, 2007*) web server was used to calculate energy and verified the 3D structure by evaluating the local compatibility of the models with the template structure *Lm*TryS (PDB ID; 2VOB). Using PROCHECK validation package the best models structure was assessed.

***2.3.4 Receptor preparation*:** The generated 3D modeled structure of *Ld*TryS was used as receptor for virtual screening of small molecular dataset of natural products. The receptor *Ld*TryS was subjected to energy minimization before screening with application of the GROMACS (*Lindahl et al., 2001; Van Der Spoel et al., 2005; Hess et al., 2008*) to remove the bad steric clashes. In this implementation, all computations were carried out in vacuo, without reaction field.

***2.3.5 Inhibitor preparation*:** The small molecular natural product dataset selected for the study contains 800 compounds was obtained from the Microsource Discovery Systems, Inc. (http://www.msdiscovery.com/natprod.html). The molecules in NatProd collection (*Vogt et al., 2005*) were selected based up on their structure and chemical scaffolds; this includes classes such as alkaloids, flavonoids, sterols, terpenes, phenones, chalcones, and coumarins. SDF file containing two dimensional co-ordinates of the compounds available online was downloaded and ChemDraw 3D ultra 8.0 software (Molecular Modeling and analysis; Cambridge Soft Corp oration, USA) was used for generation of three dimensional structure co-ordinates of small molecules.

***2.3.6 Virtual Screening*:** Virtual screening was performed with AutoDock4.2 (*Morris et al., 1998*). Lamarckian genetic algorithm considered as one of the best algorithm to identify the lowest binding energy conformation was used in the study. AutoDock is an automated docking tool that predicts protein-ligand interactions and binding energies using an empirically calibrated force field, which is projected onto a regular grid for intermolecular energy calculations and the method also considers small molecules as flexible. The model of *Leishmania donovani* TryS generated using MODELLER and crystal structure of *Leishmania*

major TryS (PDB ID: 2VOB) were used in the study. The small molecule dataset used in the study was Microsource Discovery Systems, Inc. natural product dataset (http://www.msdiscovery.com/natprod.html) containing 800 compounds; the set was selected for the study owing to presence of structurally diverse chemical entities including alkaloids flavonoids, terpenes and coumarins. Polar hydrogen's were added and grid maps were prepared using the AutoGrid utility with (80 x 80 x 102) points to cover the entire region occupying the active site residues of TryS and grid spacing set to 0.375 Å. Grid maps were generated representing all the atom types present in protein and ligand along with electrostatic and desolvation maps. All docking simulations were performed with an initial population size of 300 and docking simulations consisted of 100 LGA runs in each run. The best individual from each generation was propagated to the next generation; remaining docking parameters were set to default. The docking results from each of the hundred calculations were clustered on the basis of root-mean-square deviation (RMSD) between the Cartesian coordinates of the atoms and were ranked on the basis of free energy of binding. The top-ranked compounds were visually inspected for their predicted mode of binding. The top hits obtained from virtual screening processes were tabulated and they were observed for consensus binding patterns to identify potential inhibitors of the enzyme.

2.4 Results

***2.4.1 Analysis of sequence and homology model*:** The TryS protein is expressed in both stages of the *Leishmania* parasite, promastigotes to amastigotes forms. TryS is a unique enzyme of the parasite which is absent in mammalian hosts making it an attractive target. The complete protein sequence of *Ld*TryS (UniProt ID: Q8IFU8) from *Leishmania donovani* was used in the study. *Ld*TryS protein contains 652 amino acids. The *Leishmania donovani* TryS has 95% identity and 96% similarity with template of *Lm*TryS from *Leishmania major* (PDB ID 2VOB). Both the protein sequences of *Ld*TryS and *Lm*TryS were aligned and shown in Figure 2.1 The *Ld*TryS sequence was used to generate the 3 dimensional structures using known crystal 3 dimensional structure from *Leishmania major* (PDB ID 2VOB). A total of ten models were generated by MODELLER and one with the lowest DOPE score model of *Ld*TryS was chosen. The model was structurally visualized using PyMol (*Delano, 2002*) software to find out the secondary structural elements (helices, sheets and coils etc.) in

the protein. *Ld*TryS contain two domains, C-terminal synthetase domain and N-terminal amidase domain (Figure 2.2A). The synthetase domain which catalyzes the biological synthesis of trypanothione is further sub divided into three sub-domains A, B and C (Figure 2.2B). These three sub-domains together form the active site pocket which was then compared with the template *Lm*TryS structure (Figure 2.2C).

Figure 2.1: Protein sequence alignment of *Ld*TryS (Q8IFU8) and *Lm*TryS (PDB ID: 2VOB) which is used as template for homology modeling of *Ld*TryS. There is 96% sequence similarity between *Ld*Trys and *Lm*TryS.

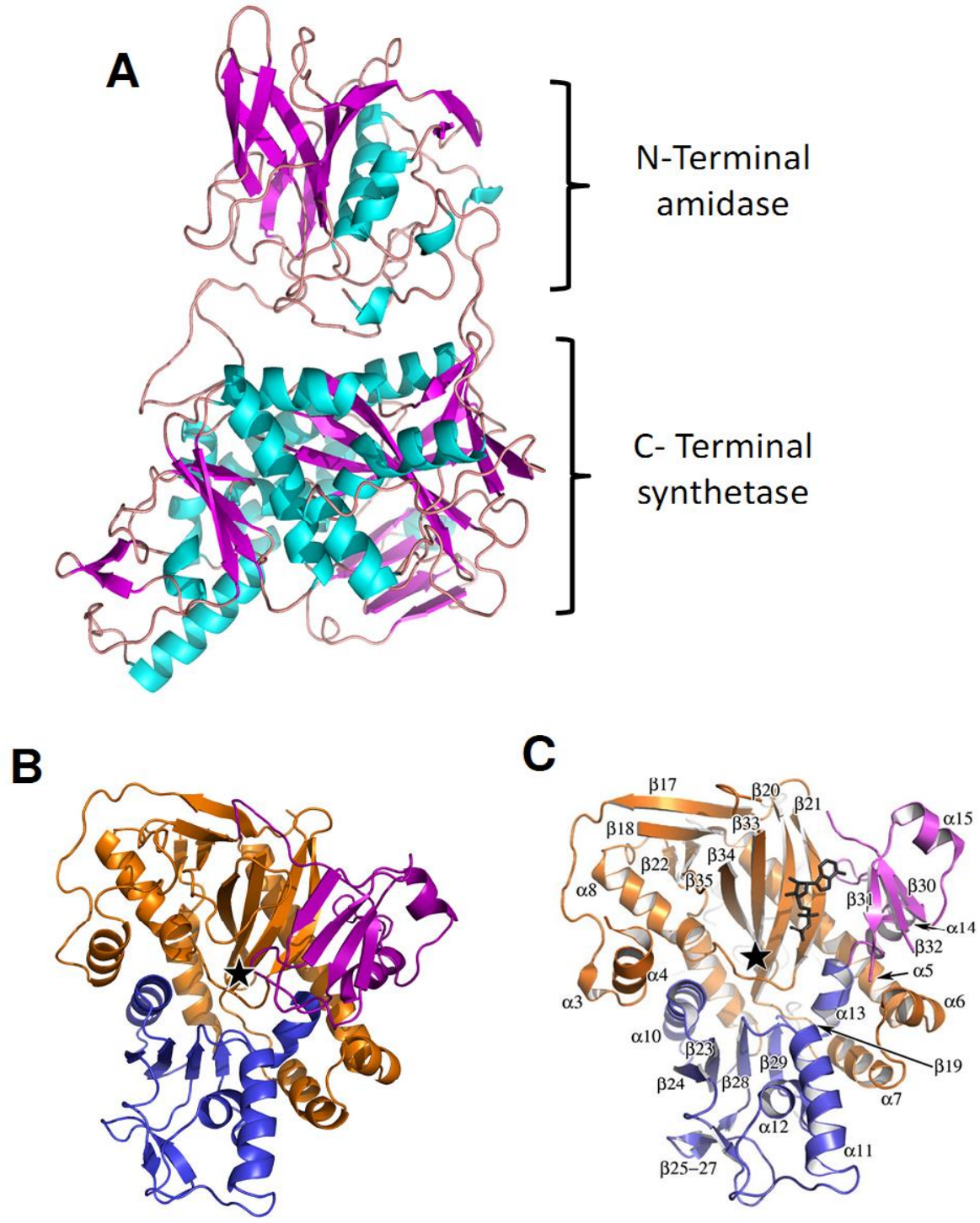


Figure 2.2: The modelled structure of LdTryS. **(A)** Predicted three dimensional structures of *Ld*TryS. It contains two domains, N-terminal amidase domain and C-terminal synthetase domain that catalysis the biosynthesis of trypanothione [$T(SH)_2$]. **(B)** Synthetase domain of modelled *Ld*TryS and **(C)** Synthetase domain of *Lm*TryS. The synthetase domain consists of three sub-domains**.** Subdomain A is colored *orange*, subdomain B is *blue*, and subdomain C is purple. A model of ADP (black sticks, based on structural comparisons) is included. (Figure C is taken from *J. Biol Chem*., 2008, 283, 17672-17680.)

***2.4.2 Validation of modelled TryS structure*:** The modeled structure of *Ld*TryS generated was validated before it was use in screening and docking studies of small molecular dataset for *Ld*TryS inhibitors. The model was validated using PROCHECK validation package. The Ramachandran plot was analyzed for stereochemical spatial arrangement. The data shows that 89.9% of residues are in most favourable region, 9.5% are in additional allowed regions, 0.4% are in generously allowed regions, and only 0.2% residues are in disallowed regions (Figure 2.3). The generated model of *Ld*TryS was validated by ProSA package in terms of Z score expressing largely the quality of the model and calculating the deviation of the total energy of the structure. The Z-score of the TryS is showed in plot with a dark black point in Figure 2.4. In this plot the Z-score value of the generated model of *Ld*TryS is −10.71, whereas the template *Lm*TryS crystal structure observed to be −9.73, signifying that the acquired model is reliable and close to experimentally resolved structure. The quality of the *Ld*TryS model was also assessed in terms of energy function of amino acid sequence. In general, folding energy of the *Ld*TryS exhibited lowest value as this accounts for the stability and nativity of the molecules. The energy profile of the homology modelled *Ld*TryS in comparison to that of the template *Lm*TryS crystal structure (PDB ID: 2VOB) is showed in Figure 2.5. The drift of the deviation of the protein folding energy in *Ld*TryS modelled structure is in good agreement with that of the template *Lm*TryS crystal (PDB ID: 2VOB).

***2.4.3 Identification of potential inhibitors of LdTryS by virtual screening*:** The virtual screening results discovered compounds with more satisfactory interactions with the active site residues of *Ld*TryS. Table 2.1, shows the top 25 hit compounds achieved through our virtual screening process, 100 LGA runs were accomplished with every compound in the dataset, and the conformations produced from each run were then clustered based up on their RMSD. The binding energy denoted in Table 2.1 is the sum of non-bonding and hydrogen bonding interactions of the ligand with key active site residues. Two distinct binding pockets were observed, one to the subdomain A (residues 695 -633) and the other spermidine binding domain of the enzyme. The key residues surrounding the cavity such as E407, E408, D403 and M459 are in interaction with ligands. Few residues are in hydrogen bonding interaction with ligand which makes them a highly favorable energy binding (Figure 2.6A). The ligands that bind to sub-domain of the protein is seen in hydrophobic interaction with residues 590 to

597 which are the anchoring residues surrounding the ATP binding site (Figure 2.6B). Consensus binding was observed among all class of inhibitors that have been studied within these two domains. Further, the top hits from two distinct binding pockets were also studied generating two dimensional ligand interaction ligplots (Figure 2.7).

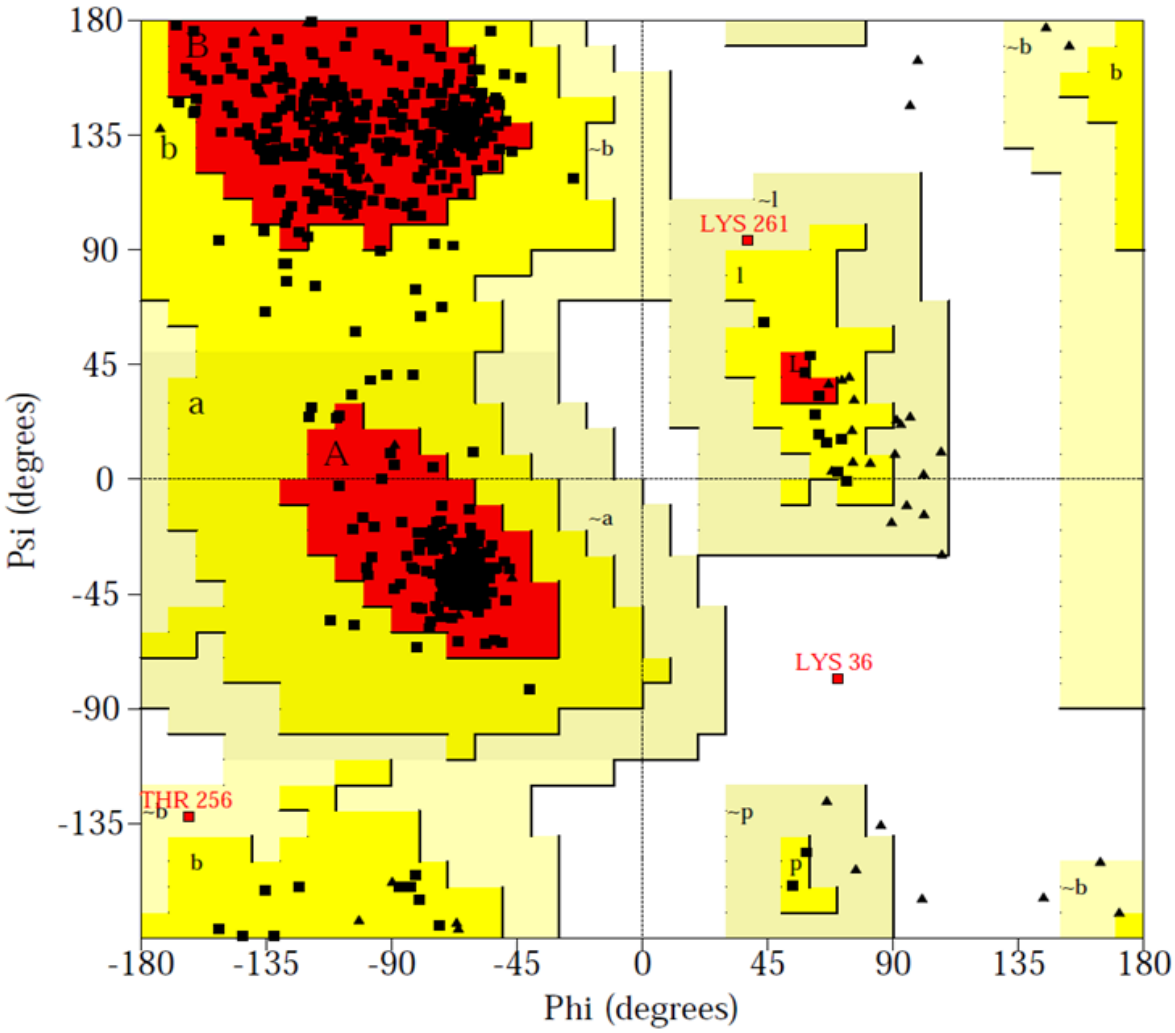


Figure 2.3: Ramachandran plot of modelled structures of *Ld*TryS model showing 89.9% amino acid residues in most favourable region; 9.5%, residues are in additional allowed regions; 0.4%, residues in generously allowed regions and 0.2% residues in disallowed regions.

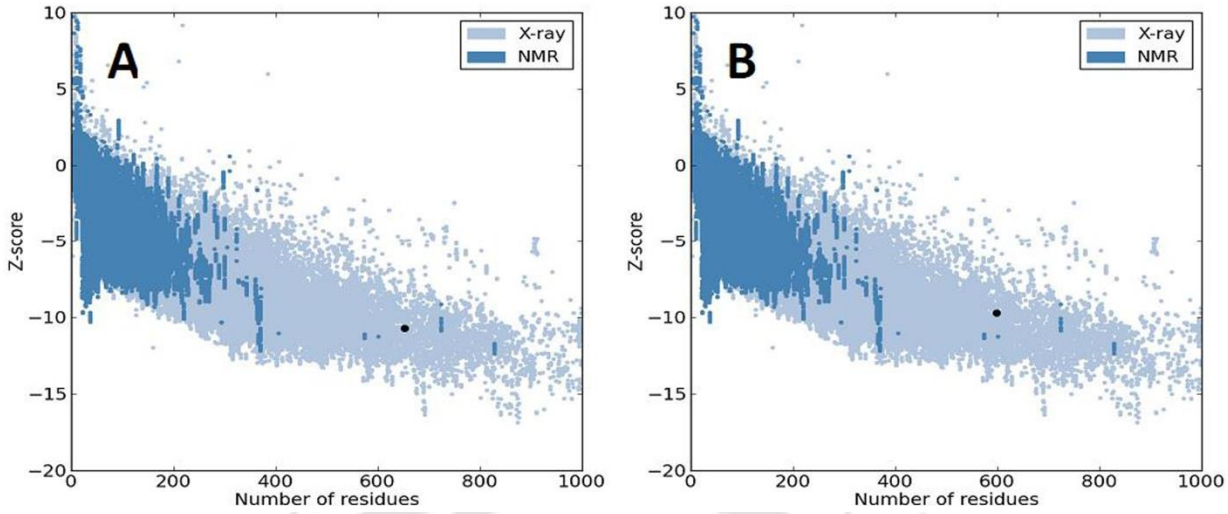


Figure 2.4: The *z*-score indicates overall model quality. In plot two dark black points represent Z-scores of the protein. **(A)** The Z score of modeled *Ld*TryS is –10.71; **(B)** The Z score of the template *Lm*TryS crystal structure is –9.73. The value is displayed in a plot (black spot) that contains the *z*-scores of all experimentally determined protein chains in current PDB. In this plot, groups of structures from different sources (X-ray, NMR) are distinguished by different colors. It is used to check whether the *z*-score of the input structure is within the range of scores typically found for native proteins.

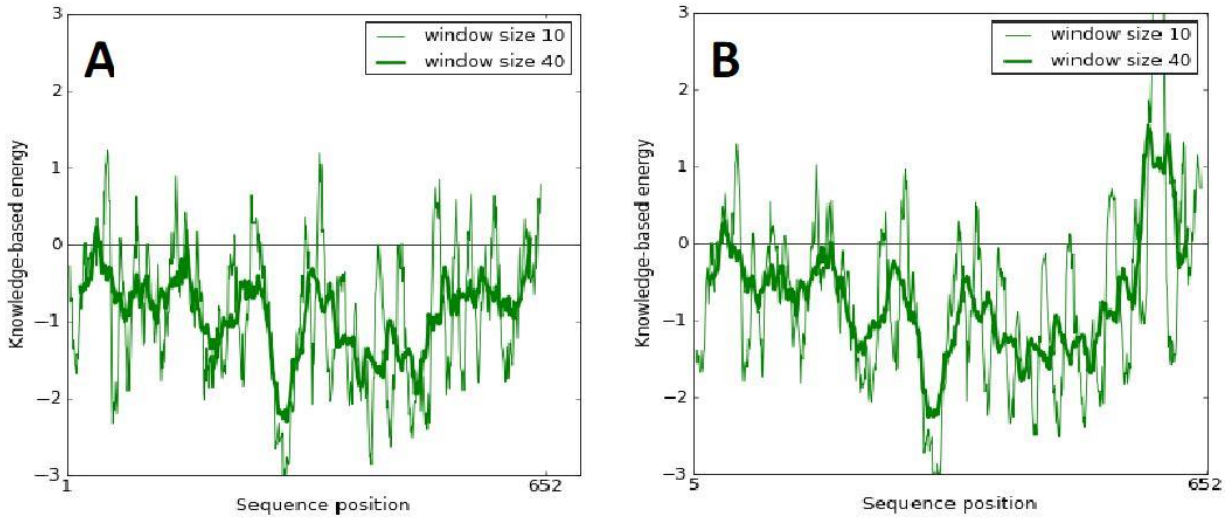
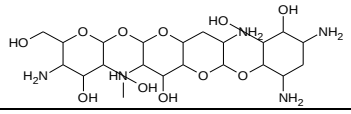
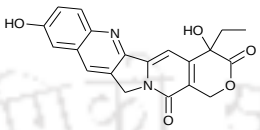
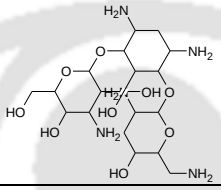
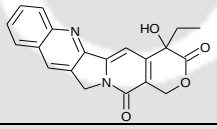
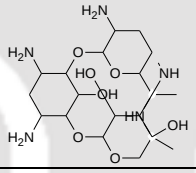
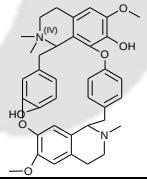
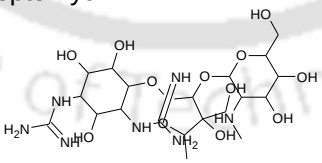
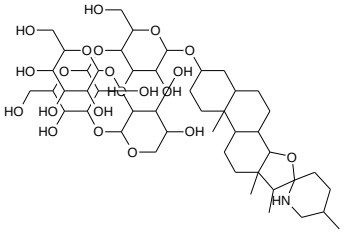
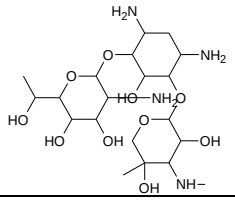
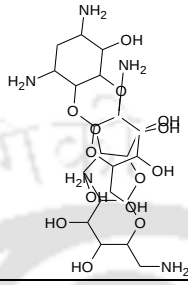
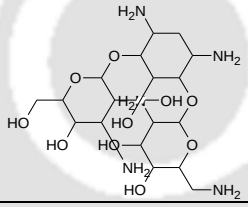
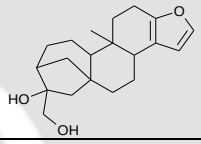
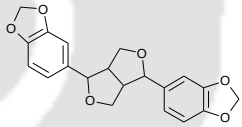
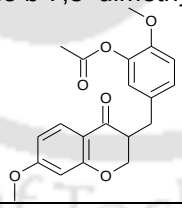
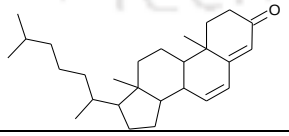
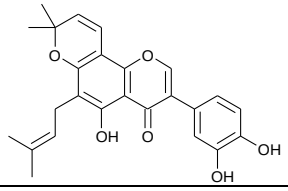
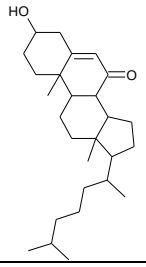
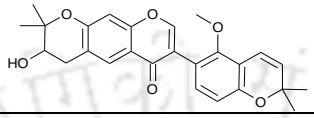
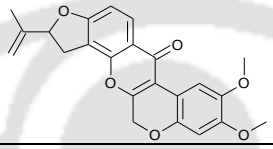
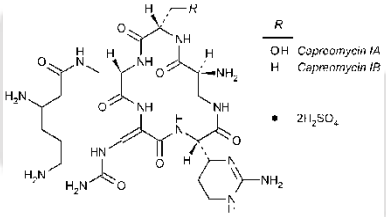
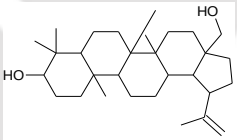
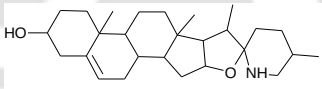
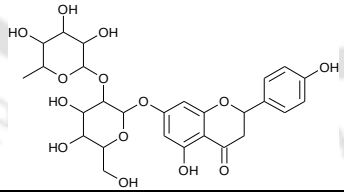
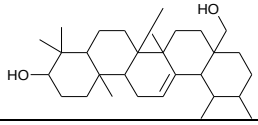
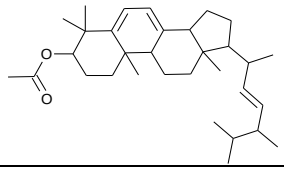


Figure 2.5: Energy profile of modeled *Ld*TryS (A) and template *Lm*TryS crystal structure (B). The plot shows protein model quality by plotting energies as a function of amino acid sequence position i. In general, positive values correspond to problematic or erroneous parts of the input structure. A plot of single residue energies usually contains large fluctuations and is of limited value for model evaluation. Hence the plot is smoothed by calculating the average energy over each 40-residue fragment s (i, i + 39), which is then assigned to the central residue of the fragment at position i + 19 (thick line). A second line with a smaller window size of 10 residues is shown in the background of the plot (thin line).

Table 2.1: Structure and docking statistics of top 25 ranked compounds**.** In the table, **E**- binding energy of the selected conformation in kcal/mol, and **T**- number of torsions, respectively.

S. No.	Compounds	E	T
1	Apramycin 	-11.64	16
2	10-hydroxycamptothecin 	-11.61	3
3	Tobramycin 	-11.44	16
4	Camptothecin 	-11.31	2
5	Gentamicin sulfate 	-11.25	13
6	Tubocurarine chloride 	-10.96	4
7	Dihydrostreptomycin 	-10.91	17
8	Tomatine 	-10.67	23

9	Geneticin 	-10.55	15
10	Paromomycin sulfate 	-10.51	22
11	Bekanamycin sulfate 	-10.5	17
12	Cafestol 	-10.27	3
13	Asarinin (-) 	-10.18	2
14	Deoxysappanone b 7,3'-dimethyl ether acetate 	-9.95	6
15	Cholest-4,6-dien-3-one 	-9.95	5
16	Pomiferin 	-9.9	6

17	7-oxocholesterol 	-9.86	6
18	Mundulone 	-9.86	3
19	Dehydrorotenone 	-9.8	3
20	Capreomycin sulfate 	-9.77	14
21	Betulin 	-9.76	21
22	Solasodine 	-9.74	16
23	Naringin 	-9.72	12
24	Uvaol 	-9.71	15
25	Ergosterol acetate 	-9.69	19

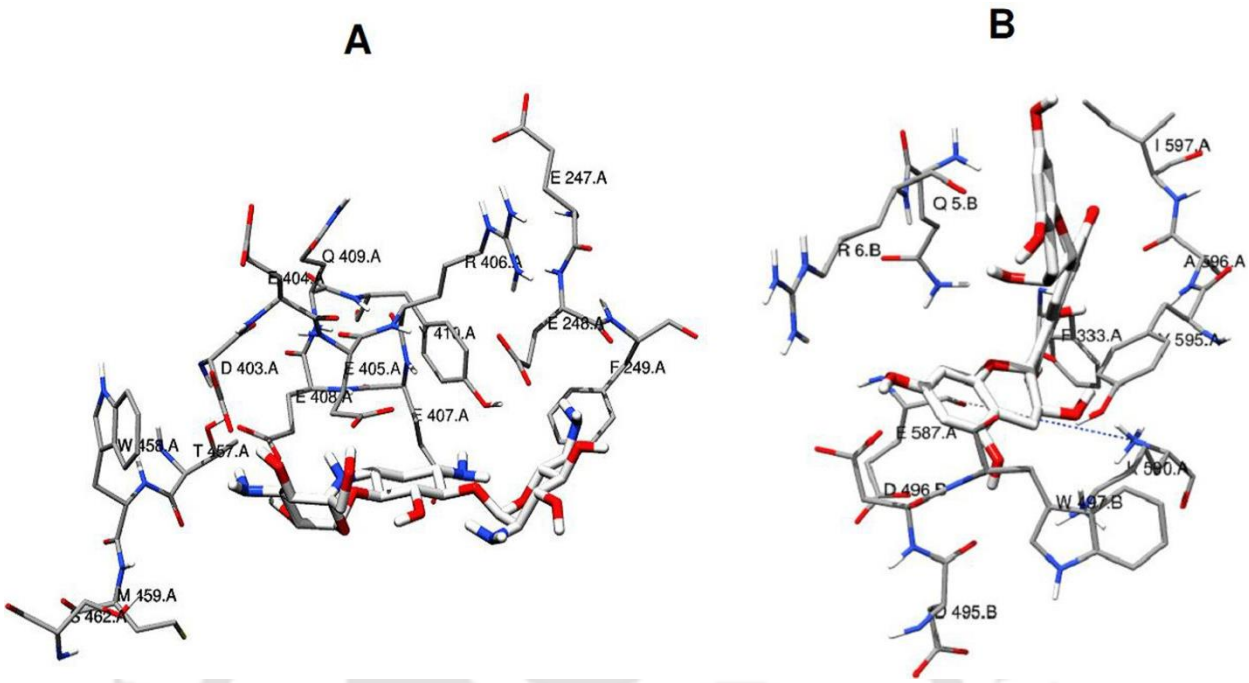


Figure 2.6: The 3D interaction of potential inhibitor with the enzyme. Inhibitors bind to the **(A)** spermidine binding domain of the enzyme or **(B)** sub-domain of the protein is seen in hydrophobic interaction with residues 590-597 which are the anchoring residues surrounding the ATP binding site.

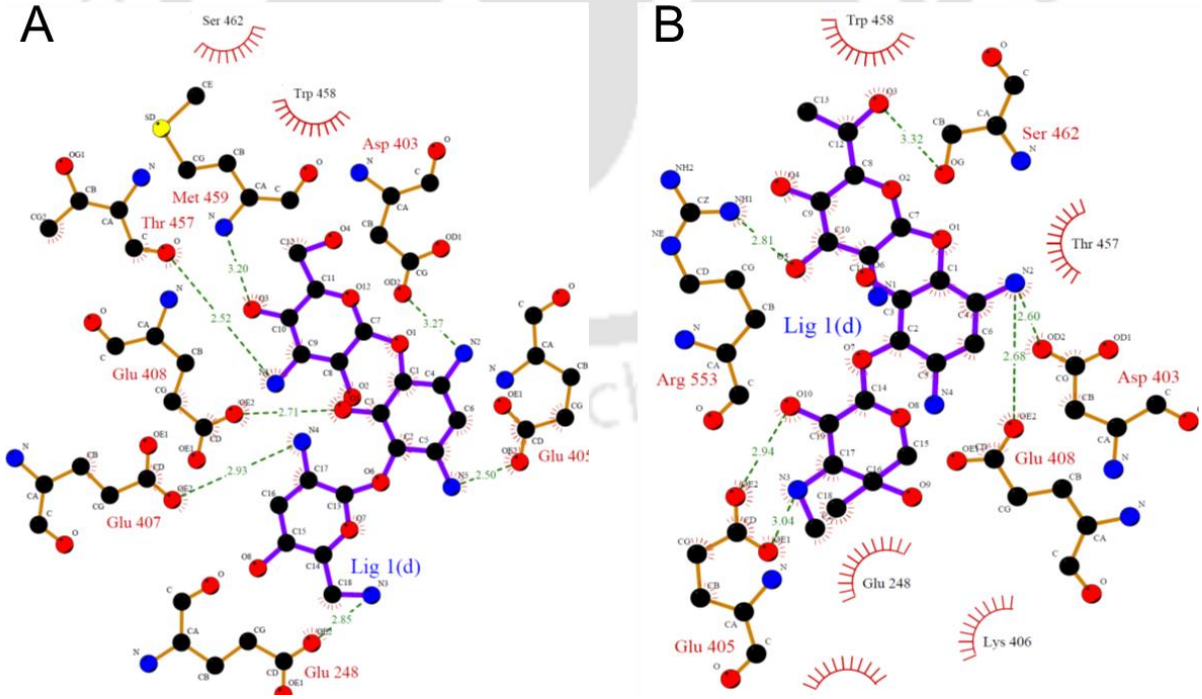


Figure 2.7: Interaction of potential inhibitor with the enzyme. Inhibitors bind to the **(A)** spermidine binding domain of the enzyme or **(B)** sub-domain of the protein is seen in hydrophobic interaction with residues 590- 597 which are the anchoring residues surrounding the ATP binding site.

2.5 Discussion

TryS of *Leishmania donovani* and *Leishmania major* were 96% similar, the active site residues were conserved and residues in the surrounding regions showed minimum divergence. The grid for docking simulations was placed over the synthase domain which comprises sub-domains. Three major sub-domains comprise the active site of synthetase enzyme- ATP binding site comprises of subdomains A and C, glutathione binding cleft is present mainly subdomain B and glutathionylspermidine binding cleft is present between subdomains A and B. Since the active site cleft is larger, the compounds bind in multiple orientations traversing all the active site domains. Two major interactions were observed at the active site region. At the end of the virtual screening process the compounds were sorted based upon their binding energy. The criterion that was set was to predict the highly favorable conformation, the run producing the lowest energy and also having highest number of conformation within the cluster. The top hits from the virtual screening processes were structurally analyzed for their potential as inhibitors of the enzymes. The top hits were structurally similar; implying that compounds with such chemical entities can be potential inhibitors of the enzyme. The interactions were also conserved; either they bind residues surrounding the ATP binding cleft which act as anchoring residues or to the residues surrounding the substrate binding site making them potential inhibitors of the enzyme. During the docking simulations, the compounds formed hydrogen bonds and were also seen in hydrophobic interaction with key residues of both the spermidine binding cleft and the ATP binding cleft, potentiating them to be possible inhibitors of the enzyme. These set of compounds can be studied in vitro for their activity against the parasite and can further chemically validated by performing inhibition studies with *Ld*TryS.

2.6 Conclusion

Homology modeling of trypanothione synthetase from *Leishmania donovani* was successfully perform using *Leishmania major* crystal structure (PDB ID: 2VOB) as template. The generated 3D model of *Ld*TryS demonstrated good overall structural quality which was validated using PROCHECK validation server. The molecular docking study with natural

product dataset was performed to discover suitable inhibitors for *Ld*TryS protein. We have identified potential anti-leishmanial compounds by targeting parasite specific enzyme TryS which is vital for survival of the pathogen. Our computational approach has provided an opportunity to identify natural products with potential anti-leishmanial activity for experimental validation, and to understand their effects on *Leishmania* parasite redox system. The identified compounds from natural sources are potential candidates for drug against leishmaniasis. The current study also points out potential of natural resources as therapeutic agents.

Chapter III

Cloning, expression, characterization and inhibition studies on trypanothione synthetase, a drug target enzyme from *Leishmania donovani.**

3.1 Abstract

Trypanothione synthetase, a validated drug target, synthesizes trypanothione from glutathione and spermidine. This chapter reports cloning, expressing, characterization and inhibition studies of trypanothione synthetase from *Leishmania donovani (Ld*TryS). The purified recombinant *Ld*TryS enzyme obeyed Michaelis-Menten kinetics. High substrate inhibition was observed with glutathione as substrate (K_m= 33.24 µM). Enzyme obeyed simple hyperbolic kinetics with fixed glutathione concentration varied other substrates. The K_m value for ATP was found to be 14.2 µM while spermidine (Spd) as substrate, the K_m value was 139.6 µM. Further, the enzyme was characterized with respect to stability and optimum activity conditions. *Ld*TryS was also screened for inhibitors. Tomatine, conessine, uvaol as well as betulin are identified as inhibitors of the enzyme. The inhibitors are tested for anti-leishmanial activity. Finally, effect of *Ld*TryS inhibitors on redox homeostasis of parasite gave a broader picture of their action against the target.

*** Part of the work has been published in *Biological Chemistry*, 2011, 392: 1113–1122.**

3.2 Introduction

Trypanosomatid protozoan of genus *Leishmania* are intramacrophage obligate parasites known to cause a vector born disease leishmaniasis, which is known for its diverse and complex characteristic infection. Leishmaniasis as endemic infection in tropics and sub-tropics regions is found in 88 countries ranging from deserts in western Asia to rainforests in the America, threatening 350 million people across the globe (*Stuart et al., 2008*). Despite of high death toll and wide spread of infection, there is a serious lack in treating leishmaniasis due to failure of present therapeutics. The serious limitation of drug against leishmaniasis includes high cost, poor efficacy, high toxicity and drug resistance (*Sundar et al., 2000*).

All living systems have adopted a mechanism to maintain cellular redox homeostasis. Likewise trypanosomatids have adopted a redox system which is unique in comparison with other living organisms. Trypanothione [bis (glutathionyl) spermidine; $T(SH)_2$], a polyamine-glutathione conjugate is used by trypanosomatids in maintaining cellular homeostasis (*Krauth-Siegel et al., 2008*). The analogous mechanism in humans and other living organisms is carried by glutathione (GSH). The pivotal role played by $T(SH)_2$ in various cellular processes include regulation of intracellular thiol redox balance, fighting chemical and oxidant stress, synthesis of deoxyribonucleotides and drug resistance (*Flohe et al., 1999; Fairlamb et al., 1992; Dormeyer et al., 2001; Krauth-Siegel et al., 1996*). $T(SH)_2$ is synthesized by trypanothione synthetase (TryS) in a sequential covalent conjugation of two GSH molecules onto spermidine (Spd). In early studies with *Crithida fasciculata*, it was assumed that two enzymes glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS) catalyzes biosynthesis of $T(SH)_2$ in a stepwise fashion (*Smith et al., 1992; Tetaud et al., 1998; Oza et al., 2002a*). But further studies on $T(SH)_2$ synthesis in *C. fasciculata* it was revealed that the TryS alone catalysis the synthesis of $T(SH)_2$ (*Comini et al., 2005*) like as in case of *Trypanosoma* species (*Oza et al., 2002b; Oza et al., 2003*). Knockout studies with TryS by RNAi in *Trypanosome brucei* revealed the absence of GspS and showed the importance of TryS for parasite survival and as a potential drug target (*Comini et al., 2004; Ariyanayagam et al., 2005*). In a study with *Leishmania major* two genes were reported which were similar to glutathionylspermidine synthetase (*GSPS*) and trypanothione synthetase (*TRYS). GSPS* in *L. major* is reported as a pseudo gene and the only

functional *TRYS* gene is known to express TryS which synthesizes $T(SH)_2$ (*Oza et al., 2005*). The uniqueness of TryS in synthesizing $T(SH)_2$ which maintains the redox system in trypanosomatids and its absence in humans makes it a potential drug target. TryS consists of two domains, N-terminal amidase domain and C-terminal synthetase domain. The amidase activity of TryS has been shown to be weak, which can hydrolyze $T(SH)_2$ back to GSH and Spd. The synthetase domain carries out the synthesis of $T(SH)_2$ in a stepwise covalently linking two GSH onto Spd by hydrolyzing two ATP molecules (*Fyfe et al., 2008*). Molecules that can inhibit TryS activity can evolve as effective drug candidates in treating leishmaniasis. TryS from *L. major, T. brucei* and *T. cruzi* is studied. There are no studies on the enzyme from *Leishmania donovani,* causative agent of visceral leishmaniasis. Only limited information of TryS inhibitors have been reported on *Trypanosoma brucei*, TryS (*Torrie et al., 2009*). In this study we report cloning, expression, purification and characterization of TryS from *Leishmania donovani* (MHOM/IN/1978/UR6). Further, we also report the kinetic properties of TryS from *Leishmania donovani* and inhibition studies with the natural compounds selected from MS discovery natural product data set. The compounds used for inhibition studies are from top hits identified early in docking studies with MS discovery natural product data set using modeled TryS of *Leishmania donovani* reported in chapter II.

3.3 Materials and Methods

***3.3.1 Organism and Reagents*:** *Leishmania donovani (*MHOM/IN/1978/UR6) promastigote used as a source for genomic DNA was provided by Dr. Hemanta K. Majumder (Indian Institute of Chemical Biology, Infectious Diseases & Immunology Division). For cloning and expression, *Escherichia coli* strains DH5α and BL21 Star (DE3) (Invitrogen) were used, respectively. All chemicals were of highest grade available from Sigma and Merck. Restriction enzymes and DNA-manipulating enzymes were from NEB or Fermentas.

***3.3.2 Parasite Culture and Genomic DNA Isolation*:** *Leishmania donovani* promastigotes were grown at 25°C in DMEM liquid media supplemented with 10% heat inactivated fetal calf serum, 40 μg/ml gentamicin. Genomic DNA was isolates using a non-toxic and versatile

protein salting-out method (*Rotureau et al., 2005*). The lysis buffer contain 10mM Tris-HCL pH 8, 5mM EDTA, 0.5% SDS, 200 mM NaCl, and 100 µg/ml proteinase K.

***3.3.3 PCR Amplification of Putative LdTRYS and Cloning in pET28a*:** A putative sequence of TRYS from *L. donovani* LV9 strain was identified from NCBI nucleotide data base with accession number AJ430863.1. Primers used to generate full length gene were: forward primer **(**LdTsF**:** 5'CAT **ATG** TCG TCT CTG CCG CGC GCG TCT3'), containing an *Nde*I site and start codon, and reverse primer (LdTsR: 5'GGA TCC **TTA** CTC GTC CTC GAC CAT CTC GTC3'), containing a *Bam*HI site and a stop codon (initiator and stop codon are in bold, restriction sites are underlined). PCR was performed in a 50 µl reaction volume containing 25 µl Dream *Taq* green PCR master mix (Fermentas), 1.0 µg each primer, 0.5µg of *L. donovani* genomic DNA and the final volume was adjusted with the nuclease free water. The PCR reaction conditions used are, initial denaturation 94°C for 4 min, 25 cycles: denaturation, 15s, 94°C; annealing, 30s, 50°C; and elongation, 2.0 min, 72°C. A final extension at 72°C was also included. PCR reaction generated a ~1.95 kb fragment which was cloned via TA vector pTZ57R/T (Fermentas) into the *Nde*I/*Bam*HI sites of pET28a to generate plasmid pET28a-*Ld*TryS. Initial conformation of the clone was done by restriction digestion (*Hind*III, which is a single cutter of *TRYS*) of PCR product amplified using pET28a-*Ld*TryS (plasmid isolated from positive colonies) as a template in PCR reaction. Two independent sequencing further conform the clone.

***3.3.4 Soluble Expression of LdTRYS*:** Typically, a 2.0 L (500 ml × 4) culture of BL21 Star (DE3)/pET28a-*Ld*TryS was grown at 37°C with moderate agitation (200 rpm) in a LB media, containing 50µg ml^{-1} kanamycin. When the culture reached an A_{600} of ~0.6, the culture was cooled to 25°C and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5mM. The cultures were grown for an additional 16 h at 25°C and then harvested by centrifugation. Using breaking buffer (30 ml) cell lysis was performed by sonication. Breaking buffer contains 50 mM Tris-HCl, pH 8.0, 0.2 M NaCl; 5 mM $MgCl_2$. Cell debris was separated and discarded after centrifugation (48,000 × *g*, 30 min, 4°C).

***3.3.5 Purification of LdTryS*:** Suspension containing soluble protein fraction was filtered and loaded onto a HIS-select high flow 6.4 ml cartridge (Sigma). Protein was eluted using imidazole, and fraction collected was dialyzed against 50 mM Tris-HCl and subjected to digestion with thrombin. Final dialysis was done against 50 mM HEPES, pH 8.0. The sample was then aliquoted and stored at 4˚C. The size and purity of *Ld*TryS was determined by SDS-PAGE 10% gel.

***3.3.6 Kinetic Analysis of LdTryS*:** The *Ld*TryS enzymatic activity was determined by monitoring release of phosphate using BIOMOL green (Enzo Life Science), which gives absorbance readout at 650 nm (*Torrie et al., 2009*). The *Ld*TryS activity assay was carried out at room temperature in a 100 µl reaction volume containing 100 mM HEPES, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 0.01% brij-35, 10 mM magnesium acetate, 20 nM recombinant TryS, and varying concentration of substrates (ATP, GSH, and Spd). The kinetic analysis studies were carried out using the above mentioned activity assay. The Michaelis constant for each of the substrate was determined in an end point assay. The pH optimum of the *Ld*TryS was determined in mixed buffer system containing 50 mM of each HEPES, MES [2-(*N*-morpholino) ethanesulfonic acid] and CHES [2-(N- cyclohexylamino) ethanesulphonic acid]. For temperature optimum studies 10 different temperature points were used in the range 15˚C- 60˚C. The effect of ionic strength was determined using assay buffer containing 100 mM HEPES buffer, pH 8.0, 0- 400 mM KCl or $(NH_4)_2SO_4$.

***3.3.7 TryS Inhibition Studies*:** *In silico* identification of hits for *Ld*TryS by studying MS discovery natural product dataset is reported in chapter II. Top hits of docking studies were screened for inhibition studies. Single point inhibition assays were carried out in flat bottom polystyrene, 96 well plates. Each assay was performed with 100 µl reaction volume containing 100 mM HEPES, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 0.01% brij-35, 10 mM magnesium acetate, 20 nM recombinant *Ld*TryS and varying concentration of substrate and test compound. When ATP was varied, GSH and Spd fixed at 0.3 and 10 mM, respectively; when GSH was varied, Spd and ATP were fixed at 10 mM each; and when Spd was varied, GSH and ATP were fixed at 0.3 and 10 mM, respectively. A nine point inhibitors curves were generated in 96 well plates. To check modality of inhibition by compounds studied, data sets were collected at six inhibitor concentrations with six varied substrate

concentrations for each of the three substrates. The data collected was individually fitted to Michaelis-Menten equation, and the resulting Lineweaver- Burk plots were examined for characteristic pattern of inhibition.

***3.3.8 In vitro Cell Toxicity Assay*:** MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a colorimetric cell proliferation assay in which formazan is measured. Formazan, an insoluble purple product produced as a result of reduction of tetrazolium dye (MTT) by mitochondrial enzymes of viable cells. The test compounds (5mM in DMSO) serially diluted in media were added to 96 well culture plates with a concentration range of 0.75-50 µM in a total volume of 200 µl. The logarithmic phase promastigote (2×10^6 cells ml^{-1}) cells grown in DMEM complete media were then added to the 96 well culture plate pre-loaded with compound. HEK cells were cultured in DMEM complete media, 2000 cells/well, were seeded and were allowed to adhere overnight. The test compounds (5mM in DMSO) serially diluted in media were added to overnight grown culture with a concentration range of 0.75-50 µM in a total volume of 200 µl. 0.5% DMSO served as negative control while 20% DMSO served as positive control for both parasite and human cell line. The cultures were incubated for 48 h in dark at 25°C for *L. donovani* promastigotes and at 37°C in an atmosphere of 5% CO_2 for HEK cell line. MTT reagent was added to the culture and 4 h incubation was given for the development of purple insoluble formazan, which was dissolve in DMSO. The absorbance was read at 570 nm which is the measure of viable cells. The IC_{50} value for each compound was calculated by plotting percentage cell viability *vs.* concentration.

***3.3.9 Analysis of Intracellular Thiol Levels*:** *L. donovani* promastigotes cultures containing 2×10^6 cells ml^{-1} were incubated with six varied concentrations (1.5-50 µM) of each compound (tomatine, conessine, uvaol and, betulin) separately in a 6 well culture plate for 48h. Promastigotes culture without compound was used as a control. The thiol contents for each of the compound treated cells were estimated using DTNB. The cells were centrifuged, washed with PBS buffer and pellet downed (900 × g, 10 min, 4°C). Pellets were dissolved in 200µl of 10 mM Tris-HCl buffer pH 2.5 and sonicated for 2 min. Acid pH is used to prevent oxidation of free thiol. Cell debris was separated by centrifugation and supernatants were collected in fresh vials. In a 96 well microtitre plate 100 µl of supernatant

and 100 µl of 500 mM phosphate buffer pH 7.4 were added to each well according to the number of supernatant samples collected. 20 µl of DTNB (1 mM) was added to each well and the observance is read at 412 nm.

3.4 Results

***3.4.1 Sequence Analysis*:** A putative *L. donovani TRYS* gene predicted to encode a protein of 652 amino acids was amplified from genomic DNA (Figure 3.1A and Figure 3.1B). The authenticity of the gene was initially confirmed by restriction digestion with *Hind* III restriction enzyme, which is a single cutter in *LdTRYS*, cuts at position 487/491, yielded a product of ~0.5 and ~1.5 Kbps fragments (Figure 3.1C). Finally two individual sequencing results conforms the *Ld*TryS clone. The sequencing result produced a 1959 kb DNA sequence which gave a 652 amino acid sequence upon *in silco* translation as predicted. NCBI protein BLAST was performed using predicted 652 amino acid sequence of *Ld*TryS UR6 strain. The BLAST result showed strong homology with *L. amazonensis* TryS (ABQ57409.1) 99% similarity and 98% identity, followed by *L. donovani* TryS (CAD23679.1) of strain LV9, unnamed protein of *L. donovani* BPK282A1 (CBZ35334.1) drug resistant strain, *L. Major* TryS (XP_848143.1) and *L. Infantum* TryS (XP_001466426.1) each showed 97% similarity and 96% identity with *Ld*TryS of avirulent strain UR6 (MHOM/IN/1978/UR6).

From the BLAST result the *Ld*TryS of UR6 (avirulent strain) showed considerable difference with TryS of both *L. donovani* strains i.e., LV9 (virulent strain) and BPK282A.1 (drug resistant strain). From the multiple sequence alignment result it was observed that all the synthetase active site residues were conserved (Figure 3.2). The model of *Ld*TryS UR6 strain was generated using MODELLER (*Marti-Renom et al., 2000*) and crystal structure of *L. major* (PBD ID: 2VOB). Quality of the 3D homology models generated was ranked by DOPE score and the best model was assed using PROCHECK validation package (*Laskowski et al., 1993*). Synthetase active was studied using PyMOL molecular viewer by selecting 4Å from centre of synthetase site. All the active site key residues came within the 4Årange indicating no change in active site residues.

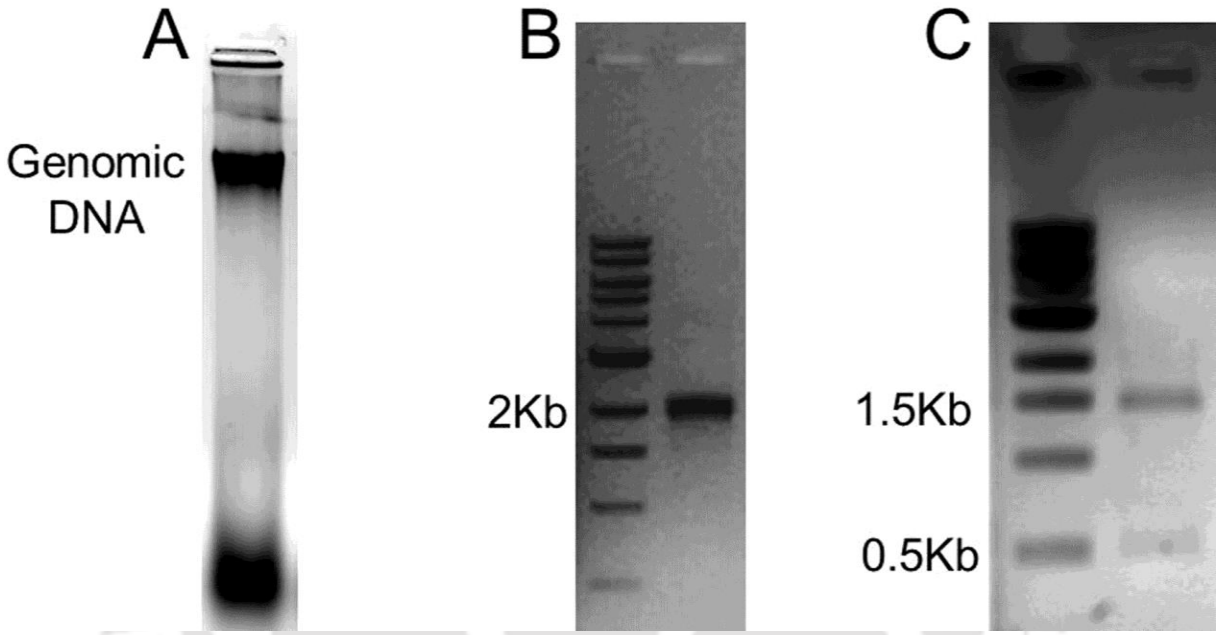


Figure 3.1: Cloning of trypanothione synthetase. (A) Genomic DNA isolated from UR6 strain of *L. donovani*; **(B)** PCR amplification of *Ld*TRYS; **(C)** Validation of TRYS by restriction digestion with *Hind*III enzyme, which is a single cutter in *Ld*TRYS, which cuts at position 487/491, yielded a product of ~0.5 and ~1.5 Kbps fragments. Finally,the clone was confirmed by two independent sequencing. The nucleotide sequence is submitted to the GenBank™ with accession number JN561695.

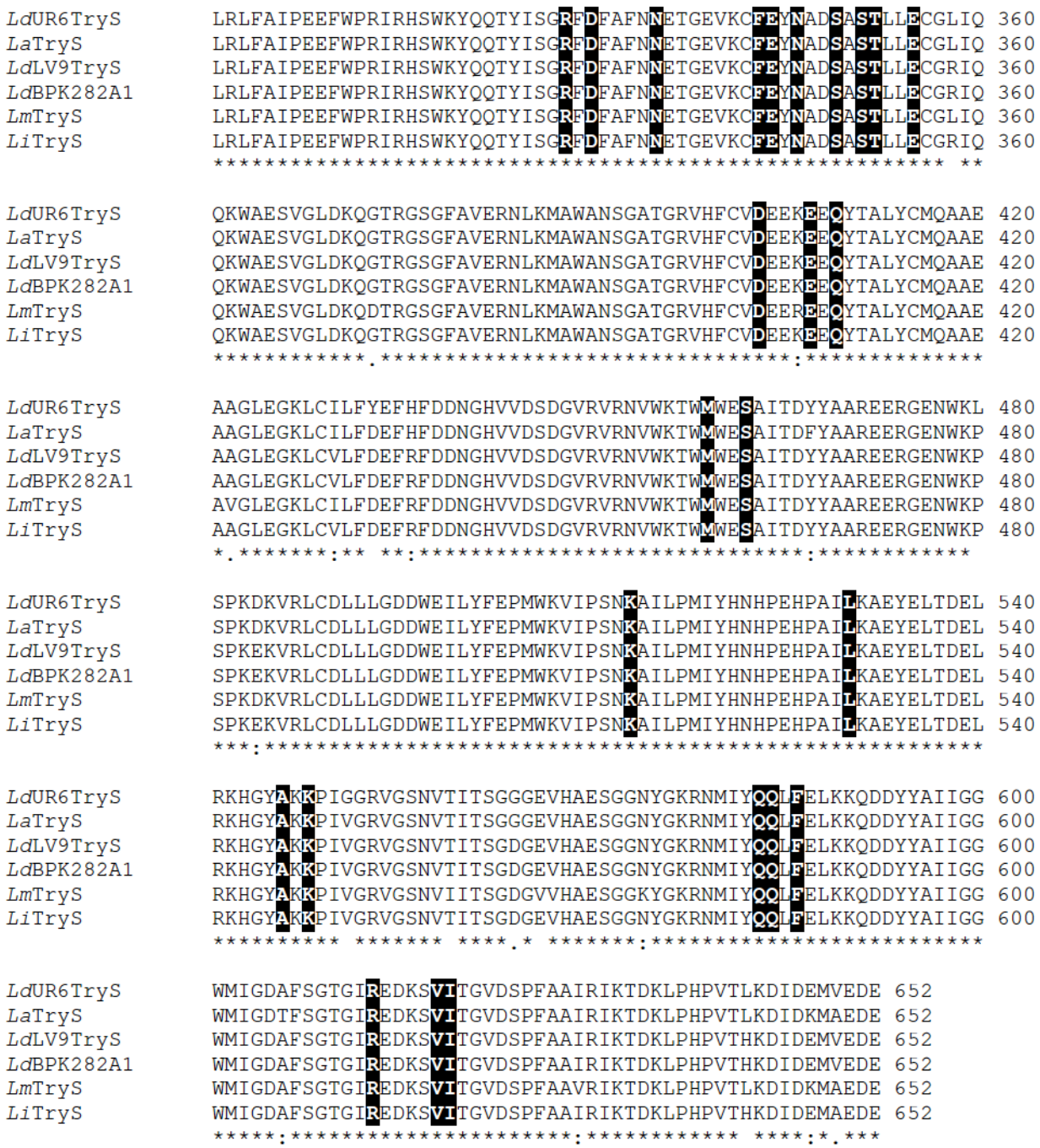


Figure 3.2: Alignment of the amino acid sequences of trypanothione synthetase. Conserved and similar residues are indicated by asterisks and dots, respectively. The protein sequences are from *L.d*UR6TryS *(*Predicted sequence of *L. donovani* UR6 strain); *La*TryS (ABQ57409.1); *Ld*LV9TryS (CAD23679.1); *Ld*BPK282A1 (CBZ35334.1); *Lm*TryS (CAC83968.1) and *Li*TryS (CAM69145.1).

***3.4.2 Expression of LdTryS in E. coli*:** pET28a-*Ld*TRYS construct transformed into BL21 star (DE3) produced soluble and enzymatically active protein. A good purification of *Ld*TryS was achieved by Ni^{2+} affinity chromatography. The purified protein was subjected to thrombin cleavage to remove $(His)_6$-tag. Migration on SDS-PAGE gave an apparent molecular mass of ~74.5 kDa (Figure 3.3). The typical yields of TryS were between 4-6 mg L^{-1}.

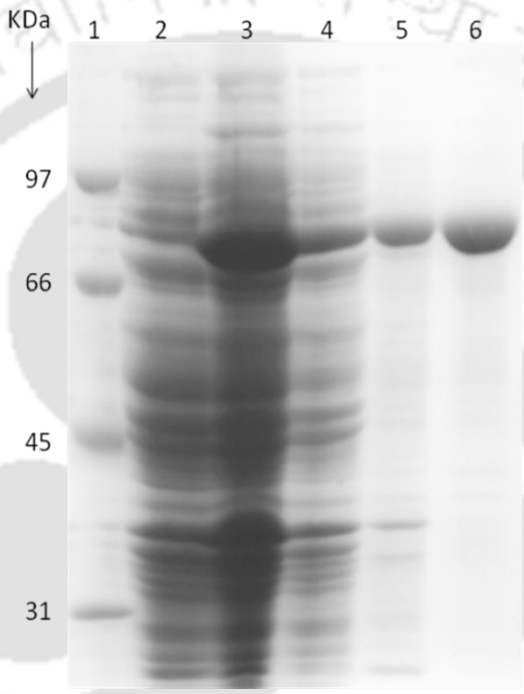


Figure 3.3: Purification of recombinant *Ld*TryS from *E. coli.* SDS-PAGE analysis: ***lane 1***, protein marker; ***lane 2***, soluble fraction of uninduced BL21 star (DE3) [pET 28a-*Ld*TRYS]; *lane 3*, soluble fraction of induced BL21 star (DE3) [pET 28a-*Ld*TRYS]*;* ***lane 4***, Unbound crude*;* ***lane 5***, purified TryS with His-tag*;* ***lane 6***, purified TryS without His-tag.

***3.4.3 Kinetic Characterization of LdTryS*:** The recombinant *Ld*TryS activity was checked by colorimetric quantification using BIOMOL green reagent, which measures free phosphate released in the reaction via measurement at $A_{650.}$ A mixed buffer system was used to determine the optimum pH range for *Ld*TryS. The pH optima studies followed a bell shaped symmetrical curve giving a pH optimum of 8.0 ± 0.1 (Figure 3.4A). *Ld*TryS has an optimum activity at 25°C. The activity gradually decreased along with increase in above optimum

temperature (Figure 3.4B). The ionic strength in presence of buffer and salt were studied and the optimum range of *Ld*TryS was found to be 40 mM KCl or $(NH_4)_2SO_4$ (Figure 3.4C).

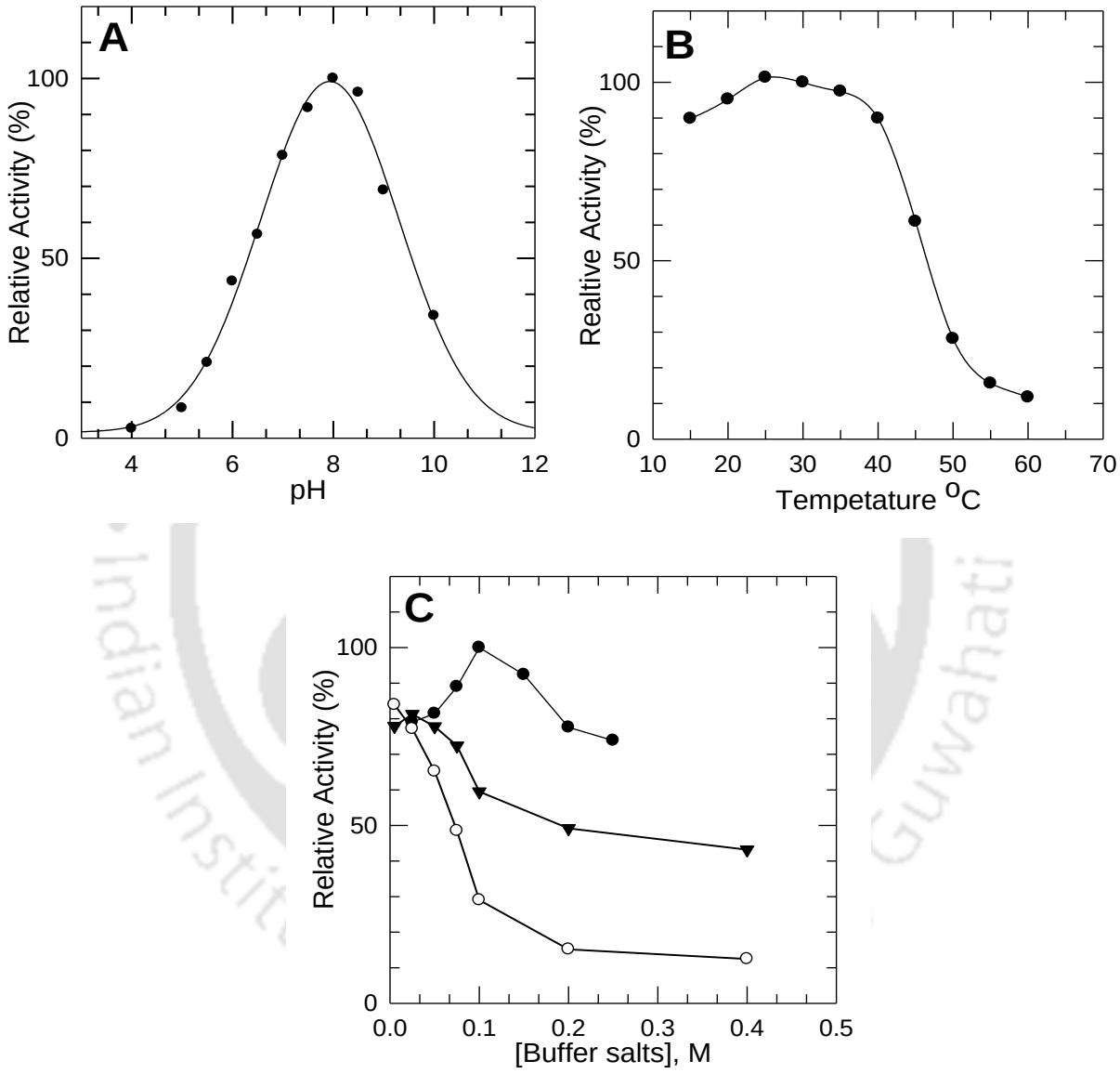


Figure 3.4: Enzymatic properties of Trypanothione Synthetase. **(A)** pH- profile. Optimum pH studies were carried out in a mixed buffer system (as described in section 2); **(B)** optimum temperature studies. Assay were carried out in different temperature conditions ranging from 15-60˚C as described in section 3.3.6; **(C)** Effect of salt and buffer concentration. Assay mixture contain either various concentration of HEPES buffer, pH 8.0 (closed circle), KCl (closed triangle), or $(NH_4)SO_4$ (open circle).

A simple hyperbolic kinetics was displaced by the enzyme with ATP and Spd as varied substrate. High substrate inhibition was observed above 0.4 mM with GSH in presence of saturating concentration of ATP (10 mM) and Spd (10 mM) with GSH as varied substrate. The substrate K_m for ATP, Spd, and GSH were determined (Figure 3.5A-C). The K_m calculate for each of the three substrate were 14.26 ± 1.8 µM for ATP, 33.24 ± 2.4 µM for GSH and 139.62 ± 8.3 µM for Spd. GSH at 0.3mM gave maximum signal and when fixed concentrations of substrate were required ATP and Spd were used at saturating concentrations with GSH fixed at 0.3mM (GSH displayed high substrate inhibition). Assay displayed linearity with respect to time under above conditions (Figure 3.5D), and was linear for at least 60 min. Hence, a 60 min time point was selected for inhibition studies.

***3.4.4 LdTryS Inhibition Studies*:** Compounds used in this study resulted to inhibit *Ld*TryS, which were depicted from the inhibition data analyzed. The inhibition study data for each of the individual compound was fitted to Michaelis-Menten equation. To assess the mode of inhibition, Lineweaver- Burk plots were studied. Effect of the inhibitors, tomatine is shown in Figure 3.6, conessine is showed in Figure 3.7, Uvaol is showed in Figure 3.8 and betulin is showed in Figure 3.9. All the four compounds displayed competitive inhibition with respect to Spd as V_{max} did not change in the presence of inhibitor and uncompetitive inhibition with ATP as the slope of Lineweaver-Burk plots was unchanged. With GSH uncompetitive inhibition was observed with tomatine and betulin. Lineweaver-Burk plots upward curvature at low reciprocal GSH is due to excess substrate inhibition. However, uvaol and conessine showed allosteric mode of inhibition. A similar allosteric inhibition pattern of Lineweaver-Burk plots was seen in case of compound prochlorperazine with GSH as varied substrate against TryS of *T. brucei* (*Torrie et al., 2009*). The K_i for each compound was calculated by considering Lineweaver-Burk plots with GSH as varied substrate concentration. The calculated value of K_i with respect to GSH for tomatine,and betulin are 12.54 ± 1.22 µM, and 6.33 ± 0.82 µM, respectively. However, as conessine and uvaol showed allosteric inhibition the K_i could not be measured reliably.

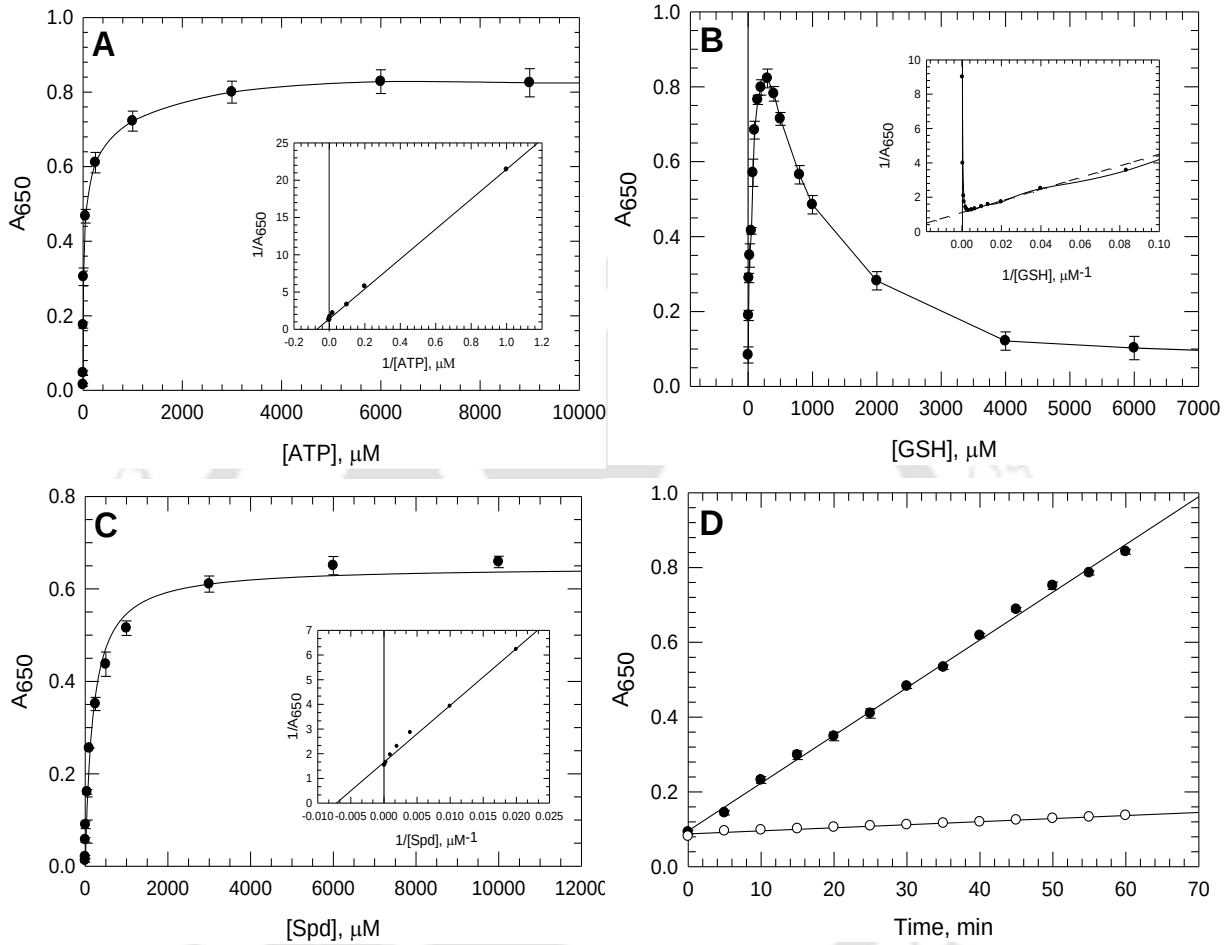


Figure 3.5: End point assay K_m determination. **(A)** K_m value for ATP as varied substrate in presence of saturating other substrates (0.2 mM GSH and 10 mM Spd) **(B)** K_m determination for GSH in presence of 10 mM of both ATP and Spd . As GSH showed excess substrate inhibition, only linear part of considered for estimation of *Km* value for GSH **(C)** K_m determination for Spd in presence of 0.2 mM GSH and 10 mM Spd **(D)** Linearity of the assay with TryS (closed circle) and without TryS (open circle) under fixed substrate concentration (10 mM ATP, 0.2 mM GSH and 10 mM Spd). Values are the mean ± S.D. of three determinations.

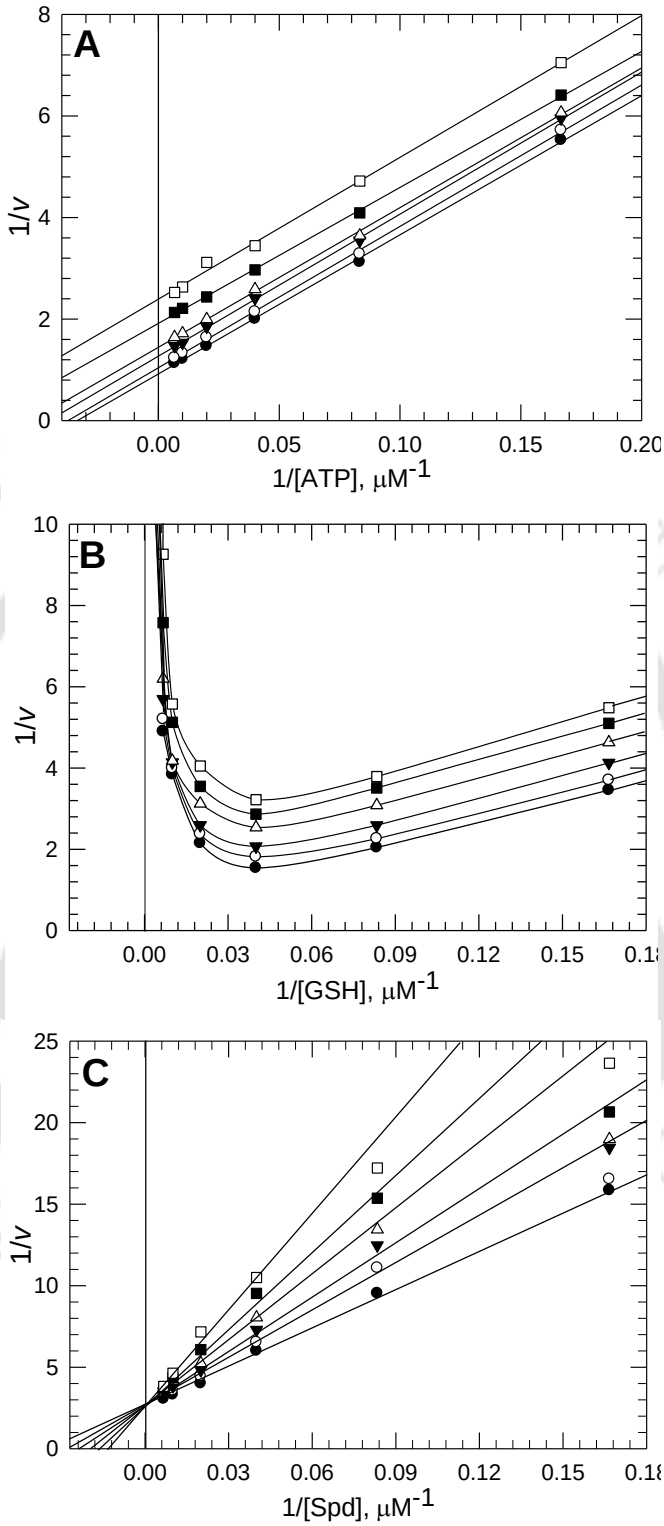


Figure 3.6: Mode of inhibition by tomatine. Uncompetitive inhibition with respect to **(A)** ATP and **(B)** GSH. As GSH showed excess substrate inhibition, only linear part of considered for estimation of *Ki* value for GSH (*Ki*= 6.8±0.8 μM) **(C)** Linear competitive inhibition with respect to Spd. Inhibition concentrations used were as follows: 50 μM (open square), 25 μM (closed square), 12.5 μM (open triangle), 6.12 μM (closed triangle), 3.0 μM (open circle) and control with DMSO (closed circle).

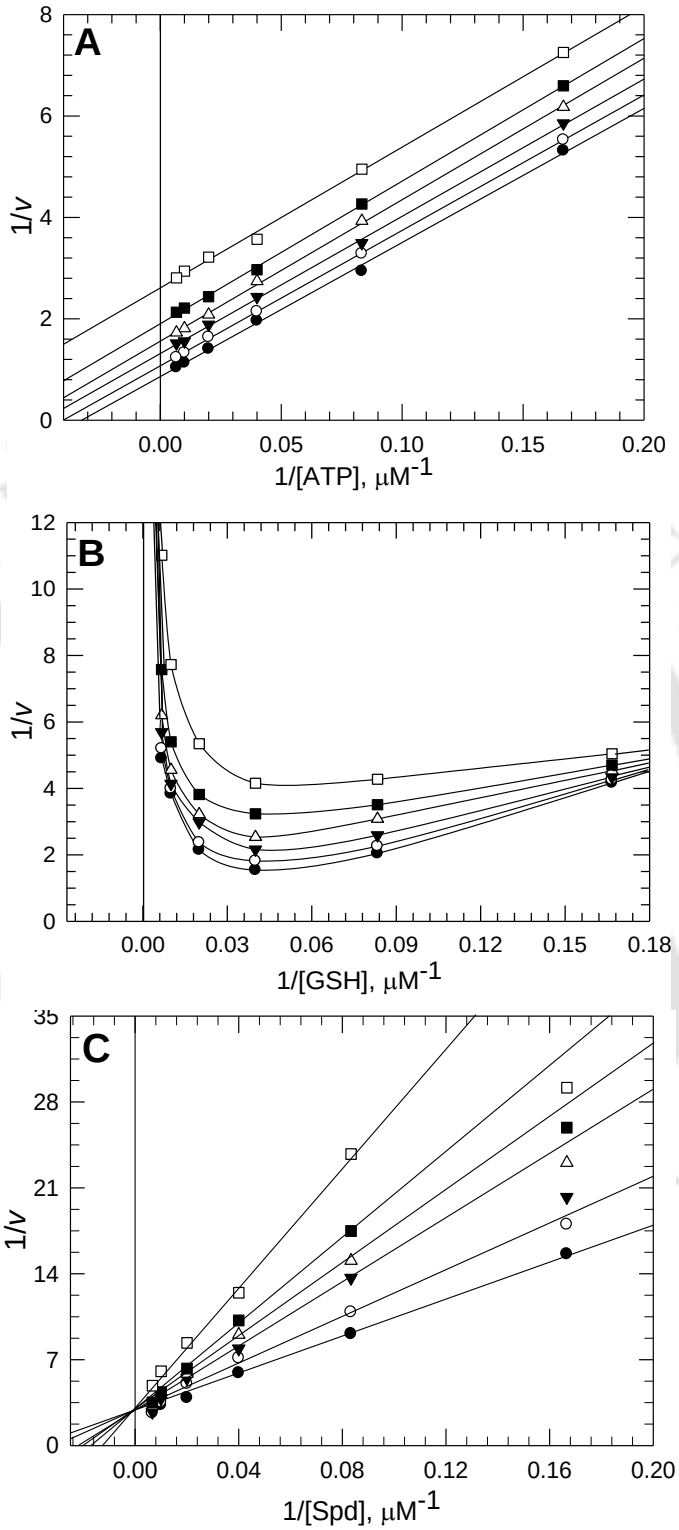


Figure 3.7: Mode of inhibition by Conessine. (A) Uncompetitive inhibition with respect to ATP **(B)** As GSH showed excess substrate inhibition and linear part of L-B plot was not parallel, it was considered due to allosterice mode of inhibition (*Torrie et al., 2009*) **(C)** Linear competitive inhibition with respect to Spd. Inhibition concentrations used were as follows: 50 µM(open square), 25 µM (closed square), 12.5 µM (open triangle), 6.12 µM (closed triangle), 3.0 µM (open circle)and control with DMSO (closed circle).

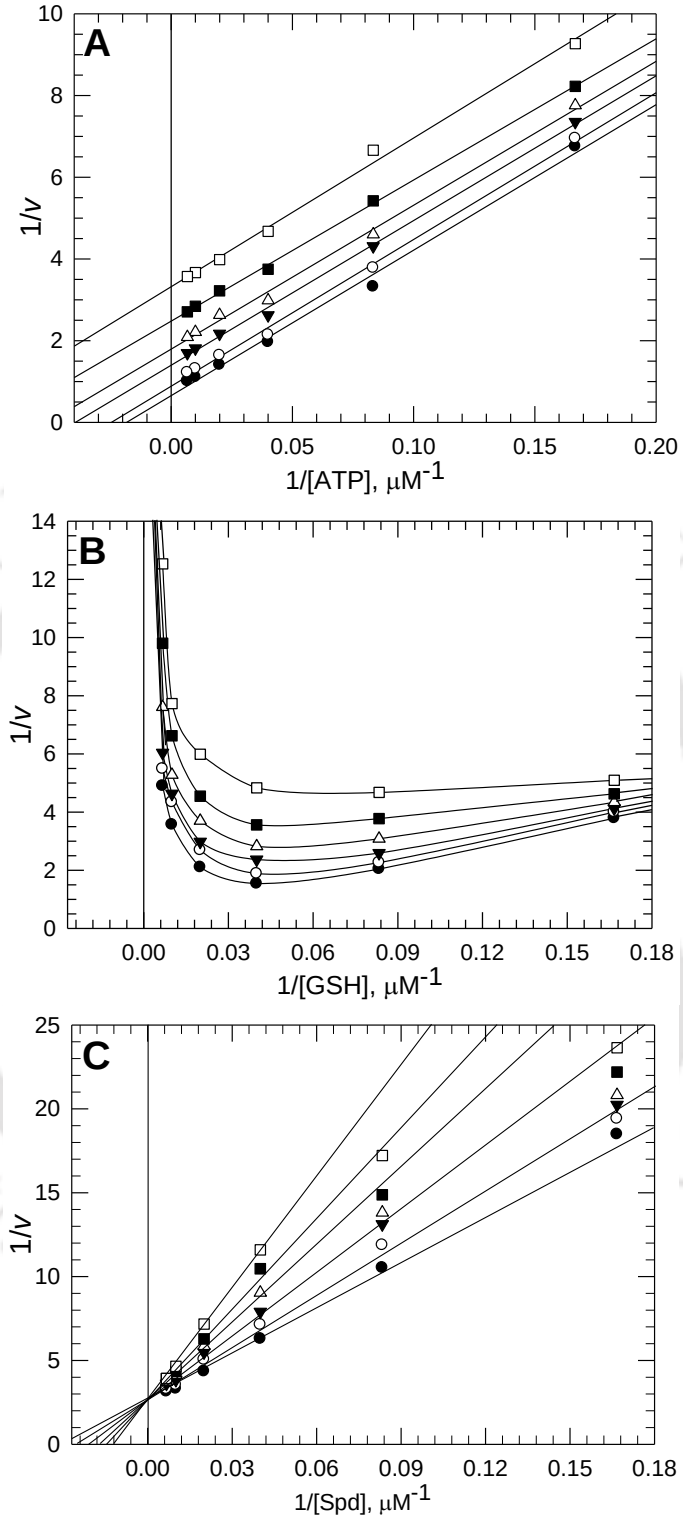


Figure 3.8: Mode of inhibition by uvaol. (A) Uncompetitive inhibition with respect to ATP **(B)** As GSH showed excess substrate inhibition and linear part of L-B plot was not parallel, it was considered due to allosterice mode of inhibition (*Torrie et al., 2009*) **(C)** Linear competitive inhibition with respect to Spd. Inhibition concentrations used were as follows: 50 µM (open square), 25 µM (closed square), 12.5 µM (open triangle), 6.12 µM (closed triangle), 3.0 µM (open circle) and control with DMSO (closed circle)

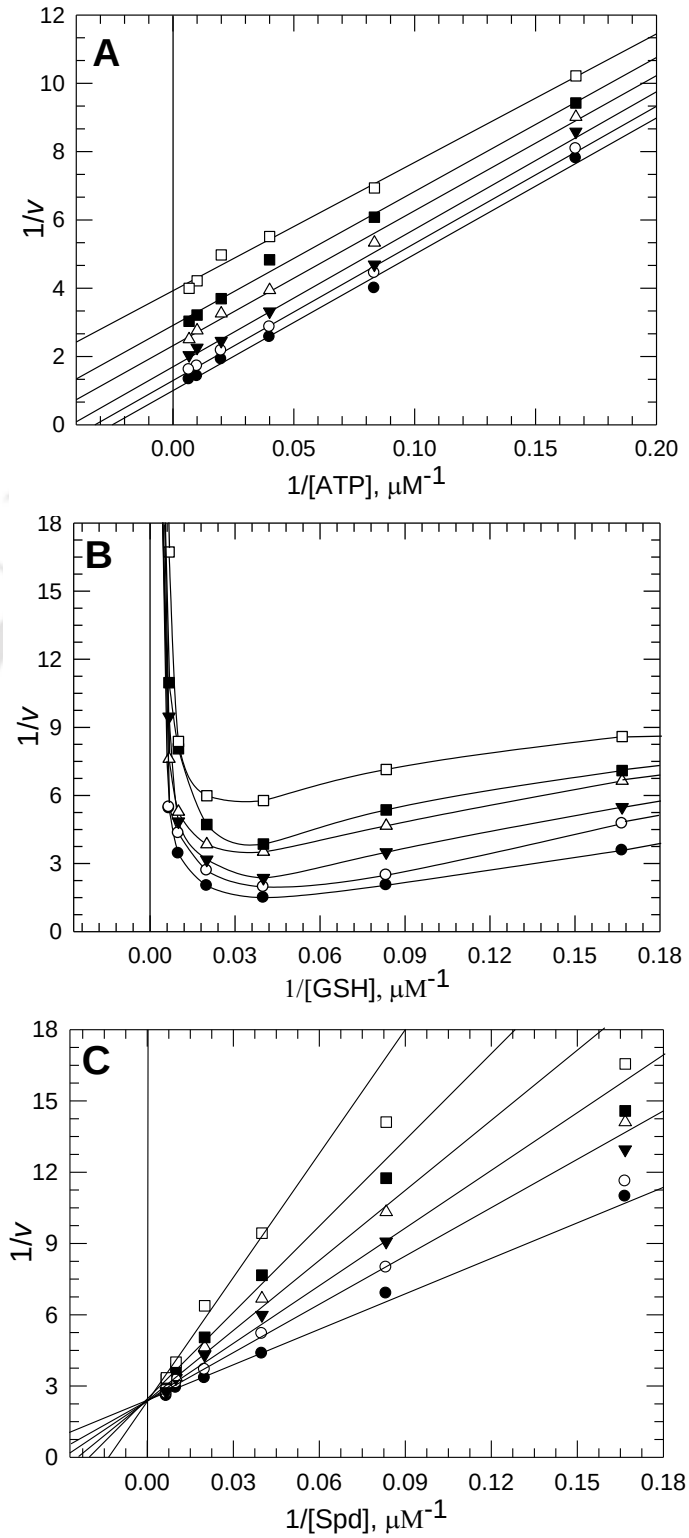


Figure 3.9: Mode of inhibition by betulin. Uncompetitive inhibition with respect to both **(A)** ATP and **(B)** GSH (*Ki*= 6.52±0.8 μM); **(C)** Competitive inhibition with respect to Spd. Inhibition concentrations used were as follows: 50 μM (Open Square), 25 μM (closed square), 12.5 μM (open triangle), 6.12 μM (closed triangle), 3.0 μM (open circle) and control with DMSO (closed circle).

***3.4.5 Significant Effect of TryS Inhibitors on L. donovani Cell Proliferation*:** The inhibition of cell proliferation was shown by all the four compounds tested in a several concentration using MTT assay. The death profile showed a characteristic significance with the increasing concentration of test compound above 6.25 µM. All the compounds showed a rapid dose response death profile between the concentrations 6.25 and 12.5 µM and reaching approximately 75% at around 50 µM of Conessine, uvaol and betulin whereas tomatine showed 60% inhibition. The death profile shown by tomatine was slow when compared to other three compounds, indicating that tomatine has less anti-leishmanial activity compare to conessine, uvaol and betulin. The IC_{50} values for the compounds tested against *L. donovani* promastigotes was calculated by plotting percentage cell viability *vs.* concentration and were found to be 18.02 ± 0.35 µM for tomatine, 13.42 ± 0.75 µM for conessine, 11.23 ± 0.48 µM for uvaol and 11.71 ± 0.56 µM betulin (Figure 3.10). In addition, cell toxicity studies of the compounds with human cell line HEK were performed to check the toxicity levels over human cell line. The compounds showed no significant effect on human cell line at concentrations up to 50 µM. In both the cells studied for toxicity, 20% DMSO is used as positive control which showed complete inhibition. The inhibition data were neutralized against positive control value read and the graph was plotted by considering 0.5% DMSO as negative control, which almost had nil effect on the cells.

***3.4.6 Decrease in Intracellular Thiol Levels*:** Inhibition of TryS will result in decrease of intracellular thiol levels. The total intra cellular thiol for compound treated *L. donovani* promastigotes were tested using DTNB assay. A clear dose dependent decrease in total free thiol was observed by all the compounds with almost ~60-70% decrease was observed at 50 µM. This decrease may be due to the inhibition of TryS and may be other enzymes of the redox system of the parasite. Trypanothione constitute the major thiol in the parasites total intracellular thiol content. Decrease of ~60-70% is a very significant result, indicating that the compounds are active against target and may be other redox enzymes of the parasite (Figure 3.11).

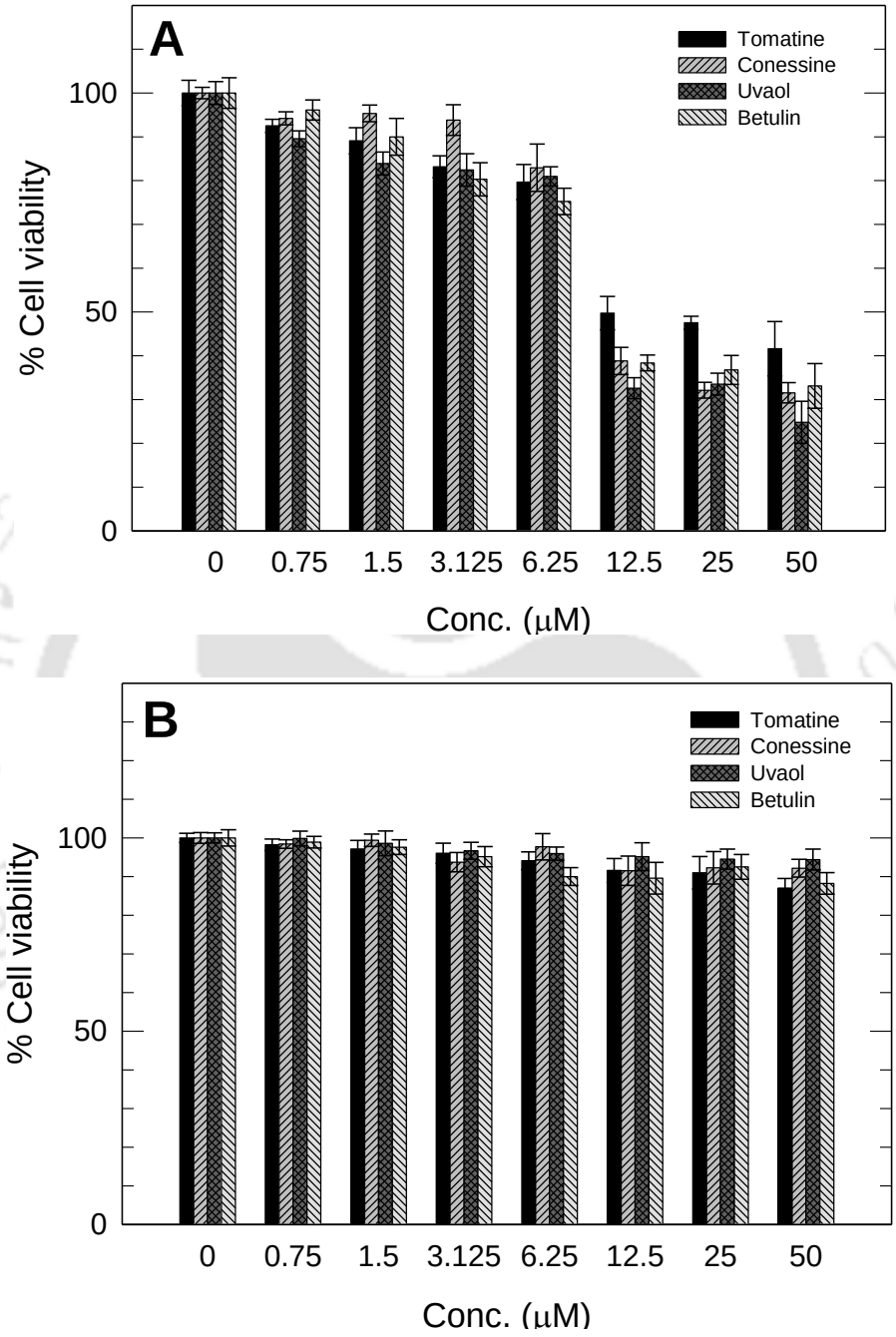


Figure 3.10: MTT cell proliferation assay. **(A)** Effect of the compounds on *L. donovani* promastigotes. The IC_{50} value for the compounds against *L. donovani* promastigotes are 18.02±0.35 µM for tomatine, 13.42±0.75 µM for conessine, 11.23±0.28 µM for uvaol and 11.71±0.56 µM betulin **(B)** Effect of compounds on human cell line HEK. Values are the mean ± S.D. of four determinations

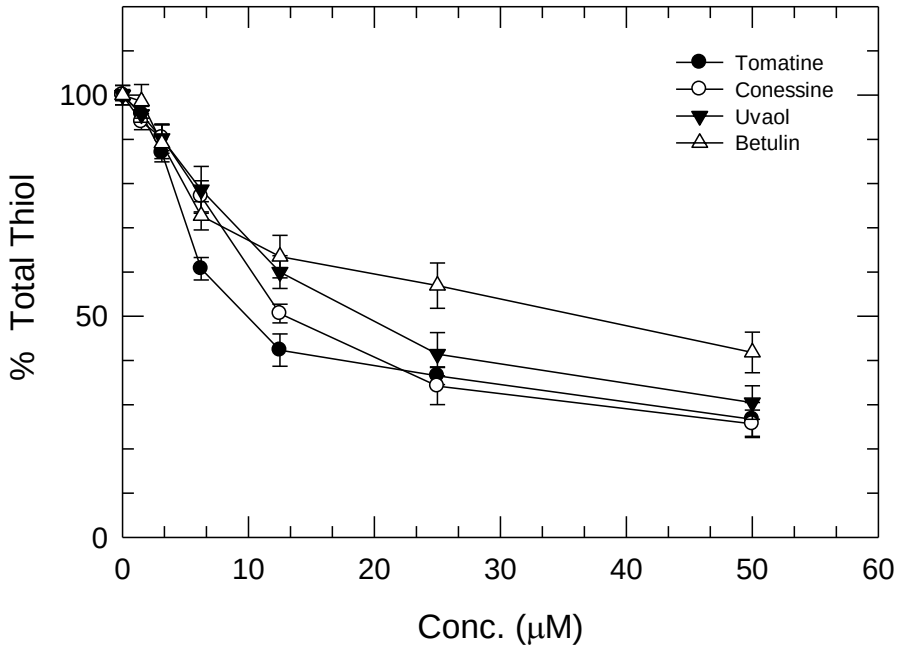


Figure 3.11: Decrease in intracellular thiol levels. *L. donovani* promastigotes treated with various concentrations of compounds showed dose dependent decrease in cell thiol levels. Values are the mean ± S.D. of three determinations.

3.5 Discussion

The uniqueness of trypanosomatids trypanothione based redox metabolism makes it an attractive target in drug discovery process. The importance of trypanothione reductase in parasite survival is known in *Leishmania (Dumas et al., 1997; Tovar et al., 1998a; Tovar et al 1998b*). The validation of enzymes involved in biosynthesis of trypanothione as drug targets is already shown in *Trypanosomes*. Knockout studies of TryS conducted with *T. brucei* proved the essentiality of TryS in parasite survival (*Comini et al., 2004; Ariyanayagam et al., 2005*). There are not many studies done on TryS from *Leishmania donovani*. This study cloning, expression, characterization and inhibition of TryS from *L. donovani* (UR6 strain) were initiated to identify the novel inhibitors of TryS enzyme. Exploring new targets with natural compounds can help in overcoming drug resistance problem (*Polonio et al., 2008*).

TRYS (CAD23679.1) sequence of Ethiopian virulent *L. donovani* LV9 strain was used for designing primers to amplify and clone TryS of Indian avirulent *L. donovani* UR6 strain.

The sequence alignment of *Ld*TryS (UR6 strain) showed 99% similarity with *L. amazonensis* TryS (ABQ57409.1) and 97% with Ethiopian virulent *L. donovani* LV9 strain TryS. There was considerable difference observed between TryS sequences of both *L. donovani* avirulent Indian UR6 strain and virulent Ethiopian LV9 strain, which made us necessary to study of the synthetase active site of the TryS (UR6 strain). From the primary sequence analysis done by using clustalW it was clear that all the active site domains were highly conserved. But the 3D analysis of the modelled structure give the clear picture of no change in the synthetase active site residues which was assessed by considering 4Å from centre of synthetase site. ATP binding region was highly conserved along with the GSH and Spd anchoring residues. The difference in the TryS sequences may be due to microheterogenicity among the *Leishmania* species which may be most likely due to geographical distributions of the organisms. The tiny differences between the trypanothione synthetases of different trypanosomatids were shown to be associated with major functional changes (*Flohé, 2012*).

Before screening inhibitors, characterization of the target is of immense important in drug discovery process. The kinetic properties of *Ld*TryS in comparison with TryS of other trypanosomatids are summarized in Table 3.1. *Ld*TryS showed substrate inhibition with respect to GSH, which was observed with the TryS of other trypanosomatids. The yield of the recombinant *Ld*TryS was very high which can be useful in screening large number of compounds from the available data sets.

Table 3.1- Comparison of kinetic properties of *L. donovani* trypanothione synthetase with *L. major*, *T. cruzi* and *T. brucei*

Substrate	Units	*Ld*TryS	*Lm*TryS[c]	*Tc*TryS[d]	*Tb*TryS[e]
K_m GSH[a]	µM	33.24 ± 2.4	89	570	56.2
K_i GSH[a]	µM	866 ± 80	1000	1200	36.5
K_m ATP[b]	µM	14.26 ± 1.8	940	625	37.8
K_m Spd[b]	µM	139.62 ± 8.3	40	53	7.1
K^a_{cat}	s^{-1}	1.34	2	3.4	2.9

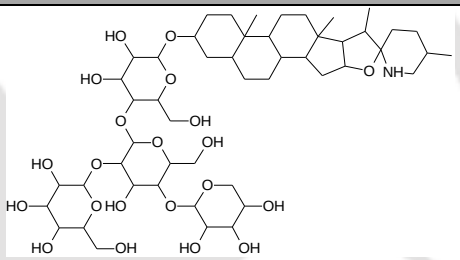
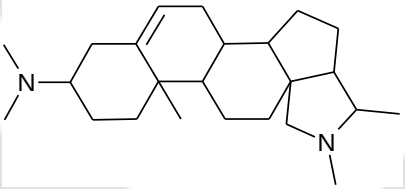
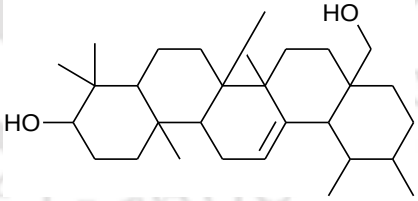
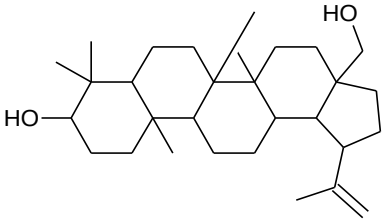
a. With 10 mM Spd and ATP, **b**. As GSH showed substrate inhibition K_m for other substrate was determined by considering peak activity with GSH at 0.2 mM, **c**. Data taken from *Oza et al. 2005*., **d**. Data taken from *Oza et al. 2002b*, **e**. Data taken from *Oza et al. 2003*.

In a docking study reported earlier, we were able to identify potent inhibitor like molecules for TryS using MS discovery natural product data set (*Venkatesan et al., 2011a*). Initially we have selected commercially available compounds from the top hits identified by docking studies reported earlier by our lab. Inhibition studies conducted with compounds showed competitive mode of binding with respect to the substrate Spd although none of the compounds show a polyamine like moiety and structural similarity (except for betulin and uvaol). However, the early report on docking studies with natural product data set showed two major binding modes at the synthetase active site. It is reported that the compounds majorly bind to the Spd binding domain of the TryS enzyme and showed interactions with key residues (*Venkatesan et al., 2011a)*. As the active site cleft is larger compounds bigger than the substrate can easily enter the active site and may show interactions with key residues and may compete with substrate though they are not closely related to the substrate both chemically and structurally. With GSH and ATP allosteric mode of bindings were observed. It is reported that physiological levels of TryS substrates are very high in trypanosomes and it could be the same with *Leishmania* also as these organism share common group. This could be disadvantageous with the inhibitors showing competitive mode of binding as presence of high concentrations of substrate may mask the inhibitors effect on TryS. Most of the compounds known to inhibit trypanothione reductase presumed to inhibit TryS also. Indeed the docking study with natural product data set was first studied with trypanothione reductase from our lab (*Venkatesan et al., 2011b*). Later considering the above comment we have done docking studies with TryS with same data set. As identifying an inhibitor with two or more targets in *Leishmania* can be an effective alternative in treating infection. Our current studies are focused on the search for compounds which have multi targets action against redox system of the parasite.

In spite of the fact that the compounds were acting on target which was shown by enzyme inhibition assay studies, MTT cell proliferation assay with *L. donovani* promastigotes cells showed a large shift in potency of the compounds there by indicating that the compounds were active against *L. donovani* promastigotes (Table 3.2). It is true that the compounds tested did not showed a typical dose response curve and most likely the calculated IC_{50} values are likely to be overestimated since they are determined with an asymptote at 0% obtained by treatment with 20% DMSO. If the compounds are acting on the

target there should be decrease in the thiol levels of compound treated cells. The thiol estimation assay showed a clear decrease in total cell thiol, which gives a clue that the compounds may be acting on the redox enzymes mainly trypanothione synthetase. The potency of these compounds has to be further validated because the effect of compounds may differ among parasite cultured in media with that of in vivo model. The studies with in vivo model will provide a better understanding of the compounds for drugs against leishmaniasis.

Table 3.2: Structures- activity relationship of compounds. The calculated IC_{50} values against *Leishmania donovani* promastigotes.

Sl. no	Compound	Structure	IC_{50}, (µM)
1	Tomatine		18.02
2	Conessine		13.42
3	Uvaol		11.23
4	Betulin		11.71

3.6 Conclusion

This chapter concludes successful cloning of *TRYS* gene from *Leishmania donovani* into the *Nde*I/*Bam*HI sites of pET28a. Further, pET28a-*Ld*TRYS construct transformed into BL21 (DE3) produced soluble and enzymatically active protein. A good purification of *Ld*TryS was achieved with the typical yields of TryS was between 4-6 mg L^{-1}.The *Ld*TryS has pH optima of 8.0 ± 0.1 and optimum temperature 25°C. The K_m was determined for all the three substrate. Tomatine, conessine, uvaol and betulin inhibit *Ld*TryS and also show anti-leishmanial activity with clear dose dependent decrease in total free thiol. Further exploring the molecular mechanism of parasite death in response to treatment of compounds will help in understanding the biochemical changes that led to parasite death.

Chapter IV

Anti-leishmanial Property of Betulin: Depicting the molecular mechanism of parasite death and its targeted delivery*

4.1 Abstract

The anti-leishmanial property of betulin (a pentacyclic triterpenoid secondary metabolite) is mediated through inhibition of parasite redox enzyme trypanothione synthetase (TryS), as discovered from the studies conducted in chapter III. In this chapter the molecular mechanism of parasite death in response to betulin treatment is investigated against *L. donovani* promastigote cells. Moreover, in vitro anti-leishmanial effect on intracellular amastigote form of parasite was also investigated. Betulin exerted ROS generation at micro-molar concentrations in *L. donovani* promastigote cells. Treatment of *L. donovani* promastigote cells with IC_{50} dose of betulin induced apoptosis, as evident by flowcytometric analysis of treated cells double stained with annexin V-FITC and PI. Further, betulin treatment also led to mitochondrial membrane damage, activation of caspase like proteases and DNA fragmentation. Further, carbon nanotube formulation of betulin (f-CNT-BET) showed better efficacy than betulin.

*** Part of the work is published in *Am J Trop Med Hyg*, DOI: 10.4269/ajtmh.13-0320.**
*** Part of the work is communicated for publication in *Colloids and Surfaces B: Biointerfaces*.**

4.2 Introduction

Leishmaniasis is a vector born disease caused by intra-macrophage obligate protozoan parasites of genus *Leishmania.* The disease is known for its diverse and complex characteristic infection. Depending on the *Leishmania* species involved in infection, there are three different clinical manifestations of leishmaniasis, visceral leishmaniasis, cutaneous leishmaniasis and mucocutaneous leishmaniasis (*Olivier et al., 2005*). Visceral leishmaniasis, also known as kala-azar is one of the severe forms of leishmaniasis infections that are most common in Indian are caused by *Leishmania donovani*. One of the major hurdles in eradicating the disease is the fact that parasite has developed resistance to most commonly used drugs. The search of successful vaccine is still elusive. The currently available drugs in the market for treating leishmaniasis have severe drawbacks (*Solomon et al., 2011; Wortmann et al., 2010; Mishra et al., 1992; Sindermann and Engel 2006; Seifert et al. 2007*). There is urgent need for discovery of new improved anti-leishmanial compound.

An ideal target is the one that is parasite specific and is essential for the parasite survival. Such targets can be identified by exploring fundamental metabolic and/or biochemical divergence between host and parasite. In trypanosomatid parasites, such as *Leishmania* and mammalian host a striking metabolic disparity is the redox metabolism that fights oxidative stress in the cell (*Lillig and Holmgren, 2007; Krauth-Siegel and Leroux, 2012*). The central key metabolite in redox system of trypanosomatid parasite like *Leishmania* is trypanothione [bis (glutathionyl) spermidine; $T(SH)_2$], which maintains the cellular redox homeostasis (*Krauth-Siegel and Comini, 2008*). Whereas functionally analogous metabolite found in mammalian host is glutathione (GSH). The biological synthesis of $T(SH)_2$ is carried out by trypanothione synthetase (TryS), one of the key enzymes of parasite's redox metabolism. Besides maintaining redox homeostasis, $T(SH)_2$ is involved in various cellular processes such as synthesis of deoxyribonucleotides, drug resistance, metal stress and chemical stress (*Dormeyer et al., 2001; Fairlamb and Cerami, 1992; Krauth-Siegel and Lüdemann, 1996; Flohe et al., 1999*). TryS has been validated as drug target by conducting knockout studies in *Trypanosome brucei* (*Comini et al., 2004; Ariyanayagam et al., 2005*). The exclusive nature of TryS in synthesizing $T(SH)_2$ in parasite and its absence in the host makes it an attractive target.

In our earlier study we have reported few natural compounds that inhibit TryS enzyme of *L. donovani* and also inhibited *L. donovani* promastigotes growth at micro-mole concentration, but the molecular mechanism underlying parasite death was not studied (*Saudagar and Dubey, 2011*). Betulin was one of the inhibitors of the TryS that we have reported earlier. In the present study, the molecular mechanisms of parasites death due to the anti-leishmanial activity shown by betulin are investigated. Betulin is an abundant naturally occurring triterpene present in the bark of white birch trees. It is known to exhibits anti-inflammatory and anti-HIV (*Reutrakul et al., 2010*), anti-malarial (*Steele et al., 1999*) and, popularly known anti-cancer agent (*Laszczyk 2009; Li et al., 2010*). Moreover, recently betulin and its derivatives are reported to inhibit type lB DNA topoisomerase of *L. donovani* (*Chowdhury et al., 2011*), and having dual target is a desirable property of a drug as it can act more effectively in treating the infection.

The main aim of the present study was to investigate more precisely the molecular mechanisms of parasite death caused by anti-leishmanial agent betulin. In this investigation, it is reported that betulin induced *Leishmania* parasites death shares numerous features with apoptotic metazoan cells such as ROS generation in the parasite, phosphatidylserine externalization, mitochondrial membrane damage, and DNA fragmentation. Our investigation also suggests the involvement of proteases in the death machinery functioning in *Leishmania* promastigotes in response to betulin. It is worth mentioning that betulin can efficiently decrease the amastigote stage of parasite in infected macrophage culture in vitro. Betulin showed selective toxicity towards intracellular *Leishmania* amastigotes, having no much effect on host macrophage J774A.1 cells. Thus, betulin can be explored for developing new anti-leishmanial therapeutic agent to treat emerging drug resistance leishmaniasis.

4.3 Materials and Methods

***4.3.1 Parasites, Cell lines and Chemicals*:** The *Leishmania donovani* (BHU-1081) promastigotes culture was obtained from Prof. Shyam Sundar, Banaras Hindu University and Macrophage cell line J774A.1 used in the study was taken from "National Centre for Cell Science" (NCCS), Pune, India. H_2DCFDA dye from Invitrogen. The apoptosis detection kit and mitochondrial membrane potential detection kit were procured from Calbiochem. Caspase 3/7 detection kit procured from Promega. Betulin (>98% pure, Sigma-Aldrich) 5

mM stock was prepared in DMSO. For all experiments<0.5% DMSO served as negative control. All the chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich or Merck.

***4.3.2 Maintenance of Cell and Parasite Cultures*:** The promastigote culture of *L. donovani* (BHU-1081) was cultivated in M199 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Macrophage cell line J774A.1 was cultured in RPMI 1640 media supplemented with 10% heat inactivated FBS, 2mM glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin.

***4.3.3 Detection of ROS*:** *L. donovani* promastigotes endogenous ROS generation was measured in betulin treated or untreated cells using a cell permeable probe 2', 7'-dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA) by flowcytometer analysis. In brief, promastigote cells (2×10^7 cells/ ml) after different periods of treatment with IC_{50} dose of betulin were washed with PBS and suspended in M199 media prior to incubation with 10 µM of H_2DCFDA probe at 25°C for 30 min in the dark. The non-fluorescent membrane permeable probe H_2DCFDA gets hydrolyzed intracellular in presence of H_2O_2 and converts to fluorogenic impermeable 2', 7'- dichlorofluorescein (DCF). Thus, production of ROS by betulin was monitored by measuring the DCF fluorescence using a BD FACSCalibur flowcytometer which was analyzed with CellQuest software.

***4.3.4 Apoptosis Detection by Annexin V-FITC and PI Staining*:** Either treated or untreated *L. donovani* promastigote cells phosphatide serine (PS) externalization was detected by Annexin V-FITC apoptosis detection kit (Calbiochem). The cells were either untreated or treated with IC_{50} dose of betulin for 6htime point. After treatment with betulin, cells were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed with cold PBS. The cells (1×10^7 cells/ ml) were suspended in 500 µl of 1× binding buffer and stained with annexin V-FITC antibody and PI as per instructions given by the manufacturer. After staining, the cells were sorted for fluorescence intensity using BD FACSCalibur flowcytometer and the fraction of cell population in different quadrants was analyzed by quadrant statistics using CellQuest software.

***4.3.5 Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)*:** The mitochondrial transmembrane potential was investigated using MitoCapture™ apoptosis detection kit (Calbiochem) by fluorescence microscopic analysis. A mitochondria-specific probe, MitoCapture™ is a fluorochrome cationic dye which accumulates in mitochondria under the influence of $\Delta\psi_m$, giving off a bright red fluorescence. Whereas in depolarized $\Delta\psi_m$ it fails to accumulate in mitochondria and exists as monomer in cytosol giving green fluorescence. In brief, promastigote cells (1×10^7 cells ml^{-1}) after 6 h period of treatment with IC_{50} dose of betulin were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed with cold PBS. The cells (1×10^6 cellsml-1) were suspended in 100 µl of incubation buffer containing MitoCapture™ reagent which was diluted as per instructions given by the manufacturer and incubated at room temperature for 30 min. After staining, the cells were washed twice with PBS, mounted on the glass slide and were photographed using fluorescent microscope.

***4.3.6 Determination of Caspase-3/7 like Protease* Activity:** The caspase like protease activity was measured fluorometrically using the Apo-1 homogenous caspase 3/7 activity assay kit (Promega). The assay was performed according to the instructions provided by the manufacturer with minor changes. In brief, *L. donovani* promastigote cells were either treated or untreated with IC_{50} dose of betulin for 6 h time period were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed with cold PBS. The cells were suspended in a 100 µl of reaction buffer containing caspase substrate Z-DEVD-R110, was incubated for 4 h in dark at room temperature. After completion of incubation period, the increase in fluorescence due to cleavage of the Z-DEVD-R110 substrate was measured by fluorometrically at excitation and emission wavelengths of 485 and 530 nm, respectively. In a parallel set of reactions, caspase inhibitor Ac-DEVD-CHO was added to the reaction mixture prior to the addition to treated cells.

***4.3.7 Determination of DNA Fragmentation by Agarose Gel Electrophoresis*:** To analyze the DNA fragmentation standard genomic DNA isolation protocol was followed (*Sambrook et al. 1989*). In brief, *L. donovani* promastigote cells were cultured in 6 well plates either treated or untreated with IC_{50} dose of betulin for 48 h time period. After incubation time point samples were harvested by centrifugation at 1000 × g for 5 min. Cells lysed in 0.5 ml of extraction buffer (10 mMTris–HCl, pH 8.0, 100mM EDTA, pH 8.0, 0.5 % SDS)

containing proteinase K (15.6 $mgml^{-1}$) were vortexed and allowed to digest at 50°C for 1 h. The lysates were then extracted using phenol- chloroform- isoamylalchol (25:24:1) and centrifuged for 10 min at 16000 × g. The upper aqueous phase was carefully collected in fresh tubes and incubated overnight with 3 M sodium acetate and 100 % ethanol at –20°C. The samples were centrifuged at 16000 × g for 10 min at 4°C, supernatant was removed and pellet was washed with 70 % ethanol. The DNA was solubilized in nuclease free water and quantified spectrophotometrically at 260/ 280 nm. On a 1.5 % agarose gel containing 0.5 µg ml^{-1} ethidium bromide, 5 µg of DNA was loaded and electrophoresed. The gel was run for 1.5 h at 60 V and visualized in a UV transilluminator.

***4.3.8 Preparation of f-CNT-BET*:** The carboxylic functionalization of CNTs were prepared by the oxidation of raw CNTs with concentrated H_2SO_4 /HNO_3 (volumetric ratio 3:1) to form a stable aqueous suspension of individual CNTs with carboxyl groups (*Sahoo et al., 2009*). The carboxylic functionalized CNTs were conjugated with BET in a carbodiimide-activated esterification reaction (*Sahoo et al., 2011*). In a distinctive procedure, 50 mg of purified carboxylic f-CNTs were dissolved in 10 ml of dimethyl sulfoxide (DMSO) and sonicated for 0.5 h to obtain a homogeneous suspension of carboxylic f-CNTs. Later, 5 ml of BET (100 mg/ml) in DMSO was added drop by drop in a stirring mixture of f-CNTs. In a separate beaker, 500 mg of N,N'-dicyclohexylcarbodiimide (DCC) and 75 mg of triethyl amine (Et_3N) were added to the suspension of f-CNT and stirred in flask for 24h. After the completion of the reaction, the DCC urea was separated by filtration. DMSO solution was washed with ethyl acetate and brine solution. Combined organic layers were collected and evaporated using rotary evaporator to obtain f-CNT-BET and dried in vacuum. The functionalization of CNTs and attachment of BET to the f-CNTs were confirmed by Fourier transform infrared (FTIR) spectroscopy and TEM.

***4.3.9 BET Loading Efficiency on f-CNTs*:** Loading efficiency of BET onto the f-CNTs was measured using spectroscopic method. In brief, BET was initially scanned to check for its maximum absorbance and the same absorbance was used in quantifying BET further. To calculate the amount of BET on to f-CNT, a suspension of f-CNT-BET dissolved in DMSO was centrifuged at 10,000 rpm for 10 min to precipitate f-CNT-BET. The absorbance of BET

in supernatant was measured using a UV-visible spectrophotometer and was subtracted from absorbance of BET in DMSO which was used as standard to calculate the relatively bound BET onto the f-CNTs. Three individual preparations of f-CNT-BET were carried out for loading efficiency to check reproducibility.

***4.3.10 In vitro Drug Release Experiments*:** The stability and rate of BET release from f-CNT-BET was determined in a two separate dialysis setups of 2 ml of f-CNT-BET against 15 ml of phosphate buffer in a two different flasks, one with pH 7.4 and the other with pH. 5.8 incubated in a 37 °C shaker for 72 h. At different time point, 2 ml of dialysate was collected out from the flask and replaced with equal volume of fresh phosphate buffer. The release of BET was quantified by spectroscopic measurement read at A_{210} nm as BET gave maximum absorbance at 210 nm.

***4.3.11 In vitro Cytotoxicity on Macrophage Cells*:** The cytotoxic effect of BET, f-CNTs and f-CNT-BET was studied using mouse macrophage cell line J774A.1 by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (*Mosmann, 1983*). In brief, mouse macrophage cell line J774A.1 were cultured in 96-well plates, 2000 cells/well were seeded and allowed to adhere overnight at 37°C in a humid atmosphere of 5% CO_2. Next morning the un-adhered cells were removed by PBS washing and replaced with fresh media containing defined concentration of either BET or f-CNTs or f-CNT-BET and incubated for 48 h. After completion of incubation period, MTT assay was performed as described previously in chapter III with some minor changes.IC_{50} values for each of the compound were calculated by plotting % cell viability vs. concentration.

***4.3.12 In vitro Anti-leishmanial Activity Assay*:** The mouse macrophage cell line J774A.1 was cultured overnight on glass cover slips. The cell density of 5×10^5 cells was maintained for proper distribution on the cover slip. After overnight incubation non-adherent cells were removed by washing with PBS and fresh media was added. Macrophages were infected with *L. donovani* promastigotes by maintaining parasite:macrophage ratio of 10:1 and incubated at 37°C in 5% CO_2 for 6 h to ensure parasite phagocytized by macrophage cells. After incubation, un-phagocytized parasites were removed by twice washing with PBS and fresh media was added and then incubated for 6 h. The graded concentrations of BET or f-CNTs or

f-CNT-BET was added and further incubated for 48 h. After completion of incubation with compounds, cells were then fixed in methanol and Giemsa stained. Anti-leishmanial efficacy of compounds towards the intracellular amastigotes was evaluated by microscopic counting of 200 infected macrophage cells and compared with untreated control. The parasite density in treated cells was expressed as a percentage of control and the IC_{50} value for each of the compound was calculated by plotting % amastigotes/100 macrophage vs. concentration.

4.4 Results

***4.4.1 Betulin Induces Generation of ROS in Parasite Causing Oxidative Stress*:** The total ROS generated in the form of superoxide anion radicals, as a result of oxidative phosphorylation during aerobic respiration and can extend up to 3 to 5% of the total oxygen utilized (*Boonstra and Post, 2004*). The results of our earlier studies show that betulin inhibits key redox enzyme, trypanothione synthetase (TryS), of *L. donovani* (*Saudagar and Dubey, 2011*). Hence, it was desirable to investigate the ROS levels in betulin treated leishmanial cells. *L. donovani* promastigote cells treated with IC_{50} concentration of betulin for 3 h resulted in significant ROS generation as evident by flowcytometric analysis (Figure 4.1). In, NAC (20mM) pretreated *L. donovani* promastigote cells, the level of ROS produced decreased drastically and was almost same as that of control cells. Thus, from the above results it is convincing to say that betulin causes oxidative stress in *Leishmania* parasite.

***4.4.2 Betulin Treated Leishmanial cells Exhibited Externalization of Phosphatidylserine*:** Cells that are undergoing apoptosis exhibit a ubiquitous change is the translocation of phosphatidylserine from the inner side of cell membrane to outer cell surface, which can be detected by using annexin V, as it has affinity to bind to exposed phosphatidylserine. Further, in late apoptosis or necrosis, there is membrane damage that permits propidium iodide (PI) to diffuse inside the cell and stain DNA. As evident from ROS studies, betulin induces ROS inside the leishmanial cells. As it is very well known that intracellular ROS is a key regulator for inducing apoptosis (*Sen et al., 2004; Roy et al., 2008*), we investigated if betulin causes apoptosis in leishmanial cells. *L. donovani* promastigote cells treated with IC_{50} concentration of betulin for 6 h were double stained with Annexin V-FITC and PI.

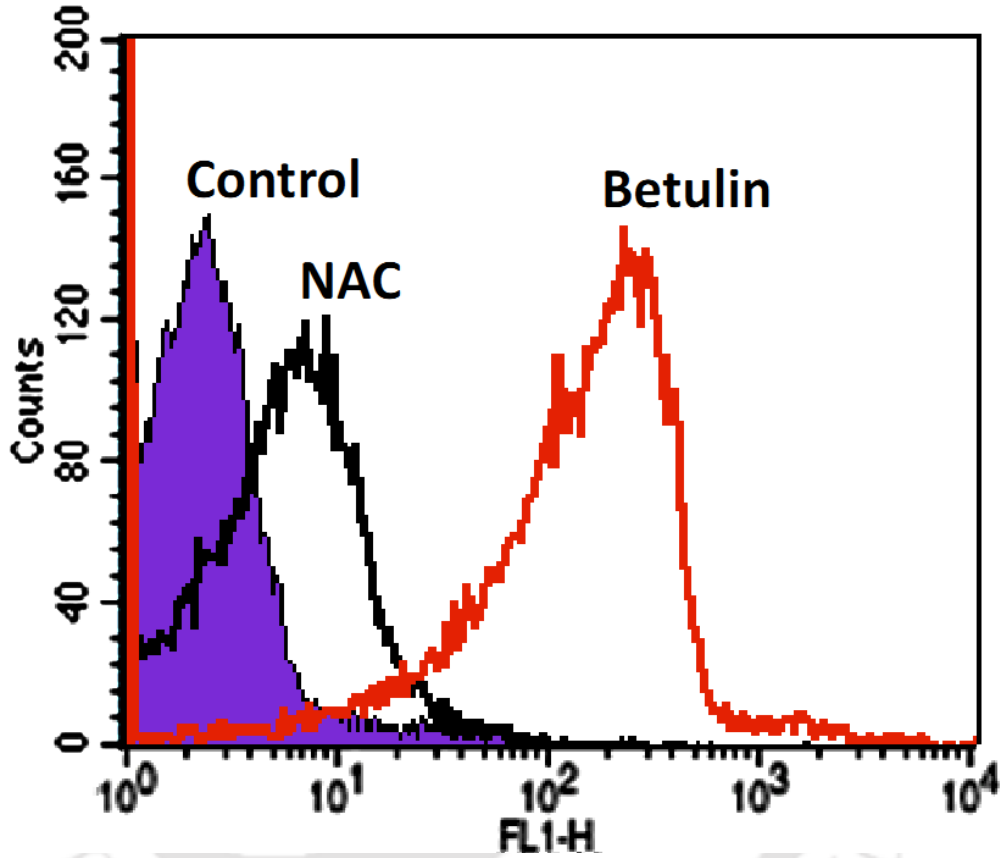


Figure 4.1: Flowcytometric analysis of ractive oxygen species. Betulin induced reactive oxygen species (ROS) is measured by DCF fluorescence. NAC represents data after N-acetyl cysteine (NAC) pre-treatment while betulin represents data after betulin treatment. The data clearly reflects increase in ROS after treatment with betulin. As NAC is scavenger of ROS, NAC pre-treatment prevented ROS accumulation to a large extent. Data shown is representative experiment of at least three experiments which gave similar results.

A significant percent of leishmanial cells stained positive for Annexin V-FITC. As shown in the Figure 4.2C, 27.78% of cells are in early apoptotic stage (lower right quadrant) when compared to control where only 0.11% cells are observed (Figure 4.2A). A clear cut difference in cells that are in late apoptotic stage (upper right quadrant) was observed in betulin treated and control cells. In betulin treated leishmanial cells 55.59% of cells are in late apoptotic stage (Figure 4.2C), whereas only 0.31% of cells were present in control (Figure 4.2A). When the cells were treated with NAC (20 mM) before treatment with betulin, phosphatidylserine externalization was suppressed and cells behaved normally as that of control (Figure 4.2B). From this data it can be concluded that betulin induced ROS triggers apoptosis processes in leishmanial cells.

***4.4.3 Betulin Induces Depolarization of Mitochondrial Membrane Potential ($\Delta\psi_m$) in Leishmania*:** Depolarization of mitochondrial membrane potential is the characteristic feature observed in cells that are undergoing programmed cell death. Betulin treated *Leishmania* cells were investigated for depolarization of $\Delta\psi_m$. The detection of $\Delta\psi_m$ in betulin treated/untreated *Leishmania* cells was done using MitoCapture™ apoptosis detection kit (Calbiochem). MitoCaptur™ is a cationic mitochondrial specific dye that gets aggregated in normal mitochondria, whose $\Delta\psi_m$ is retained, giving red fluorescence. Whereas in cells with altered $\Delta\psi_m$, MitoCapture™ fails to aggregate in mitochondria and accumulation in cytosol in its monomeric form, giving green fluorescence. Thus, the fluorescence of MitoCapture™ reagent can be regarded as an indicator of $\Delta\psi_m$ energy state. The cells were treated with IC_{50} dose of betulin for 6 h and were analyzed using fluorescent microscope. A green fluorescence was observed under fluorescent microscope in 6 h treated cells due to complete loss in the mitochondria $\Delta\psi_m$ as detected by MitoCapture™ reagent. Whereas in control untreated cell MitoCapture™ reagent gave red fluorescence indicating retention of membrane potential in cells (Figure 4.3). The data indicate that betulin treatment lead to mitochondrial membrane damage that leads to apoptosis as monitored by Annexin V-FITC and PI staining (Figure 4.2).

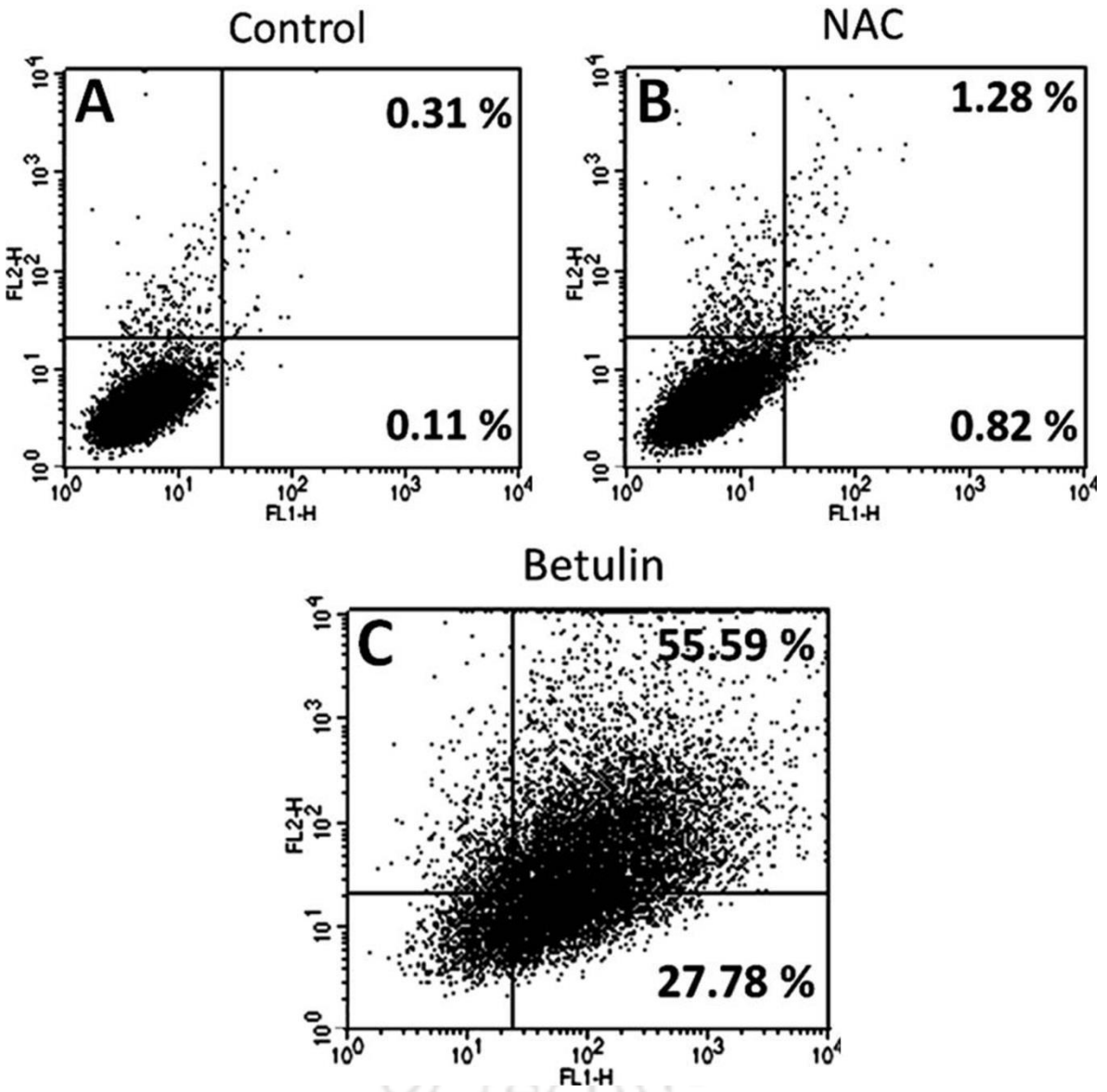


Figure 4.2: Betulin elicits apoptosis in *Leishmania* promastigotes. **(A)** The *Leishmania* promastigotes treated with 0.2% DMSO for 6 h were double stained with PI and annexin V-FITC and used as control. **(B)** N-acetyl cysteine (NAC) pre-treated *Leishmania* promastigotes were treated with IC_{50} dose of betulin for 6 h were double stained with PI and annexin V-FITC. **(C)** *L. donovani* promastigotes cells treated with IC_{50} dose of betulin for 6 h were double stained with PI and annexin V-FITC. All data shown are representative experiments of at least three experiments which gave similar results.

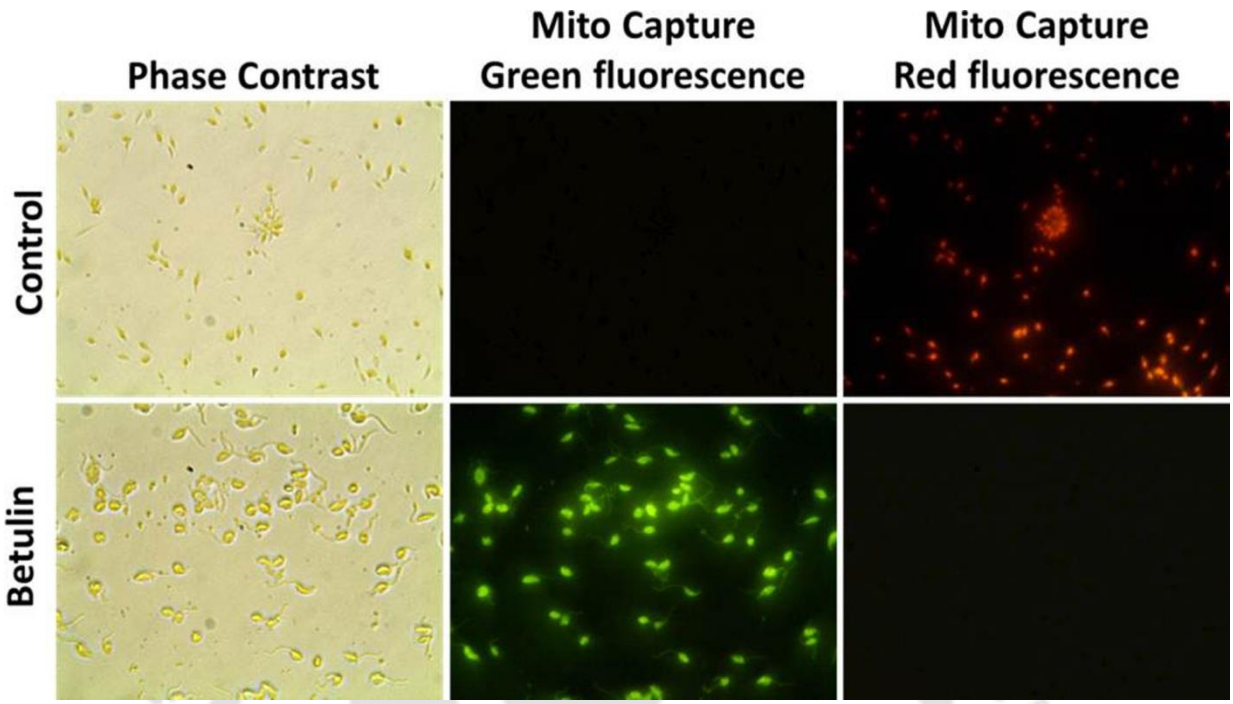


Figure 4.3: Betulin induces mitochondrial membrane injury. *Leishmania* promastigotes were treated with IC_{50} dose of betulin for 6 h, stained with mitochondria- specific cationic dye, MitoCapture™. Untreated *Leishmania* cells were used as control. The promastigotes cells were photographed for control and for betulin treated cells with excitation wavelengths of ~570 nm (red) and ~ 500 nm (green), respectively, using fluorescence microscope (60x). Normal mitochondrial membrane potential is indicated by the red fluorescence whereas green fluorescence indicates depolarization mitochondrial membrane. In the optimization process of incubation time with the dye, we noticed that 20 minutes incubation time recommended by manufacturer, does not give intense red fluorescence in control *Leishmania* cell and image quality was not good. Moreover, some green fluorescence was also seen in control cell. Thus, a longer incubation time was used to insure maximum uptake of the dye by mitochondria. After 30 minutes of the dye incubation and subsequent wash with PBS, control cells gave good red fluorescence intensity.

***4.4.4 Betulin Triggers Activation of Caspase-Like Protease and DNA Fragmentation in Leishmania*:** The altered $\Delta\psi_m$ may consequence in release of cytochrome *c* into the cytosol which leads to the activation of caspases in cell which triggers the downstream events leading to apoptosis (*Kupchan et al. 1969; Zou et al. 1997*). *L. donovani* promastigotes treaded with IC_{50} dose of betulin for 6 h time point was studied by spectrofluorometric method using Apo-ONE Homogeneous Caspase 3/7 assay kit (Promega). An increased caspase-like activity was observed in betulin treated leishmanial promastigotes cells (Figure 4.4A). *Leishmania* promastigotes treaded with IC_{50} dose of betulin with caspase inhibitor (DEVD-CHO inhibitor) was very much similar to that of control cells. The control betulin

untreated leishmanial promastigotes cells did not show any caspase-like protease activity. In control, *L. donovani* promastigotes were treated with 0.2% DMSO to compensate the effect of DMSO that was used for dissolving caspase inhibitor or substrate solution. Fragmentation of genomic DNA is considered as the hallmark of apoptotic cell death (*Compton 1992*). To establish betulin induced genomic DNA fragmentation, we assayed agarose gel electrophoresis of genomic DNA isolated from betulin treated/untreated leishmanial promastigotes cells as described in the *materials and methods* section. It was observed that *L. donovani* promastigote treated with IC_{50} dose of betulin for 48 h time point showed fragmentation of DNA (Figure 4.4B). It is predicted that the action of both ROS and caspase like proteases may be responsible for DNA fragmentation in betulin treated leishmanial promastigotes cells.

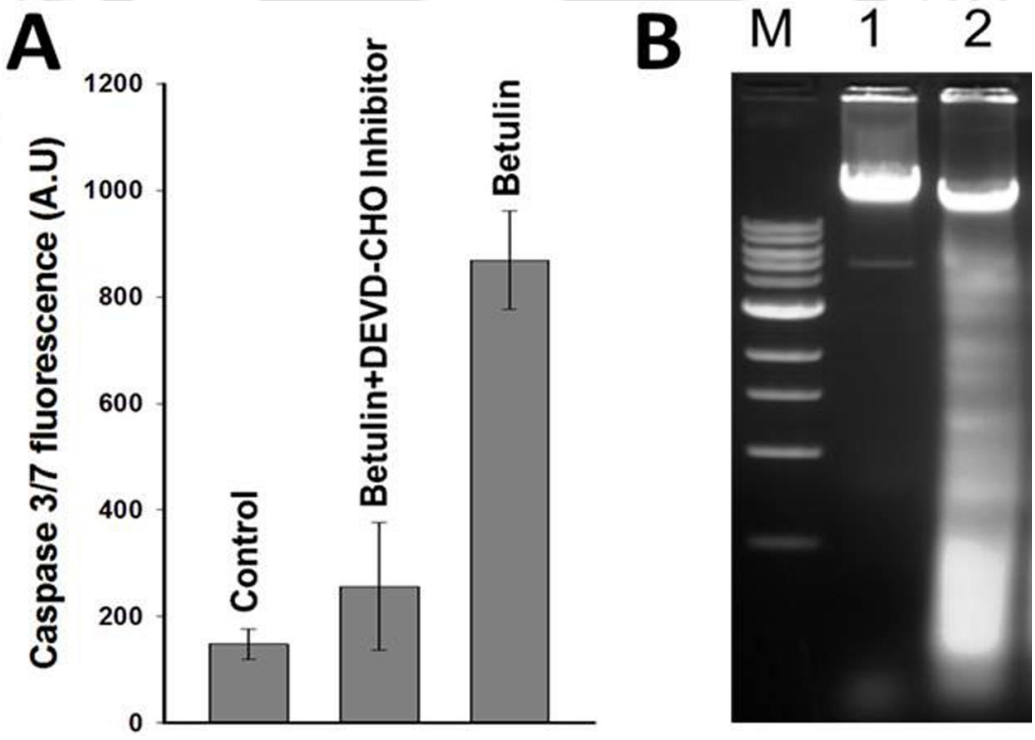


Figure 4.4: (A) Activation of Caspase- 3/7 like protease inside *Leishmania* promastigote cells after treatment with IC_{50} dose of betulin and in absence or presence of protease inhibitor (DEVD-CHO inhibitor) for 6 h time point. Untreated l *Leishmania* promastigote cells are used as control. Data represent the mean ± SD of three independent experiments. **(B)** *Leishmania donovani* promastigotes genomic DNA fragmentation after treatment of betulin compared to control (untreated promastigote). Lane M represents DNA molecular mass marker (1 kb DNA ladder), lane 1 represents control while lane 2 indicates cells after treatment with IC_{50} dose of betulin for 48 h, respectively.

4.4.5 Conformation of f-CNT-BET Construct as Characterization by FTIR and TEM:

The f-CNT-BET were synthesized by carbodiimide-activated esterification reaction and the schematic representation of the same is shown in Figure 4.5. The carboxylic acid functionalization of CNTs (f-CNTs) and attachment of BET on to the f-CNTs was successfully achieved as evident from FTIR spectroscopic analysis. The Figure 4.6A corresponds to the FTIR spectra of CNTs. The strong band at wavelength 3426 cm^{-1} corresponds to the presence of –OH moieties on the surface of CNTs. In Figure 4.6B, represents the FTIR spectra of carboxylic group functionalized CNTs. As a result of functionalization, the new peak at wavelength 1768 $cm^{-1}$is due to C=O stretching of carboxyl functional groups (–COOH). The peaks at wavelength 2920 cm^{-1} and 2849 cm^{-1} were assigned to O–H stretch of carboxyl functional groups. The distinctive peaks observed in FTIR spectra of f-CNTs have confirmed the carboxylic functionalization of CNTs. The Figure 4.6C represents the FTIR spectra of BET. The attachment of BET on to f-CNT was achieved by carbodimide based esterification reaction. The synthesis of f-CNT-BET was confirmed by the characteristic ester C=O asymmetric stretching as studied by FTIR spectra. In Figure 4.6D, the peak at 1741 is due to the C=O asymmetric stretch of the ester linkage present in synthesized f-CNT-BET. The physical characterization of f-CNTs and f-CNT-BET was done by studying micrograph structures using TEM (Figure 4.7). The micrograph of f-CNTs as seen figure 4.7A & C shows the length and slight rough surface caused due to functionalization with acid treatment. The functionalization of CNTs improves its solubility nature and also provides the surface anchors to attach drug molecules. The micrograph of f-CNT-BET as seen in figure 4.7B & D were also characterized by TEM using the same image scale to understand the morphological changes. The formation of very rough surface on the exterior and interior walls of the f-CNT-BET can be very clearly seen (Figure 4.7D). The diameter of f-CNT-BET is remarkable increased due to attachment of BET on to f-CNT surface, forming a layer. The presence of dark spots indicated by arrow marks shows the drug attachment sites. The successful synthesis of f-CNT-BET is evident from FTIR and TEM results.

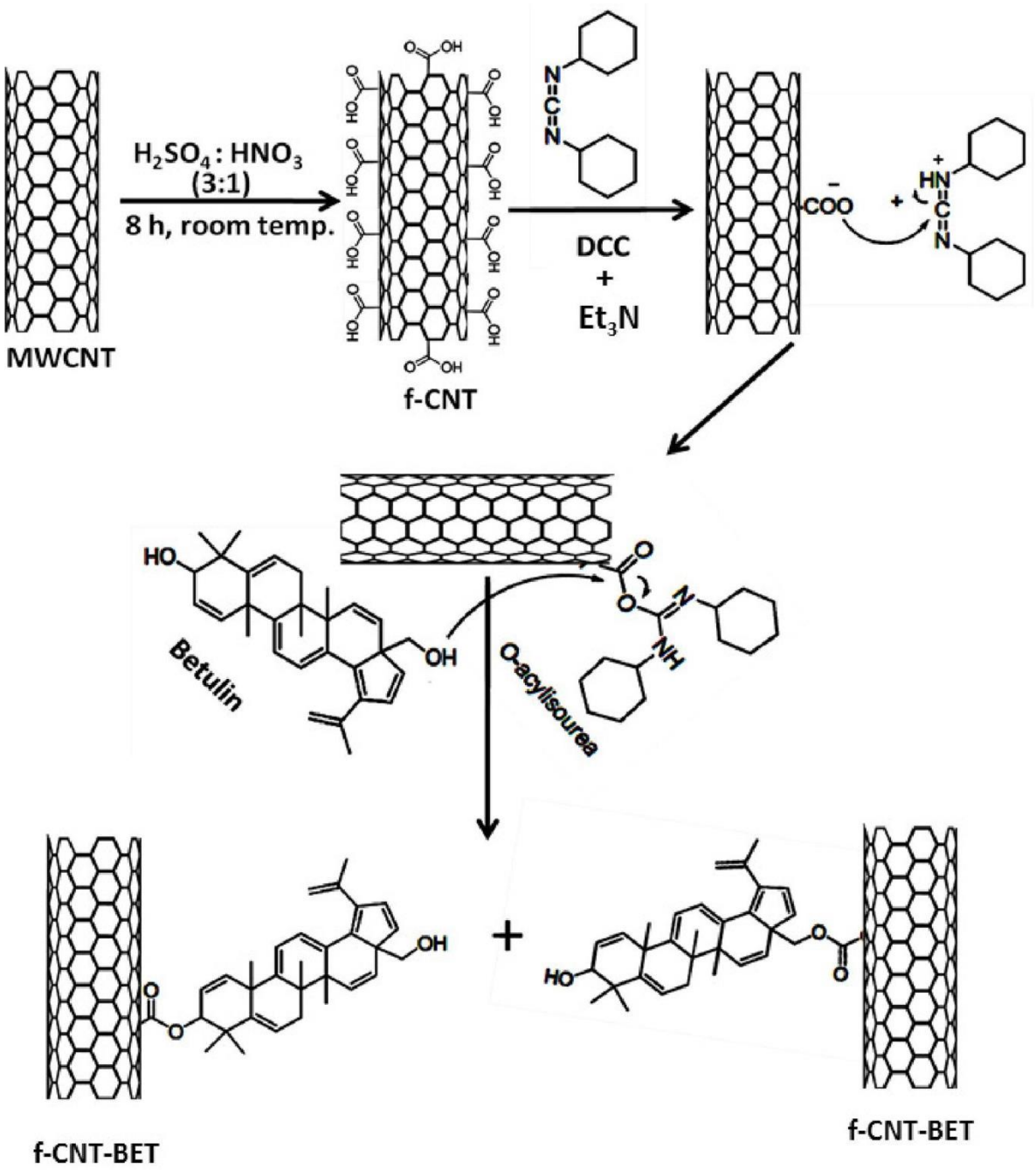


Figure 4.5: Schematic representation of the synthesis of f-CNT attached BET: The carboxylic f-CNTs were prepared by acid oxidation of CNT. The f-CNT-BET were synthesized by carbodiimide-activated esterification reaction in which the carboxyl-reactive chemical groups (–COOH) present on f-CNTs are made to cross-linked with alcohol group (–OH) of betulin in presence of N,N'-dicyclohexylcarbodiimide (DCC) and of triethyl amine (Et_3N). The insoluble DCC urea is separated by filtration.

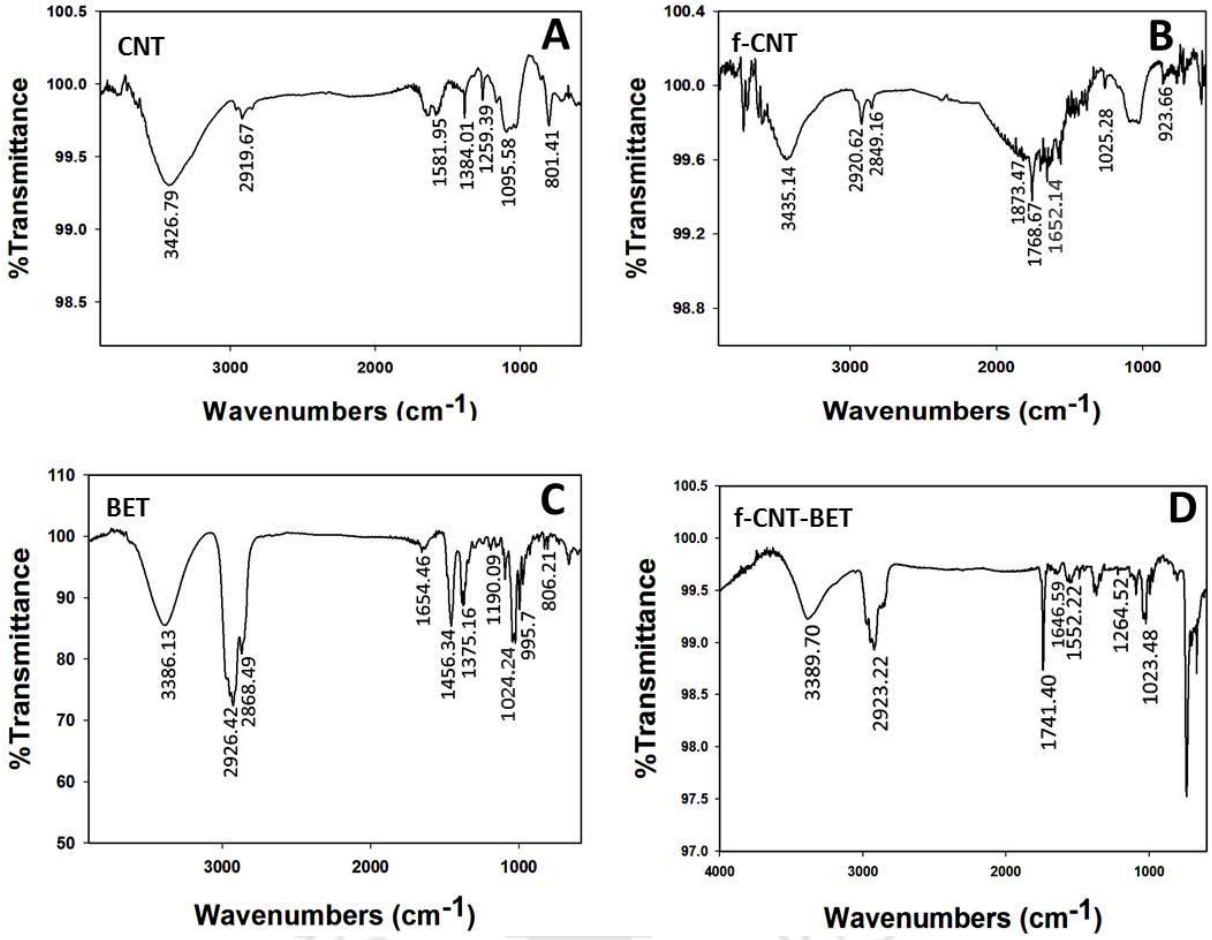


Figure 4.6: Characterization of f-CNT–BET by FTIR spectroscopy: FTIR spectra of **(A)** CNTs **(B)** f-CNTs **(C)** BET **(D)** f-CNT-BET. In f-CNT spectra the peaks at 1768 represent C=O of COOH f-CNT. In f-CNT-BET spectra peak at 1741 C=O stretching of the ester group indicates the attachment of BET to f-CNT to form f-CNT-BET.

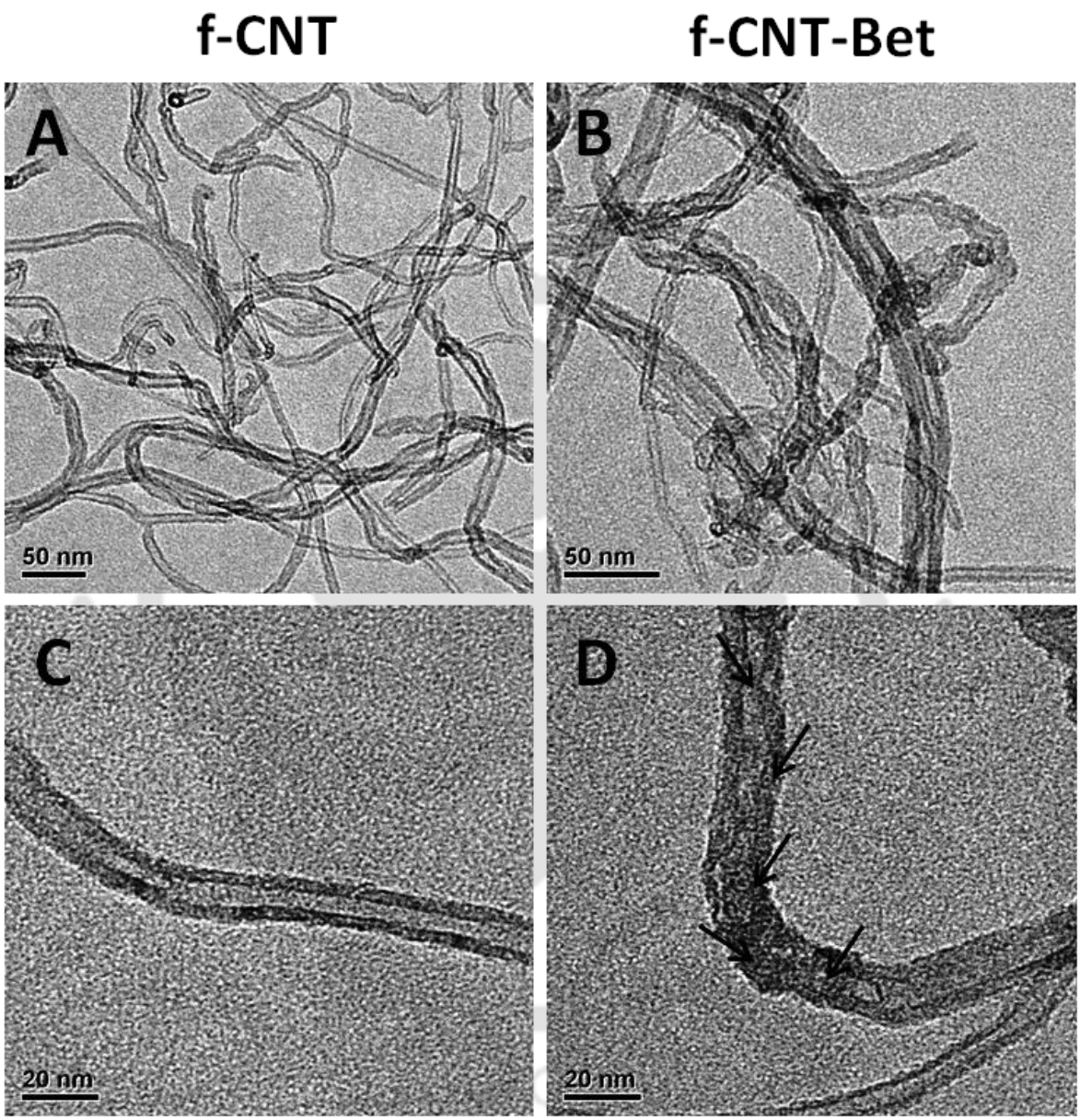


Figure 4.7: Physical characterization of the f-CNT–BET by using TEM: (A) Carboxylic f-CNTs **(B)** BET attached to carboxylic f-CNT **(C)** Surfaces of functionalized CNTs **(D)** Surfaces of f-CNT-Bet attached (the arrows show the rough surface which is due to attachment of BET to a carboxylic f-CNT).

***4.4.6 BET Loading Efficiency onto f-CNTs*:** BET gave the maximum absorbance of 210 nm (Figure 4.8A). Hence, the loading efficiency of BET was spectroscopically measured at same wavelength. The absorbance of 3.846 and 0.833 at 210 nm was recorded by 1mg/ml BET and unbound BET from the supernatant of f-CNT-BET, respectively. Thus, the percentage of BET loaded on to the f-CNTs was calculated to be 78.3% (Figure 4.8B). The percentage of BET loading onto the f-CNTs was reproducible.

***4.4.7 In vitro Release of BET*:** The Figure 4.8C summarizes the in vitro drug release profiles of BET from f-CNT-BET in phosphate buffer with pH 7.4 and 5.8 at 37 °C. The drug release was tested under acidic and neutral pH conditions to mimic the cytosol and lysosome conditions of the macrophage cells. The release of BET reached up to 38.4 ± 1.8 % in acidic pH 5.8 in a 72 h incubation period, whereas only 12.5 ± 1.1 % of BET release was observed in neutral pH 7.4 condition with same incubation time. The more release of BET in acidic condition is observed compared to neutral pH condition because the ester bond degrades rapidly under acidic condition and slowly in the neutral pH condition.

***4.4.8 In vitro Cytotoxicity*:** In host, *Leishmania* parasite thrives and proliferates inside the macrophage cells and hence, it is the main site for drug delivery. Nano formulation mediated drug delivery is specific for macrophages due to their phagocytosis nature. Therefore, it is important to assess the cytotoxicity of f-CNT-BET formulation to the macrophage cells themselves. The cytotoxicity was studied using standard MTT assay which is also used by other groups to assess the toxic response on CNTs to cells. To understand the cytotoxic effect of BET, f-CNT and f-CNT-BET on host cells, the in vitro cytotoxicity studied on macrophage cell line J774A.1 was evaluated at different concentrations and the fifty percent inhibition concentration was calculated for all the three compounds (Figure 4.9). The IC_{50} value of BET, f-CNT and f-CNT-BET was calculated to be 211.05 ± 7.14 µg/ml, 24.67 ± 3.11 µg/ml and 72.63 ± 6.14 µg/ml, respectively. The data clearly indicates that BET, f-CNT and f-CNT-BET did not any significant toxicity and there by indicating their safe application in anti-leishmanial drug formulation.

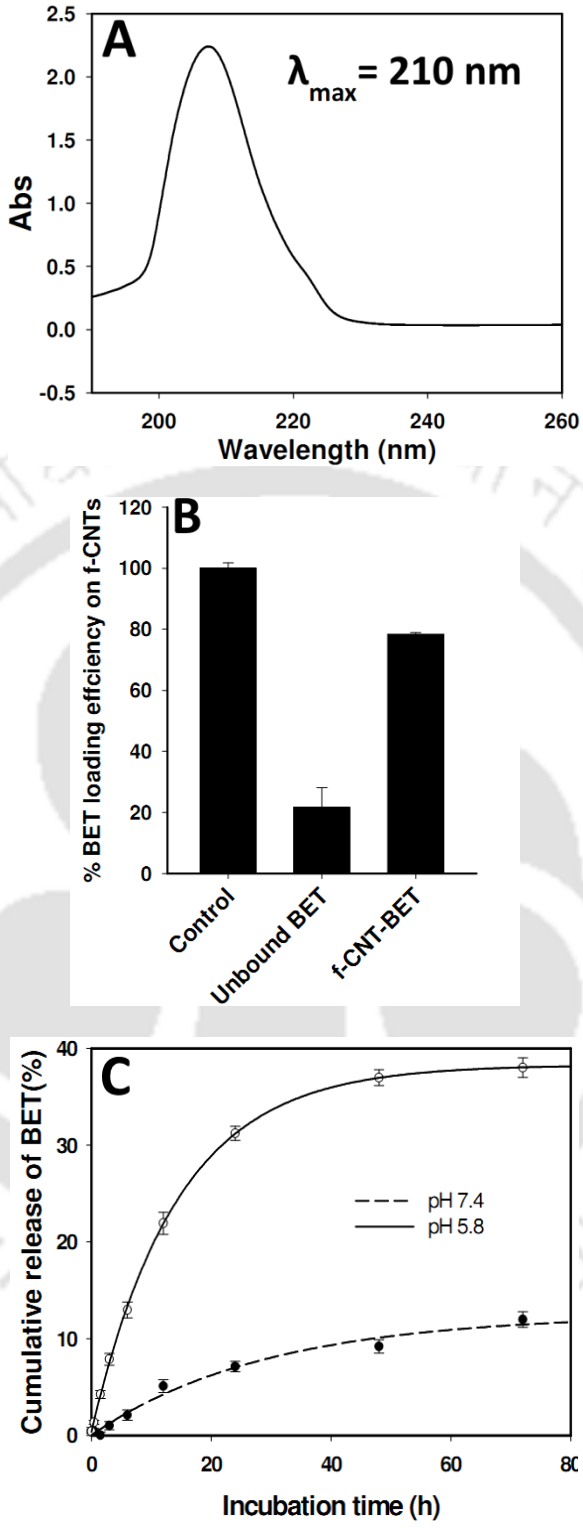


Figure 4.8: Measuring BET loading efficiency onto the f-CNT and its release: **(A)** spectral scan of BET compound. The maximum absorbance read by BET is 210 nm; **(B)** Loading efficiency BET on to f-CNT. Around 78.3% of the drug loaded efficiency was achieved; **(C)** The drug release profile of BET studied at acidic and neutral pH conditions. Around 38.4±1.8 % of BET was released in acidic pH condition whereas only around 12.5±1.1 % of BET release in neutral pH condition.

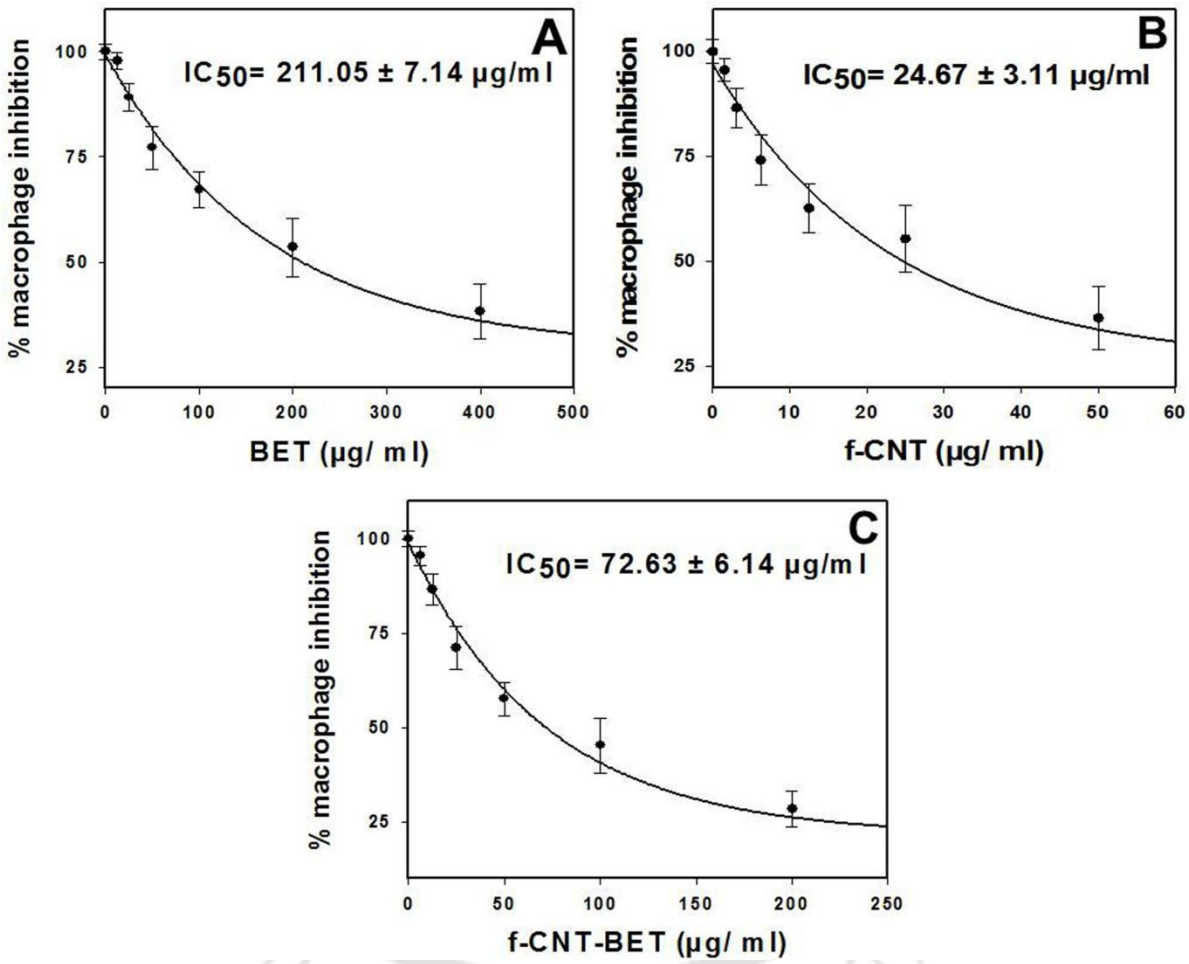


Figure 4.9: In vitro assessment of cytotoxicity studied on macrophage cells (J774A.1 cell line). (A) Cytotoxic effect of BET on macrophage cells and the IC_{50} value observed to be 211.05±7.14 µg/ml **(B)** The Cytotoxic effect of f-CNTs on macrophage cells and the IC_{50} value observed to be 24.67±3.11 µg/ml **(C)** The cytotoxic effect of f-CNT-BET on macrophage cells and the IC_{50} value observed to be 211.05±7.14 µg/ml. Values are the mean ± S.D. of three determinations.

***4.4.9 The f-CNT-BET has Better Efficacy than BET*:** The in vitro anti-leishmanial activity of BET and f-CNT-BET was evaluated at different concentrations against intracellular *Leishmania donovani* amastigote cell for 48 h of treatment. The anti-leishmanial activity in the form parasite burden in parasite infected macrophage cells was studied microscopically. There is a significant difference observed in parasite burden in cells treated with BET and f-CNT-BET. It was observed that nearly 80 % of the amastigotes were inhibited at 3.91 µg/ml of f-CNT-BET whereas only ~30 % inhibition was observed with BET at same concentration (Figure 4.10). The IC_{50} value for BET and f-CNT-BET calculated was 8.33 ± 0.41 µg/ml and 0.69 ± 0.08 µg/ml, respectively. There is more than tenfold difference in the IC_{50} value for BET and f-CNT-BET, which clearly indicate the increased efficacy of drug due to their targeted site of delivery. The anti-leishmanial activity of f-CNTs was also tested and there is marginal anti-leishmanial activity detected at higher concentration (Figure 4.10). Thereby, revealing that the improved efficacy is mainly due to the action of the drug attached to f-CNT but not by f-CNT alone.

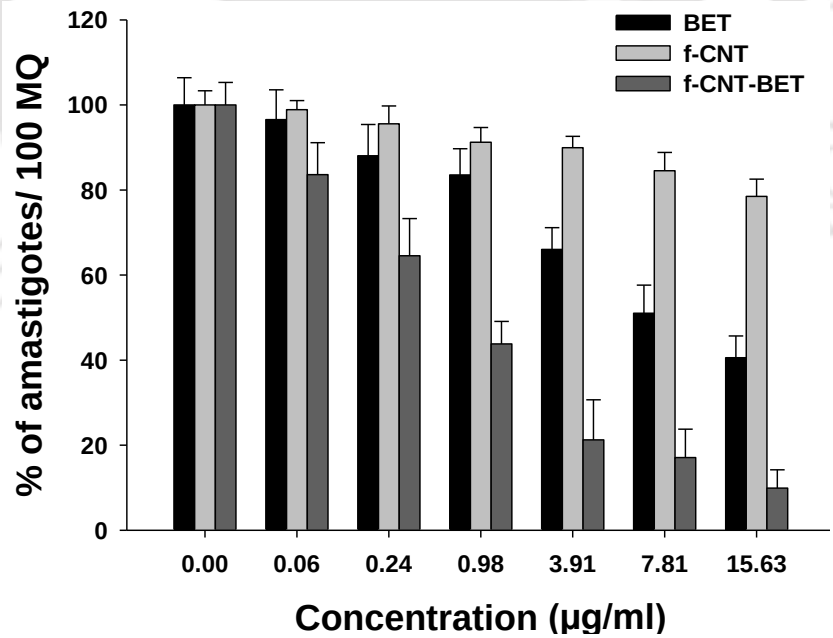


Figure 4.10: In vitro anti-leishmanial activity on intracellular amastigote cells: The anti-leishmanial activity of BET, f-CNTs, and f-CNT-BET was studied using microscopic counting of amastigote cell inside the infected macrophage cells. A dose dependent response is observed with BET and f-CNT-BET, whereas the f-CNT showed marginal anti-leishmanial effect on parasite. This reveals that the f-CNTs alone are not anti-leishmanial and the improved efficacy is due to impact of the drug attached to f-CNT-BET. The IC_{50} value for BET and f-CNT-BET is found to be 8.33 ± 0.41 µg/ml and 0.69 ± 0.08 µg/ml respectively.

4.5 Discussion

Leishmaniasis is potentially emerging as drug resistance infection, it is essential to study the disease to find effective anti-leishmanial drugs. Anti-leishmanial drugs that originate from natural sources have benefits including low toxicity, less or no side effects, high activity and abundant variety in nature. Betulin, a triterpene present in the bark of white birch trees is abundant naturally occurring compound. In our previous report, we have identified betulin as an inhibitor of TryS of *L. donovani* and as potent anti-leishmanial compound (*Saudagar and Dubey, 2011*), but the possible molecular mechanism and type of cell death induced by betulin was not studied. In this study, we attempted to understand the molecular mechanisms underlying the anti-leishmanial property of betulin. We were interested to know whether betulin induced parasites death process resembles metazoan apoptosis involving death machinery or not. Betulin, a known anti-cancerous agent that induces apoptosis in human cancer cells through the intrinsic apoptotic pathway (*Li et al., 2010*). Betulin and its derivatives are reported to inhibit type IB DNA topoisomerase of *L. donovani* (*Chowdhury et al., 2011*). As betulin inhibits redox enzyme of *Leishmania* parasites, we were interested to study the ROS accumulation in betulin treated cells. *L. donovani* promastigotes treated with betulin exerts ROS generation efficiently. The *N-acetyl cysteine* (NAC), pre-treated *L. donovani* promastigotes failed to accumulate ROS upon treatment with betulin indicating the effective removal of ROS by NAC. Inhibition of TryS by betulin can decrease the $T(SH)_2$ levels drastically in the parasites, thereby increasing the ROS accumulation due to lack of required amount of $T(SH)_2$ for scavenging ROS, resulting in destabilization of cellular redox homeostasis. Further, $T(SH)_2$ is known to participate in many metabolic processes, such as reduction of glutathione disulphide (GSSG), ovithiol disulphide (*Ariyanayagam and Fairlamb, 2001; Steenkamp, 2002*), and as well as direct reduction of dehydriascorbate (DHA), by at least two times faster than GSH (*Bocedi et al., 2010*). $T(SH)_2$ is also known to interact with nitric oxide (*Tovar et al., 1998*), involved in detoxification of ketoaldehydes, display defense against toxic xenobiotics and transferring its reducing equivalent to proteins such as ribonucleotide reductase (*Dormeyer et al., 2001*), and many other functions. Therefore, the metabolic processes that are $T(SH)_2$ dependent are also affected due to the decrease levels of $T(SH)_2$ and may lead to enormous stress.

The accumulation of ROS acts as a primary source in eliciting programmed cell death process. Betulin induced ROS, elicits apoptosis process in *Leishmania* promastigotes was studied by staining *Leishmania* promastigotes with annexin V-FITC and PI. Annexin V is a Ca^{2+} dependent phospholipid binding protein which has an affinity for phosphatidylserine. As annexin V is also known to label necrotic cells, another stain PI was used to distinguish between apoptotic cells, stain annexin V- positive and PI- negative, necrotic calls, stain annexin V- negative and PI- positive whereas normal cells stain both annexin V and PI negative. Our results indicated that betulin induces apoptotic in *Leishmania* promastigotes as confirmed by FACS analysis of externalization of phosphatidylserine in betulin treated cells. It appears that betulin induced ROS generation caused parasite death by apoptosis.

Apoptosis is a cascade process involving physical and biochemical changes in the cell. Mitochondria are the power house of the cell where the cellular ATP is generated by oxidative phosphorylation process and are the easy targets in a cell to induce apoptosis under stress conditions. Mitochondrion is an important organelle for cell survival, any damage or dysfunction to it leads to cell death. Thus, this makes mitochondria a prime target in order to achieve programmed cell death or necrosis. Betulin treatment lead to the accumulation of ROS, thereby increased cell stress which resulted in depolarization of mitochondrial membrane potential in *Leishmania* promastigotes, as observed in a classical programmed cell death process. The depolarization of mitochondrial membrane potential acts as a switch in activation proteases which play an important role in apoptosis process.

We have found that betulin treatment in *Leishmania* promastigotes resulted in apoptotic death of the parasite is mediated through caspase like proteases activity as cell lysates of apoptotic cells have cleaved caspase substrate. Few other reports also indicate caspase like activity in *Leishmania* (*Das et al., 2001; Lee et al., 2002; Das et al., 2008*). Caspases that has essential role in apoptosis in higher eukaryotic organism are absent in genome of *Leishmania* (*Castanys-Muñoz et al., 2012*). Metacaspases are cysteine proteases distinctly related to caspase are found in *Leishmania* (but absent in mammal) was initially thought to be responsible for caspase like activity. However, recently, they are reported to have trypsin like activity rather than caspase like activity (*Moss et al 2007*). Thus, it remains crucial to

rigorously analyze possible enzyme that is responsible for caspase like activity. This may provide fundamental insights into apoptotic pathways in *Leishmania*.

The damaged mitochondrial membrane releases apoptotic eliciting signals that switches on the cascade for caspase like proteases, which causes genomic DNA fragmentation leading to apoptotic death of the cell. Betulin induced DNA fragmentation was observed in *Leishmania* promastigotes. Betulin inhibits TryS, a redox enzyme of the parasite which is involved in synthesis trypanothione [$T(SH)_2$]. Further, the reduced $T(SH)_2$ helps in deoxyribonucleotides (dNTPs) synthesis by transferring reducing equivalence to ribonuclease reductase which is involved dNTPs synthesis (*Krauth-Siegel and Comini, 2008*). Thus, inhibition of TryS may hinder the DNA synthesis leading to the formation of DNA suicidal complexes that are necessary events of apoptotic DNA fragmentation (*Liu et al., 1997; Liu et al., 1998*).

In the last decade several drug formulations like liposomal, emulsions, micelles and nanoparticles have been employed for targeted delivery of drug to the particular tissue. Unfortunately, most of these formulations fail to reach the target as they are readily engulfed by the macrophage cell that recognizes them as foreign particles. Fortunately this nature of macrophages facilitates the anti-leishmanial drug delivered to the parasite infected site which is macrophage cells itself. Although the most successful drug formulation for leishmaniasis liposomal amphotericin B is available in the market, its use is limited due to its high cost, in spite of preferential pricing of US$18/50 mg negotiated by the WHO. A new drug with cost effective formulation can change the present drawbacks with chemotherapeutics against leishmaniasis. BET, a pentacyclictriterpenoid widely found in the bark of white birch trees is a widely studied anticancer compound can emerge as new anti-leishmanial drug due to its selective toxicity towards parasite cells. We have explored the properties of f-CNTs and BET to construct f-CNT-BET which was achieved by carbodimide based esterification reaction. The drug release was more at acidic conditions, thereby favoring high release of drug at targeted site i.e. lysosome complex inside the macrophage cells where the parasite thrives and proliferates.

We have selected CNTs as the nanocarriers for the attachment of BET, primarily due to their ability to cross the cell membrane and have large surface area where a large quantity of

drug can be loaded and thereby increasing the drug efficacy. This work shows a new approach to leishmaniasis cure. By employing f-CNTs as nanocarriers for the drug delivery system of new anti-leishmanial drug BET, its efficacy is significantly increased as observed in in vitro settings. There is no obvious toxicity measured when tested on macrophage cell, thus indicating their biocompatibility at cellular level. The f-CNTs have several advantages as nanocarriers of drug compared to other materials as the surface of CNTs can be designed according to our need for anchoring the drug stably onto them. The chemical synthesis of f-CNT-BET is rather cost effective then macromolecules, which can be produced in low cost compared to existing liposomal formulation.

4.6 Conclusion

In conclusion, the study in this chapter demonstrated for the first time the molecular mechanism of leishmanial cell death caused by betulin. In *Leishmania* promastigotes, betulin elicit an apoptotic response involving mitochondrial membrane damage and further activation of caspase-like proteases and DNA fragmentation which are hallmark events observed in the apoptosis process. Hence, understanding the molecular mechanism underlying the anti-leishmanial property of betulin provides the opportunity to exploit it for development of anti-leishmanial therapeutics to treat leishmaniasis. Further this study also reports the successful synthesis of f-CNT-BET by carbodimide based esterification reaction and investigated loading efficiency and release prolife of the drug, BET. Studies on the cytotoxicity of BET, f-CNT and f-CNT-BET on macrophage cell which demonstrated their biocompatibility nature at cellular level. The f-CNT-BET showed high anti-leishmanial activity with increased efficacy compared to BET as evident from in vitro anti-leishmanial studies on intracellular amastigote. Taken together these results, f-CNT-BET can emerge as a new therapy in treating leishmaniasis.

Chapter V

Molecular mechanism underlying antileishmanial effect of oxabicyclo derivatives: Inhibition of key redox enzymes of the parasite*

5.1 Abstract

This chapter report oxabicyclo[3.3.1]nonanones as inhibitors of key redox enzymes, trypanothione synthetase (TryS) and trypanothione reductase (TryR) of *Leishmania*. Further, detailed cellular effects of 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester, a oxabicyclo[3.3.1]nonanones, on the parasite were investigated. As these compounds inhibit key redox enzymes (TryR and TryS), treatment of these compounds resulted in increased reactive oxygen species (ROS), mitochondrial membrane damage, activation of caspase like proteases and DNA damage that finally leads to apoptotic death of the parasite. The in vitro and in vivo studies on parasite showed anti-leishmanial effect. Although the compound has modest activity, they identify a novel chemical space to design and develop drugs based on these compounds against the *Leishmania* parasite. This is first report of oxabicyclo[3.3.1]nonanones as anti-leishmanial.

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*** Part of the work is Communicated for publication in *Eur J Med Chem*.**

5.2 Introduction

Leishmania, which belongs to the order *kinetoplastida*, is a dimorphic protozoan parasite. It is an etiologic agent of broad infection spectrum from self-healing cutaneous leishmaniasis (CL) to a devastating and life claiming visceral leishmaniasis (VL), commonly called kala-azar in India. *Leishmania* shuttles between vector sand fly and mammalian host during its life cycle (*Murray et al., 2005*). Search for a successful vaccine against the parasite is still elusive (*Kaye and Aebischer, 2011*). The main stream of treatment solely relies on chemotherapy. The current scenarios of chemotherapeutics against leishmaniasis pose a huge fall due to their high toxicity, high cost, tiresome to administer, and the emergence of drug resistance (*Croft et al., 2006*). By exploiting fundamental metabolic and/or biochemical divergence between host and parasite, rationally designed, specific and potent anti-parasitic drug is a significant strategy in drug discovery process. Conceivably the populous striking metabolic disparities between mammalian host and *Leishmania* is the redox metabolism to fight oxidative stress (*Krauth-Siegel and Leroux, 2012; Krauth-Siegel and Comini, 2008*). In mammalian host, glutathione (GSH) performs several crucial metabolic processes, such as maintaining cellular redox homeostasis and scavenging oxidative stress, induced by either extracellular or intracellular factors. In contrary, *Leishmania* is equipped with a unique defense mechanism to fight reactive oxygen species (ROS). Unlike mammalian host, in which the main thiol is GSH, *Leishmania* depends on a dithiol peptide called trypanothione [$T(SH)_2$] (*Krauth-Siegel and Leroux, 2012; Krauth-Siegel and Comini, 2008*). *Leishmania* redox system lacks glutathione reductase and thioredoxin reductase, the redox fighting enzymes found in mammalian host. It adopted an analogous redox system, consisting of trypanothione reductase, an enzyme analogous to glutathione reductase, which keeps $T(SH)_2$ in reduced form (*Krauth-Siegel and Comini, 2008*). Reduced $T(SH)_2$ plays vital role in various metabolic processes in both *Leishmania* and other trypanosome (*Krauth-Siegel and Comini, 2008*).

In our computational screening processes for inhibitors against redox enzymes of *Leishmania*, we have identified a new class of molecules that inhibit trypanothione synthetase (TryS), an enzyme which catalysis the synthesis of $T(SH)_2$ and trypanothione reductase (TryR). Subsequently, these compounds were tested against TryS and TryR using

experimental methods. The compound 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester showed the highest anti-leishmanial effect among all compounds tested. For ease presentation the compound was designated as PS-203. PS-203 was chosen as representative compound for mode of action studies. These compounds are basically oxabicyclo[3.3.1]nonanones which are our in house creation (*Saha et al., 2010*). Oxabicyclo[3.3.1]nonanones have shown anti-cancerous and other biological active features (*Wang et al., 2008*). We report for the first time, 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester, an oxabicyclo[3.3.1]nonanones, as anti-leishmanial compound and the mechanism of action by inhibition of two key redox enzymes TryS as well as TryR that induces increased reactive oxygen species and apoptotic death of parasite. Further, in vivo cytotoxicity and anti-leishmanial activity was studies Swiss albino mice and hamsters, respectively.

5.3 Materials and methods

***5.3.1 Parasites, Cell lines and Chemicals*:** The *Leishmania donovani* (BHU-1081) was obtained from Prof. Shyam Sundar, Banaras Hindu University, India and cultivated in M199 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. *L. donovani* LEM 138 (MHOM/IN/00/DEVI) stationart-stage promastigotes were used for in vivo work. Macrophage cell line J774A.1 used in the study was taken from "National Centre for Cell Science" (NCCS), Pune, India and cultured in RPMI 1640 media supplemented with 10% heat inactivated FBS, 2mM glutamine, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Cloning expression and purification of TryS, used in the study has already been reported in chapter III. *TRYR* gene inserted in pET28b was generously donated by Dr. Andrea Ilari, Universita` ''La Sapienza'' Rome, Italy. The details of the cloning, expression and purification of TryR is already reported (*Baiocco et al., 2009b*). Synthetic procedure and the spectral data for all the compounds in this study were previously reported (*Saha et al., 2010*). All the chemicals used in the experiments were of the highest grade procured from Sigma-Aldrich and Merck.

***5.3.2 Anti-leishmanial Activity Assay*:** Anti-leishmanial effect of synthesized oxabicyclo derivatives (PS compounds) were investigated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide] assay as described previously with some minor changes (*Saudagar and Dubey, 2011*). Exponentially growing *L. donovani* promastigotes (2×10^6 cellsml-1) were freshly transformed in M199 media and incubated with graded concentrations of PS compounds at 25°C for 24 h. After treatment, cells were centrifuged and subsequently washed with 1× ice-cold phosphate-buffered saline (PBS) and incubated in fresh M199 media with 15% heat-inactivated FBS and 0.5 mg/ml MTT for 4 h. The cell viability was determined by measuring the optical density at 570nm for reduced formazan (*Mosmann, 1983*), compared to untreated cells.

***5.3.3 Docking Studies with Redox Enzymes of Leishmania*:** The modeled structure of TryS from *L. donovani* which is already reported in chapter II and crystal structure of TryR (*Baiocco et al., 2009b*) from *L. infantum* (PDB ID: 2JK6) were used for molecular docking studies. The active site of TyrS and TyrR were kept rigid and non-flexible docking was carried out. The docking parameters were set in default values with the exception of the following: trials of 20 Lamarckian Genetic Algorithm runs with each case (ga run, 20), initial population size of 300 (ga pop size, 300), random starting position and conformation, and 250,000 (ga num evals, 250,000) energy evaluations. Each docking simulation produced 20 different docked conformations, which were then clustered based up on Root Mean-Square Deviation (RMSD) of the different bound conformations, the RMSD difference between conformations within each cluster will be less than 2 Å. The binding energy of each cluster is the mean binding energy of all the conformations present within the cluster, the cluster with the lowest binding energy and higher number of conformations within it was selected as the docked pose of that particular ligand.

***5.3.4 Inhibition Studies with Redox Enzymes of Leishmania*:** The two redox enzymes, trypanothione synthetase (TryS) from *L. donovani* and trypanothione reductase (TryR) from *L. infantum* were assayed for inhibition with PS compounds. The TryS inhibition studies were carried out using a 60 minutes time point inhibition assay at room temperature, in a clear flat bottom 96 well polystyrene plate. In brief, TryS assay was performed with 100 µl reaction volume containing 100 mM HEPES, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 0.01% brij-35, 10 mM magnesium acetate, 20 nM recombinant *Ld*TryS and varying concentration of substrate (GSH) and test compounds. The reaction was started by addition

of GSH and stopped by the addition of BIOMOL Green reagent (BIOMOL International UK). BIOMOL Green reagent which measures the release of free phosphate was read at 650 nm (*Torrie et al 2009; Saudagar and Dubey, 2011*). TryR assay was performed with 50 µl reaction volume containing 40 mM HEPES, pH 7.5, 0.15 mM NADPH, 1.0 mM EDTA, 25.0 µM DTNB, and varying concentration of substrate trypanothione disulfide [$T(SH)_2$] and test compounds. The assay mixture was pre-incubated at 26 °C with NADPH for 5 min. The reaction was started with the addition of $T(S)_2$. The reaction was monitored by measuring the conversion of DTNB into yellow colored TNB, which is read at 412 nm. The data collected was individually fitted to Michaelis-Menten equation, and the resulting Lineweaver-Burk plots were examined for characteristic pattern of inhibition.

***5.3.5 Analysis of Intracellular Thiols*:** The thiols were analyzed using HPLC method (*Mukhopadhyay et al., 1996; Bhattacharya et al., 2009*). *L. donovani* promastigote culture containing 1×10^6 cells ml^{-1} was incubated with IC_{50} dose of PS-203 compound. Following incubation for 24 h, ten milliliters of cell (1×10^6 cells ml^{-1}) were harvested by centrifugation (1000 × g, 10 min, 4°C), washed twice with PBS, suspended in 50 mM HEPES (pH 8.0) containing 5 mM EDTA taken in a dark tube and 0.1 ml of 2 mM monobromobimane in ethanol was added and suspension was incubated at 70°C for 3 minutes. An ice cold 0.2 ml of 25% trichloroacetic acid was added to the suspension and incubated on ice for 20 minutes. The denatured protein and cell debris were removed by centrifugation. Acid-soluble thiols were separated by ion-paired, reverse phase HPLC on an ion-paired Ultrasphere C18 column with linear gradient of 0–90% methanol in 0.25% acetic acid (pH 3.5). Thiols were identified form bimane fluorescence with an excitation and emission of 360 nm and 450 nm, respectively, using a Dionex Ultimate 3000 instrument fitted with a Dionex RF-2000 fluorescence detector.

***5.3.6 Flowcytometric Studies*:** Flowcytometric studies were carried out using BD FACSCalibur flowcytometer and analyzed using CellQuestPro software (Becton Dickinson). *Leishmania donovani* promastigotes endogenous ROS generation was measured using a cell permeable probe 2', 7'- dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA) by flowcytometer analysis. The apoptosis in *L. donovani* was studied using annexin V-FITC apoptosis detection kit (Calbiochem) using staining with annexin V-FITC antibody and

propidium iodide (PI) as per instructions given by the manufacturer. For cell cycle analysis, *L. donovani* promastigote cells treated or untreated with PS-203 were analyzed for DNA content using PI. In brief, the cells were harvested, washed with PBS twice and fixed in 70% methanol (diluted in 1× PBS). After being incubated with 500 µg ml^{-1} RNase A, the cells were stained with PI (50 µg ml^{-1}), incubated for 45 minutes in dark at room temperature and were immediately analyzed using flowcytometer. For all flowcytometric studies cells were washed twice in PBS before flow analysis.

***5.3.7 Measurement of Mitochondrial Membrane Potential ($\Delta\psi_m$)*:** The investigation of mitochondrial transmembrane potential was done using MitoCapture™ apoptosis detection kit (Calbiochem) by fluorescence microscopic analysis. MitoCapture is a fluorochrome cationic dye which is mitochondria-specific probe. In mitochondria this dye accumulates under the influence of $\Delta\psi_m$, giving off a bright red fluorescence. Whereas in depolarized $\Delta\psi_m$ it fails to accumulate in mitochondria and exists as monomer in cytosol giving green fluorescence. In brief, promastigote cells (1×10^7 cells ml^{-1}) after 24 h treatment with IC_{50} dose of PS-203 cells were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed with cold PBS. The cells (1×10^6 cells ml^{-1}) were suspended in 100 µl of incubation buffer containing MitoCapture™ reagent which was diluted as per instructions given by the manufacturer and incubated at room temperature for 30 min. After staining, the cells were washed twice with PBS, mounted on the glass slide and were photographed using fluorescent microscope. In the optimization process of incubation time with the dye, we noticed that 20 minutes incubation time recommended by manufacturer, does not give intense red fluorescence in control *Leishmania* cell and image quality was not good. Moreover, some green fluorescence was also seen in control cell. Thus, a longer incubation time was used to insure maximum uptake of the dye by mitochondria. After 30 minutes of the dye incubation and subsequent wash with PBS, control cells gave good red fluorescence intensity. Thus, in all experiments that involves fluorescent microscope, 30 minutes incubation time was used.

***5.3.8 Determination of Caspase-3/7 like Protease Activity*:** Caspase 3/7 protease like activity was measured fluorometrically using Apo-1 homogenous caspase 3/7 activity assay kit (Promega). The assay was performed according to the instructions provided by the manufacturer with minor changes. In brief, *L. donovani* promastigote cells were either treated

or untreated with IC_{50} dose of PS-203 for different period of time were harvested by centrifugation at 1000 × g for 5 min at 4°C and washed with cold PBS. The cells were suspended in a 100 µl of reaction buffer containing caspase substrate Z-DEVD-R110, was incubated for 4 h in dark at room temperature. After completion of incubation period, the increase in fluorescence due to cleavage of the Z-DEVD-R110 substrate was measured fluorometrically at excitation and emission wavelengths of 485 and 530 nm, respectively. In a parallel set of reaction, caspase inhibitor Ac-DEVD-CHO was added to the reaction mixture prior to the addition to treated cells.

***5.3.9 Determination of DNA Fragmentation by Agarose Gel Electrophoresis*:** To analyze the DNA fragmentation standard genomic DNA isolation protocol was followed (*Sambrook et al 1989*). In brief, *L. donovani* promastigote cells were cultured in 24 well plates either untreated or treated with IC_{50} dose of PS-203 for different period of time. At requisite time point samples were harvested by centrifugation at 3000 × g for 5 min. Cells were lysed in 0.5 ml of extraction buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, pH 8.0, 0.5 % SDS) containing proteinase K (15.6 mg/ ml) was vortexsd and allowed to digest overnight at 50 °C. After overnight incubation the lysates were then extracted using phenol- chloroform- isoamyl alchol (25:24:1) and centrifuged for 10 min at 16000×g the upper aqueous phase was carefully collected in a fresh tubes and incubated overnight with 3 M sodium acetate and 100% ethanol at –20°C. The samples were centrifuged at 16000×g for 10 min at 4°C, supernatant was removed and pellet was washed with 70 % ethanol. The DNA was solubilized in nuclease free water and quantified spectrophotometrically at 260/ 280 nm. On a 1.5 % agarose gel containing 0.5 µg/ ml ethidium bromide, 5 µg of DNA was loaded and electrophoresed. The gel was run for 1.5 h at 60 V and visualized in a UV illuminator.

***5.3.10 In vitro Effect of PS-203 on Amastigote Stage of Parasite*:** The mouse macrophage cell line J774A.1 was cultured overnight on glass cover slips. The cell density of 5×10^5 cells was maintained for proper distribution on the cover slip. After overnight incubation non-adherent cells were removed by washing with PBS and fresh media was added. Macrophages were infected with *L. donovani* promastigotes by maintaining parasite:macrophage ratio of 10:1 and incubated at 37°C in 5% CO_2 for 6 h to ensure parasite phagocytized by macrophage cells. After incubation, un-phagocytized parasites were removed by twice

washing with PBS and fresh media was added and then incubated for 24 h. The macrophage infectivity was checked by microscopic observation of Giemsa stained cells. After conforming infectivity, graded concentrations of PS-203 was added and further incubated for 24 h. After completion of incubation with PS-203, cells were then fixed in methanol and Giemsa stained. Anti-leishmanial activity of PS-203 towards the intracellular amastigotes was evaluated by microscopic counting of 200 infected macrophage cells and compared with untreated control. The IC_{50} value for PS-203 was calculated by plotting no. of amastigotes/200 macrophage *vs.* concentration. In an alternate experiment the *L. donovani* promastigote cells pre-treated with IC_{50} dose of PS-203 for different time period were used for infection with macrophage cells. The cells were Giemsa stained and randomly 200 macrophage cells were counted and infectivity index was calculated as percentage of infected macrophages × average number of amastigotes in infected macrophages.

***5.3.11 In vivo Assessment of Toxicity*:** To evaluate the in vivo toxicity, thirty Swiss albino mice were studied in a 5 day course of daily intraperitonial injections of PS-203 using 5 mg/kg, 10 mg/kg and 20 mg/kg dose regimens using earlier reported procedure (*Prajapati et al., 2011*). The compound PS-203 was tested in 12 mice with 4 mice for each concentration. The control group consists of 4 mice injected with PBS. On day 5 the mice were euthanized and serum was collected for assessment of serum glutamic-pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT) levels for hepatic function and renal function is measured by checking urea and creatinine levels

***5.3.12 In vivo Assessment of Anti-leishmanial Activity*:** The in vivo anti-leishmanial activity was studied in hamsters using method described earlier (*Prajapati et al., 2011*). PS-203 dissolved in DMSO at 2.5 mg/ml was used as stock solution which was reconstituted for in vivo administration in 1x PBS at 1.5 mg/ml (injection volume ranging from 200-400 µl according to animal weight). Twenty hamsters were infected by intracardiac injection of 1×10^8 *L. donovani* promastigotes. At day 30, the infection was confirmed by splenic biopsy and performance of splenic dab of four randomly selected hamsters. The PS-203 was injected intraperitoneally at 5 mg/kg body weight per day for 5 days and in controls PBS was used. The splenic dab performed on all animals was analysed by calculating Leishmania Donovani

units (LDUs) and the percentage inhibition of parasites. This experiment was repeated for reproducibility with a further 20 hamsters and the results were collated.

5.4 Results

***5.4.1 Effect of Compounds on L. donovani Promastigotes and Macrophage Cells*:** The in vitro anti-leishmanial activity of oxabicyclo derivatives were determined after 24 h treatment of *L. donovani* promastigotes (Figure 5.1A). A dose-dependent death profile was seen with all PS compounds (Table 5.1) tested in the range of 0.75-100 µM. Approximately 60% inhibition was observed at 12.5 µM with PS-202 and PS-203, whereas PS-192, PS-193 and PS-197 showed only around 40% inhibition at this concentration. The PS-202 and PS-203 compounds were more effective as anti-leishmanial compared to PS-192, PS-193 and PS-197. The IC_{50} values for each of the compounds were calculated by plotting % viable promastigotes *vs.* concentration. The IC_{50} were found to be 14.9 ± 0.9 µM for PS-192, 16.6 ± 0.7 µM for PS-193, 19.5 ± 0.9 µM for PS-197, 7.1 ± 0.5 µM for PS-202 and, 4.9 ± 0.4 µM for PS-203. In addition, the effect of compound PS-203 on macrophage cell line J774A.1 was tested. There was no significant toxicity shown by the compound PS-203 on the macrophage cells upto 100 µM indicating PS-203 selectively inhibit *Leishmania* cells (Figure 5.1B). Maximum anti-leishmanial property was exhibited by PS-203. Thus, this compound was chosen for detailed study.

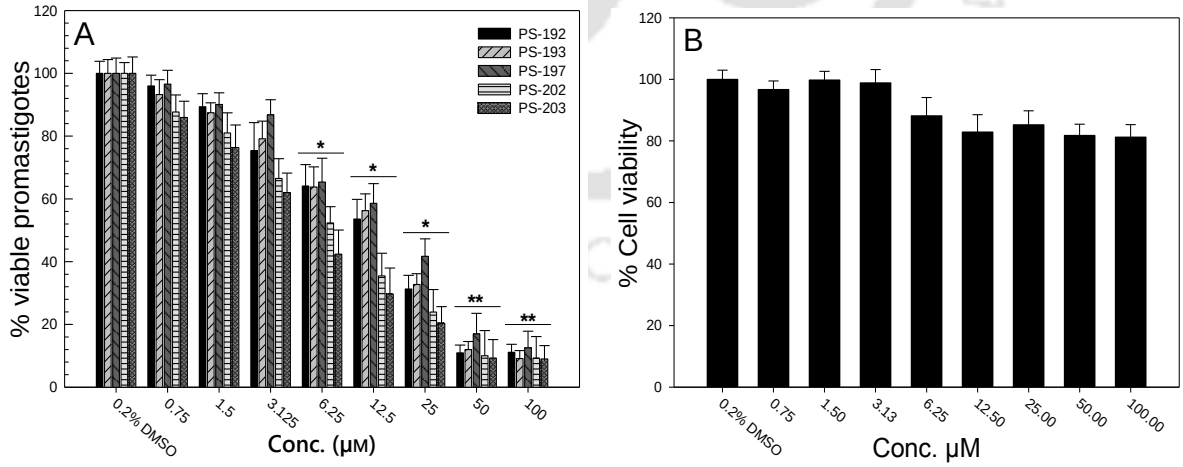
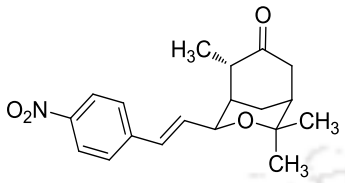
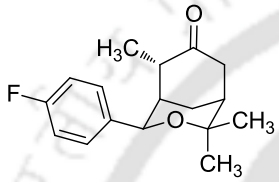
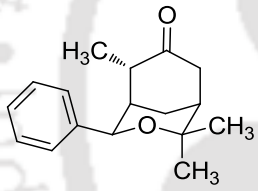
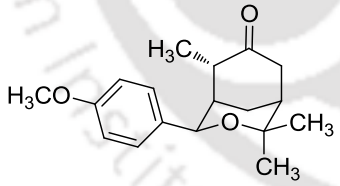
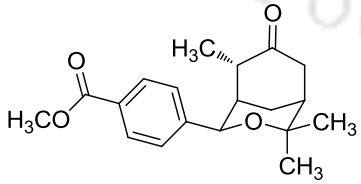


Figure 5.1: (A) The effect of PS compounds on *L. donovani* promastigotes. *L. donovani* promastigotes treated with different concentrations of PS compounds. **(B)** Effect of PS-203 compound on mouse macrophage cell line J774A.1. Data with *P* values of <0.05 (*) and <0.001 (**) were considered statistically significantly different compared to the 0.2% DMSO control. Data represent the mean ± SD of three independent experiments.

Table 5.1: Structures of the oxabicyclo derivatives (PS compounds) used in the study

S.No	Compound Structure	Compound ID
1		PS-192
	2,2,6-Trimethyl-4-[2-(4-nitrophenyl-vinyl)]-3-oxabicyclo[3.3.1]nonan-7-one	
2		PS-193
	4-(4-Fluorophenyl)-2,2,6-trimethyl-3-oxabicyclo[3.3.1]nonan-7-one	
3		PS-197
	2,2,6-Trimethyl-4-phenyl-3-oxabicyclo[3.3.1]nonan-7-one	
4		PS-202
	4-(4-Methylphenyl)-2,2,6-trimethyl-3-oxabicyclo[3.3.1]nonan-7-one	
5		PS-203
	4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester	

***5.4.2 Molecular Docking and Analysis of Binding Site*:** Docking studies with PS compounds yielded acceptable docking statistics (Table 5.2) with both TryS and TryR redox proteins of the parasite. The preliminary lead form experimental and docking studies prompted us for further experimental studies with PS-203 which has shown better anti-leishmanial activity among all the PS compounds and also yielded acceptable docking statistics with both TryS (binding energy -6.81 kcal/mol) and TryR (binding energy -7.14 kcal/mol) redox proteins of the parasite. These passable docking results further encouraged us for chemical validation. With TryS, PS compounds showed a highly conserved interaction with the active site residues. The active site residues of TryS that are in hydrogen bonding interaction are Arg328, Ser349, Met459, Ser462, Arg553 and Lys616. The PS compounds interaction with TryR active site showed less hydrogen bonding interactions compared to TryS active site. The TryR active site residues making hydrogen bonding interaction are Thr65, Thr397, Leu399 and Met400. The hydrogen bonding interaction of PS-203 with active site residues of both TryS and TryR is shown in Figure 5.2. For better understanding of PS-203 binding in active site of TryR, we have compared the binding mode of PS-203 with TryR with that of the available crystal structure complexed with trypanothione from *L. infantum* (PDB ID: 4ADW). As the PS-203 binds in the same pocket very similar to the natural substrate trypanothione (Figure 5.3B) it was predicted that the mode of inhibition most likely to be competitive which was further conformed by inhibition studies.

Table 5.2: Docking statistics of PS compounds studied with TryS and TryR. In the table **C**- Number of Clusters, **CL**- Number of conformations within the selected cluster, **E**- Binding energy of the selected conformation in kcal/mol, **T**- Number of torsions.

S. No	Compound ID	Trypanothione synthetase (TryS)				Trypanothione reductase (TryR)			
		CL	C	E	T	CL	C	E	T
1	PS-192	48	5	-7.50	6	66	9	-7.57	16
2	PS-193	35	8	-6.81	3	30	7	-7.14	3
3	PS-197	32	7	-7.01	7	27	3	-7.26	13
4	PS-202	53	5	-11.31	12	23	7	-7.25	2
5	PS-203	69	4	-6.81	16	46	6	-7.14	8

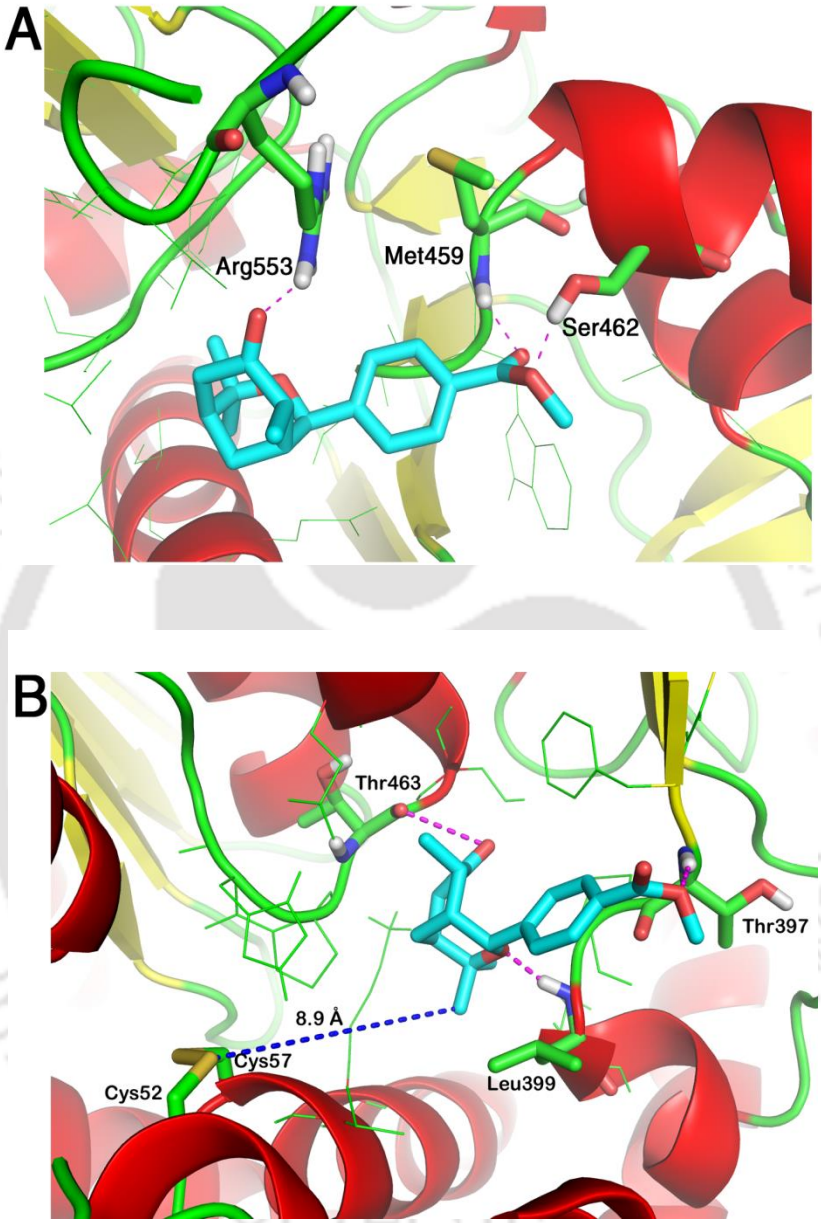


Figure 5.2: Chemical structure of4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester. The compound is named as PS-203 for easy presentation. Docking of the compound, PS-203, with TryS and TryR. The hydrogen bond interaction of PS-203 with TryS and TryR are shown. **(A)** The TryS active site residues Arg553, Met459 and ser462 shows hydrogen bond interaction with PS-203. **(B)** The TryR active site residues Thr463, Leu399 and Thr 397 show hydrogen bond interaction with PS-203 and the distance between PS-203 and two catalytic cysteine's (Cys52 and Cys57) is 8.9 Å is shown in blue broken line.

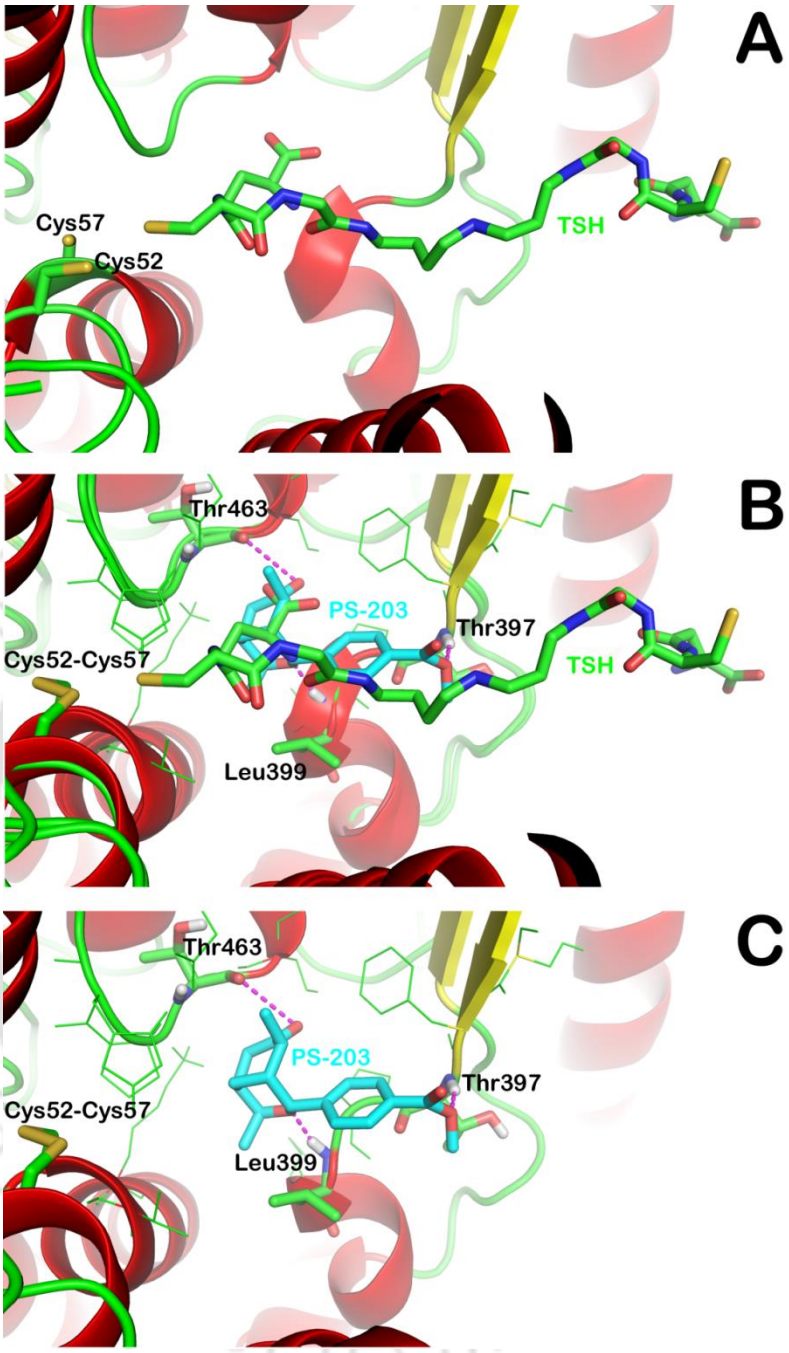


Figure 5.3: Comparison of the binding mode of PS-203 and trypanothione in the active site of TryR. **(A)** The binding of trypanothione with *L. infantum* TryR (PDB ID: 4ADW) in the close proximity of the catalytic Cysteine (Cys52 and Cys57). **(B)** Alignment of PS-203 complex with *L. infantum* TryR reveals that the potential inhibitor PS-203 binds in the same pocket as that of trypanothione, indicating the possibility of competitive inhibition. **(C)** The binding mode of PS-203 in the active site of TryR (PDB ID: 2JK6). The hydrogen bonds between PS-203 and the active site residues (Thr397, Leu399 and Thr463) of TryR are depicted with pink dashed line. TryR is represented with cartoon representation while the key residues are shown as sticks and contact residues in lines.

***5.4.3 PS-203 Inhibits TryS and TryR of Leishmania*:** The PS compounds inhibit both TryS and TryR enzymes. The inhibition data was fitted to the Michaelies-Menten equation and Lineweaver-Burk plots were generated to check the mode of inhibition The K_i for each of the PS compound assayed for inhibition of TryS and TryR was calculated and are compared with the IC_{50} (Table 5.3). The compound, PS-203 which showed best anti-leishmanial activity among the PS compounds, also showed better inhibition of two enzymes TryS and TryR among the PS compounds studied. PS-203 showed competitive inhibition with both TryS and TryR as no change in V_{max} was observed (Figure 5.4). The K_i calculated for PS-203 was found to be 14.2 ± 0.8 µM with *Ld*TryS and 26.1 ± 1.7 µM with TryR.

***5.4.4 Effect on Intracellular Thiol*:** The intracellular thiol levels were investigated in PS-203 treated *L. donovani* promastigotes. The cells treated with IC_{50} dose of PS-203 for 24 h showed significant decrease in the levels of intracellular $T(SH)_2$. A decrease of up to ~80% of $T(SH)_2$ was observed in PS-203 treated cells compared to untreated control. In contrast, an increase of ~75% of GSH was observed compared to untreated control (Figure 5.5).

Table 5.3: Comparison of IC_{50} and K_i values of PS compounds. Among all the PS compounds PS-203 has the least IC_{50} and also shows better K_i. The least K_i shown by PS compounds against TryS is PS-203 where PS-202 showed least K_i against TryR. Considering the IC_{50} and K_i values of PS compounds PS-203 has the optimum values, hence was preferred for further studies on *Leishmania* parasite.

S. No	Compound ID	IC_{50} µM	TryS inhibition K_i (µM)	TryR inhibition K_i (µM)
1	PS-192	14.9 ± 0.9	26.5 ± 0.8	75.1 ± 1.1
2	PS-193	16.6 ± 0.7	23.7 ± 0.5	36.5 ± 0.7
3	PS-197	19.5 ± 0.9	71.2 ± 2.2	26. 4 ± 0.8
4	PS-202	7.1 ± 0.5	36.7 ± 0.4	16.3 ± 0.4
5	PS-203	4.9 ± 0.4	14.2 ± 0.8	26.1 ± 1.7

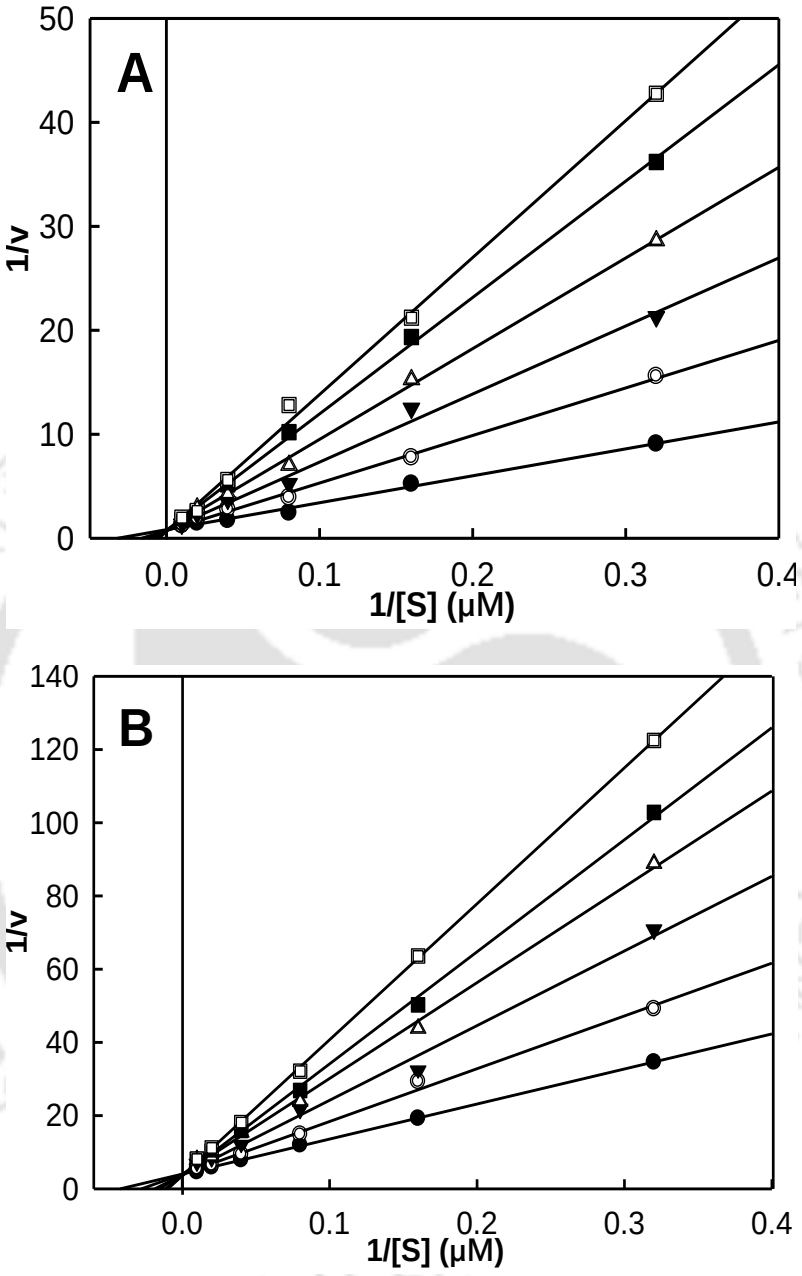


Figure 5.4: Inhibition studied with PS-203. (A) Inhibition of TryS shown in form of Lineweaver-Burk plot with glutathione (GHS) as a varied substrate concentration. The calculated K_i for PS-203 is 14.2 ± 0.8 μM. **(B)** Inhibition of TryR is shown in the form of Lineweaver-Burk plot with trypanothione [$T(SH)_2$] as a varied substrate concentration. The calculated K_i for PS-203 is 26.1 ± 1.7 μM.

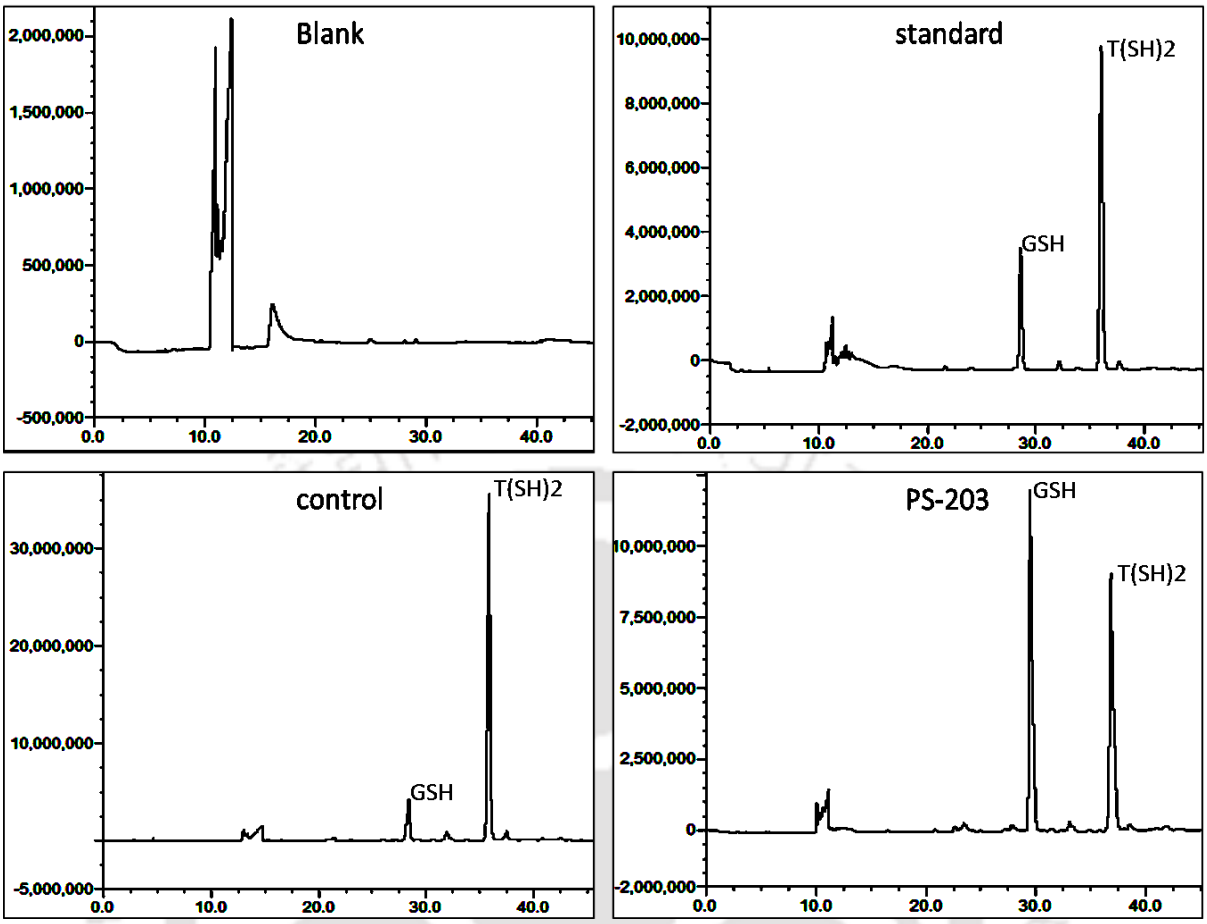


Figure 5.5: Thiols (GSH and TSH) estimation by HPLC. Traces showing levels of reduced TSH and an increase in the GSH content in the promastigotes treated with IC50 dose of PS 203 for 24 hr. The thiols were derivatized with monobromobimana. Purified derivatized GSH and $T(SH)_2$ were run to identify the representing reduced peaks.

***5.4.5 Inhibition of TryS and TryR Induces ROS Accumulation in Parasite Causing Oxidative Stress*:** The generated ROS is scavenged by the redox metabolite such as GSH in all living systems and $T(SH)_2$ in trypanosomatids like *Leishmania*. As PS-203 inhibits key redox enzymes TryS and TryR in *Leishmania* parasite, the levels of $T(SH)_2$ are decreased. Hence, it was desirable to check the levels of ROS in PS-203 treated cells. The IC_{50} dose of PS-203 was used for *L. donovani* promastigotes treatment at different time period and the results obtained showed a significant increase in ROS levels. The maximum ROS was obtained at 3 h time point and with time the parasite managed to reduce the ROS levels (Figure 5.6). In NAC pre-treated *L. donovani* promastigotes cells tested for ROS at 3 h time point showed significant decrease in the ROS levels as compared to N-acetyl cysteine (NAC)

untreated cells for the same time point. The results suggest that inhibition of TryS and TryR by PS-203 induces ROS generation which further leads to parasite death. NAC pre-treatment prevented ROS accumulation.

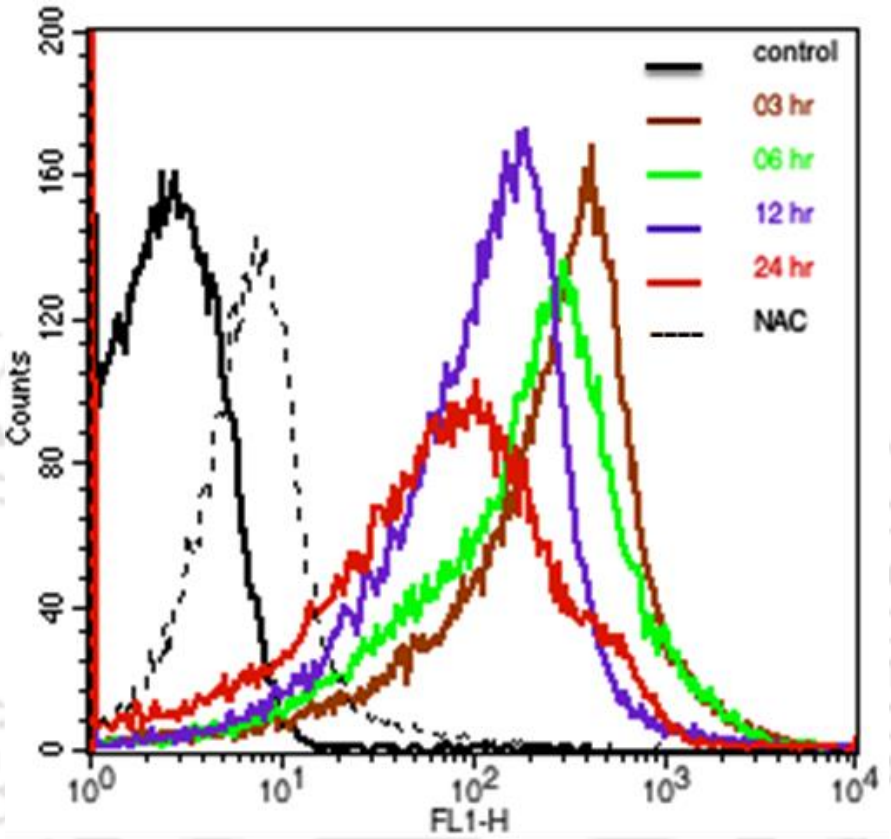


Figure 5.6: PS-203 induced ROS is measured by DCF fluorescence as assayed by flowcytometry. *L. donovani* promastigotes were either untreated, treated or pretreated with NAC (20 mM) before treatment with IC_{50} dose of PS-203 for 03 h, 06 h, 12 h and 24 h were stained by H2DCFDA and analyzed using flowcytometry. Data shown is representative experiment of at least three experiments which gave similar results.

***5.4.6 PS-203 Induces Apoptosis in L. donovani and Cell Cycle Analysis*:** Externalization of phosphatidylserine is an identity for cells undergoing apoptosis, which can be detected using annexin V, as it has affinity to bind to exposed phosphatidylserine. In PS-203 treated *L. donovani* promastigotes cells, phosphatidylserine externalization was studied using apoptotic kit (Calbiochem) and analyzed by flowcytometry. The *L. donovani* promastigotes cells treated with IC_{50} concentration of PS-203, untreated with PS-203 or NAC pre-treated followed by IC_{50} concentration of PS-203 treatment, for different time point, were double

stained with annexin V and Propidium iodide (PI) to check whether cells are undergoing apoptosis or necrosis (Figure 5.7). The results clearly indicated that PS-203 induced apoptosis in *L. donovani* promastigotes. In 12 h treated sample, 86.25% cells were in early apoptotic stage and in 24 h treated sample, 95.57% of cells were in late apoptotic stage (Figure 5.7A). In the NAC pre-treated cells, 12 h and 24 h treated samples showsd no apoptotic signal (Figure 5.7B). Thus, from the result it can be concluded that NAC pre-treatment prevents ROS accumulation and parasite survives to large extent. Analysis of cell population at different cell cycle stages is possible after staining with PI and subsequent flowcytometric analysis. Cells at G1 stage of cell cycle will have one copy of DNA while G2 stage will have two copies of DNA. Thus, the fluorescence of G2 phase cells will be double of G1 phase after PI staining. Synthesis phase (S-phase) will have intermediate fluorescence intensity. The data suggests that after treatment of PS-203, there is substantial increase in apoptotic cell population and G1 phase cells remained constant, as a function of time of PS-203 treatment, compared to untreated control (Figure 5.8). This indicates that the *Leishmania* promastigote cells are locked at G1 phase of cell cycle and are being driven to apoptosis.

***5.4.7 PS-203 Induced Depolarization of Mitochondrial Membrane Potential ($\Delta\psi_m$)*:** Depolarization of mitochondrial membrane potential is the characteristic feature of apoptosis. PS-203 treated cells were investigated for depolarization of $\Delta\psi_m$. The detection was done using MitoCapture™ apoptosis detection kit (Calbiochem). MitoCaptur™ is a cationic mitochondrial specific dye that gets aggregated in mitochondria, whose $\Delta\psi_m$ is retained, giving red fluorescence. Whereas in cells with altered mitochondrial $\Delta\psi_m$, MitoCapture™ fails to aggregate in mitochondria leading to its accumulation in cytosol in its monomeric form, hence giving green fluorescence. The cells were treated with IC_{50} dose of PS-203 for 24 h and analyzed using fluorescence microscope (Figure 5.9). Exposure of *Leishmania* promastigotes cells to PS-203 for 24 h caused a loss of red fluorescence indicating loss in the mitochondrial $\Delta\psi_m$ as detected by MitoCapture™ reagent. Whereas in control untreated cell stained with MitoCapture™ reagent gave red fluorescence indicating retention of membrane potential in cells as evident from flowcytometric analysis. There was no green fluorescence observed in control sample which may be due to longer incubation with MitoCapture™.

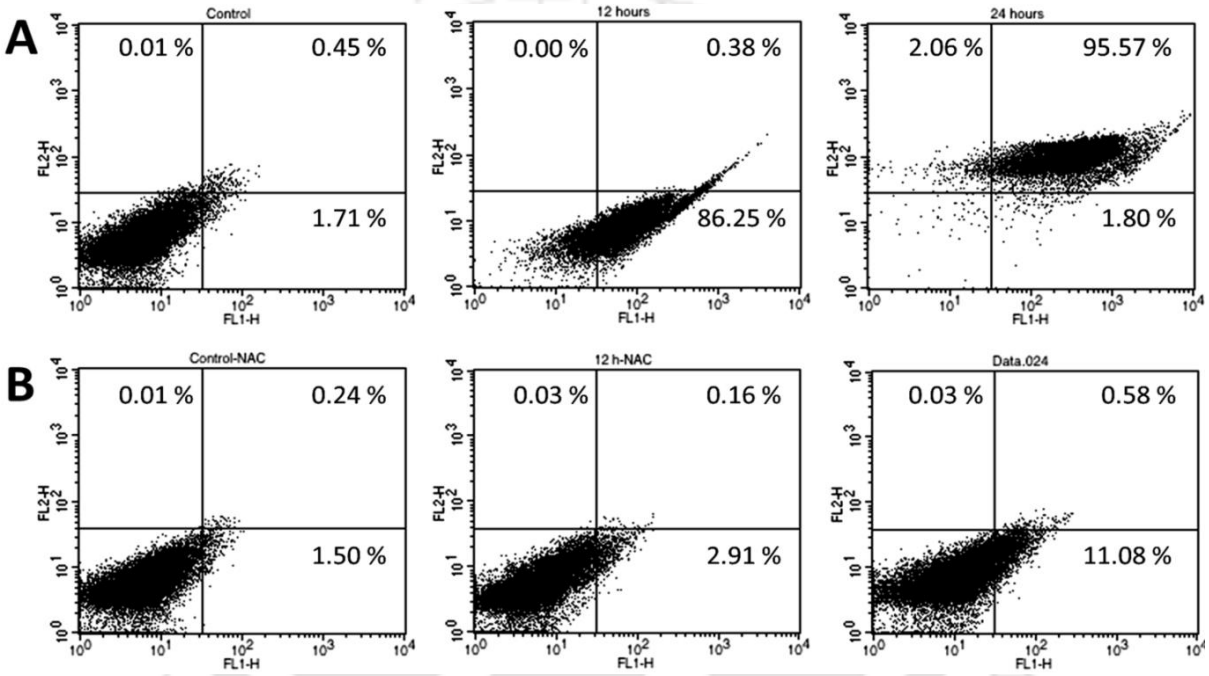


Figure 5.7: Flowcytomatric analysis **(A)** *Leishmania* promastigotes were treated with IC_{50} dose of PS-203 for 12 h and 24 h were double stained with PI and annexin V-FITC. The *Leishmania* promastigotes untreated with PS-203 were also double stained with PI and annexin V-FITC and used as control. **(B)** NAC pre-treated *Leishmania* promastigotes were treated with IC_{50} dose of PS-203 for 12 h and 24 h were double stained with PI and annexin V-FITC. 0.2% DMSO is used as control.

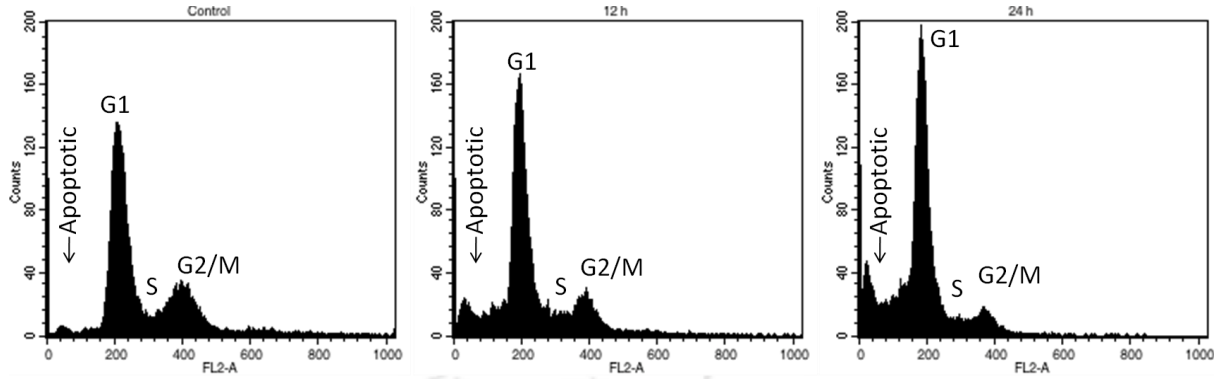


Figure 5.8: Cell cycle analysis using Flowcytometry after treatment with IC_{50} dose PS-203. *L. donovani* promastigotes cells treated with IC_{50} dose for different time point and stained with propidium iodide. 0.2% DMSO is used as control. All data shown are representative experiments of at least three experiments which gave similar results.

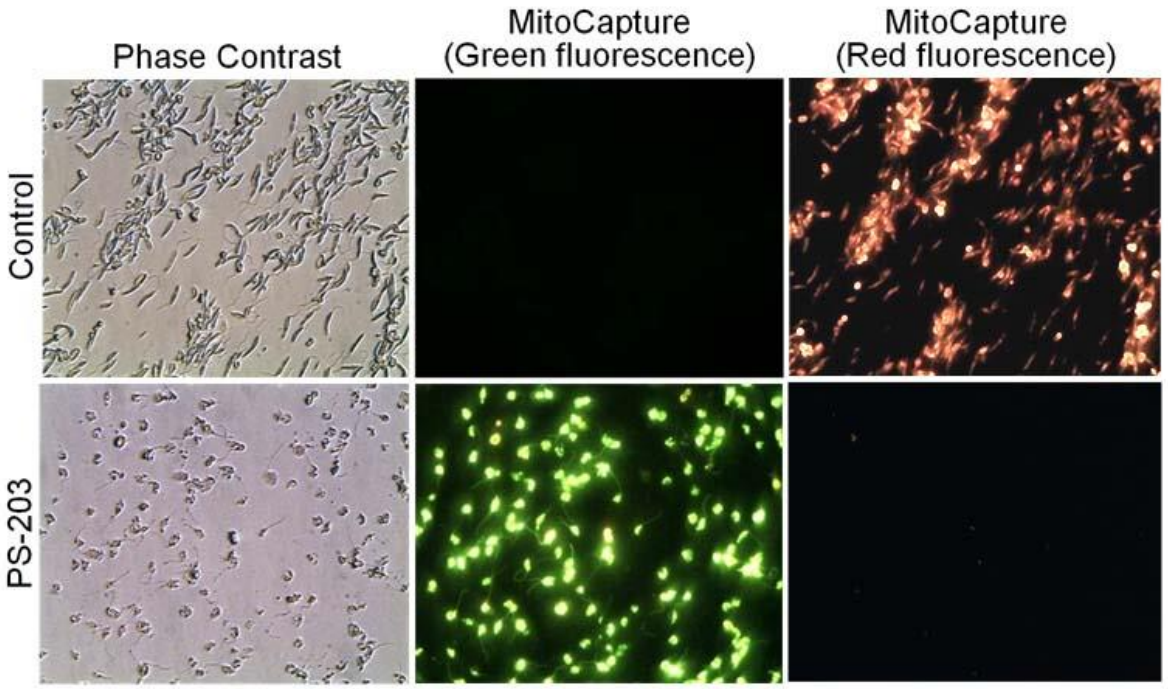


Figure 5.9: PS-203 induces apoptosis as measured by mitochondrial membrane injury. *Leishmania* promastigotes were treated or with IC_{50} dose of PS-203 for 24 h, stained with mitochondria- specific cation dye, MitoCapture™ (Calbiochem). Longer treatment of MitoCapture™ (30 minutes) was given to ensure maximum update of dye by mitochondria for intense fluorescence as explained in method section .Untreated *Leishmania* promastigotes were used as control. The promastigotes cells were photographed for control and for PS-203 treated cells using fluorescence microscope (60x). Normal mitochondrial membrane potential is indicated by the red fluorescence where as green fluorescence indicates depolarization mitochondrial membrane. Data shown are representative experiment of at least three experiments which gave similar result.

***5.4.8 Activity of Caspase Like Proteases in PS-203 Treated Leishmania Promastigote Cells*:** The activation of caspase-3/7 like proteases in *L. donovani* promastigote treated with IC_{50} dose of PS-203 for different time point were studied using Apo-ONE Homogeneous Caspase 3/7 assay kit (Promega). An increased caspase like activity was observed with increase in time of treatment with PS-203 (Figure 5.10). *L. donovani* promastigote treated with IC_{50} dose of PS-203 with caspase inhibitor (DEVD-CHO inhibitor) did not show any activity. Control *L. donovani* promastigotes i.e. without PS-203 or caspase inhibitor, also showed no activity. In control, *L. donovani* promastigotes were treated with 0.2% DMSO to compensate the effect of DMSO that was used for making caspase inhibitor or substrate solution.

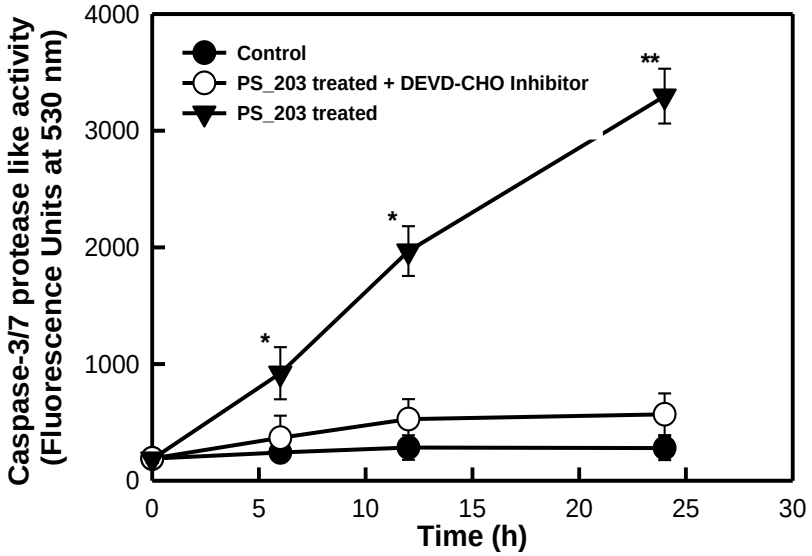


Figure 5.10: Activation of Caspase- 3/7 like protease inside leishmanial promastigote cells after treatment with IC_{50} dose of PS-203 and in absence or presence of protease inhibitor (DEVD-CHO inhibitor) at different time point. Untreated leishmanial promastigote cells are used as control. Data represent the mean ± SD of three independent experiments. Data with *P* values of <0.05 (*) and <0.001 (**) were considered statistically significantly different compared to the 0.2% DMSO control, assessed by the unpaired student *t* test.

***5.4.9 PS-203 Induces Genomic DNA Fragmentation*:** Genomic DNA fragmentation is considered as the hallmark of apoptotic cell death. The genomic DNA fragmentation assay was carried out using agarose gel electrophoresis as described in the *materials and methods*

section. *L. donovani* promastigote treated with IC_{50} dose of PS-203 for different time point were analyzed for DNA fragmentation. In 24 h treated samples, there was remarkable DNA fragmentation observed (Figure 5.11). It is predicted that the action of both ROS and caspase like proteases may be responsible for DNA fragmentation in PS-203 treated cells.

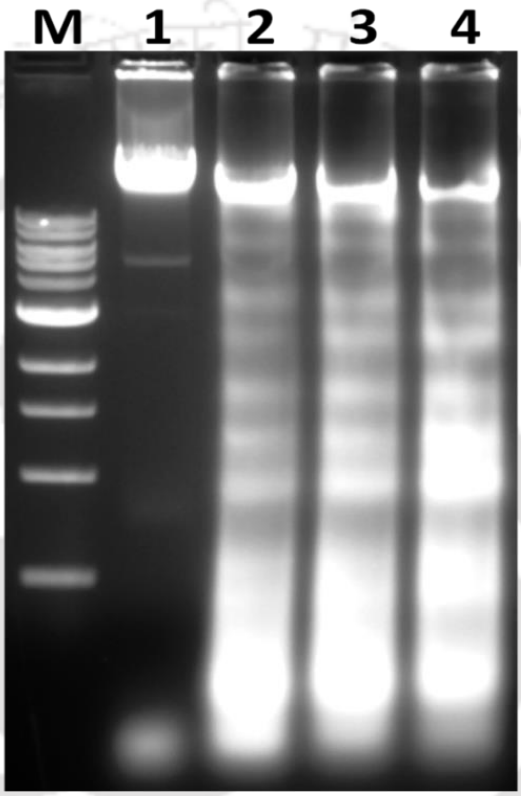


Figure 5.11: *Leishmania donovani* promastigote genomic DNA fragmentation after treatment of PS-203 compared to control (untreated promastigote). Lane M represents DNA molecular mass marker (1 kb DNA ladder), lane 1 represents control while lane 2-3 indicates cells after treatment with IC_{50} dose of PS-203 for 06 h, 12 h and 24 h, respectively.

***5.4.10 PS-203 Decreases Proliferation of Intracellular Amastigotes and the Infectivity Index of Promastigotes*:** The effect of PS-203 on amastigote stage of the parasite inside macrophages was studied by manual counting of 200 Giemsa stained cells. The *L. donovani* infected macrophage cells either treated or untreated with graded concentration of PS-203 for 24 h time period were studied. The dose dependent decrease in the intracellular parasite was

observed in response to PS-203 treatment (Figure 5.12A). The IC_{50} of PS-203 against intracellular amastigotes was found to be 6.28 ± 0.41 µM. The microscopic observation of infected Giemsa stained macrophage showed a decrease in the parasite burden with an increased dose of PS-203. Effect of PS-203 pre-treated *L. donovani* promastigotes in infecting the macrophage was studied by random counting of 200 macrophage cells and infectivity index was calculated. The data indicates that the *L. donovani* promastigotes shows lower infectivity index as a function of time when treated with IC_{50} dose of PS-203 (Figure 5.12B). Further, the infected macrophage cells treated with IC_{50} dose of PS-203 for different time period were Giemsa stained and photographed (Figure 5.13).

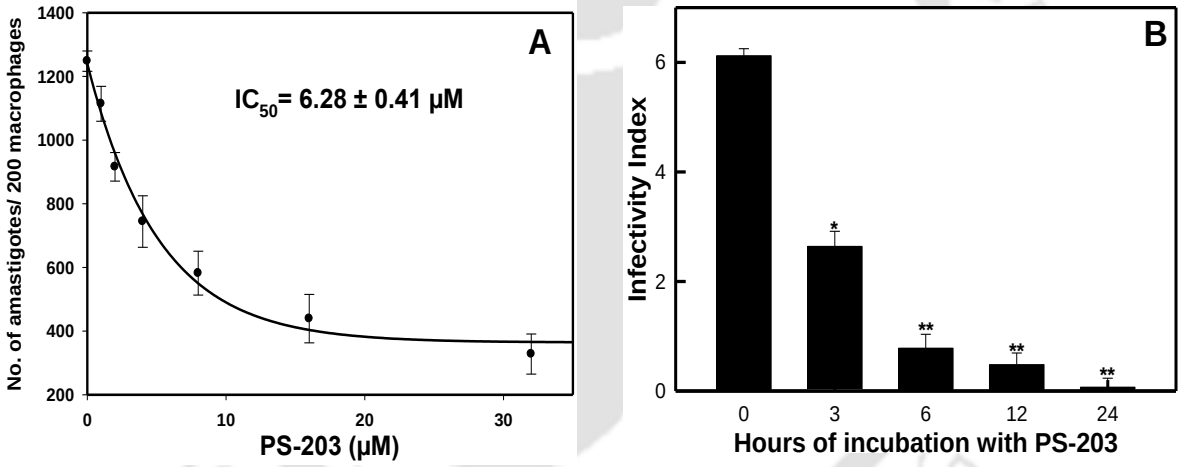


Figure 5.12: Effect of PS-203 on Intracellular amastigotes: (A) Anti-leishmanial activity of PS-203 on intracellular *Leishmania* amastigotes treated with graded concentration of PS-203 for 24 h time period. **(B)** *Leishmania* promastigotes pretreated with IC_{50} dose of PS-203 for different time period and used for macrophage infection. The infectivity index (calculated by using the formula percentage of infected macrophage × number of amastigotes per infected macrophage) was Calculated and compared with control. The data clearly shows decrease in infectivity index with time after treatment of IC_{50} dose of PS-203 **(**For all macrophage infectivity assays the cells were stained with Giemsa stain for counting by using light microscope under 100x lens. Data with *P* values of <0.05 (*) and <0.001 (**) were considered statistically significantly different compared to the 0.2% DMSO control, assessed by the unpaired student *t* test.

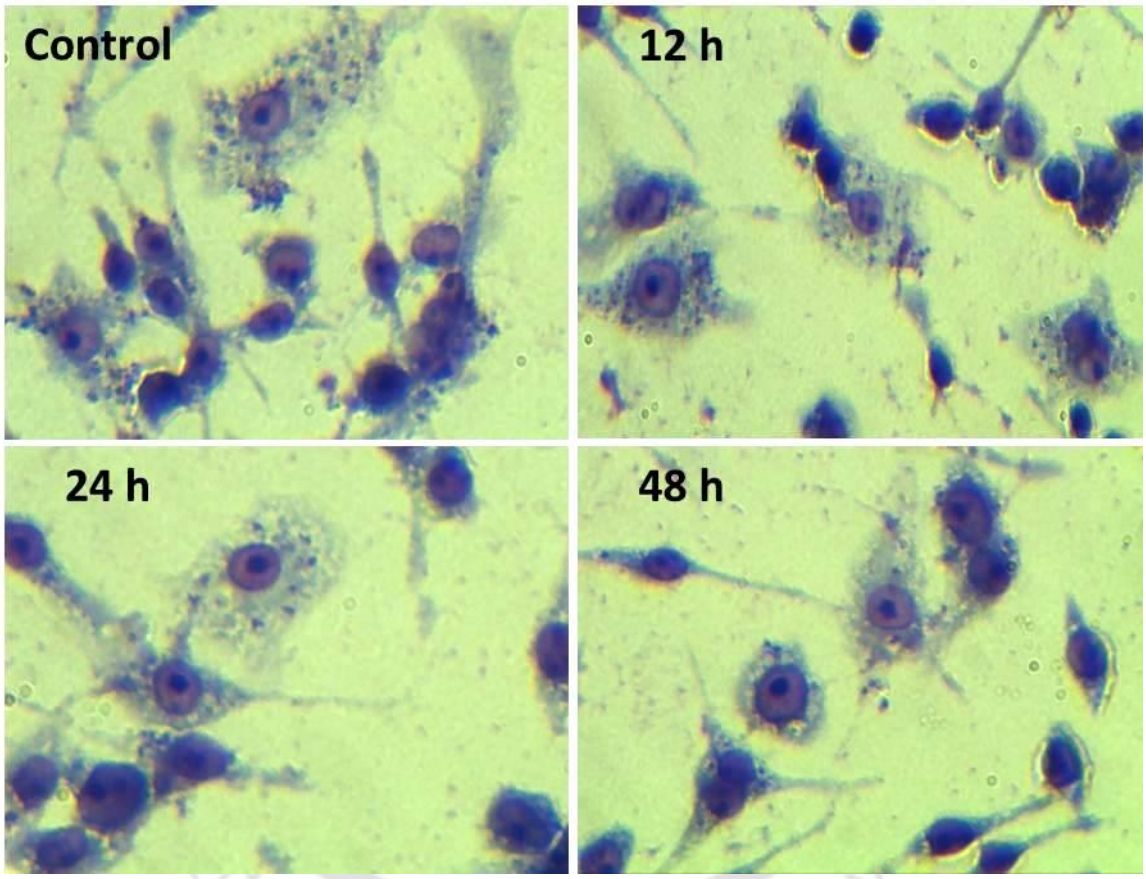


Figure 5.13: Microscopic images of mouse peritoneal macrophages (J774A.1) infected with *L. donovani* amastigotes. The infected macrophage treated with IC 50 dose of PS-203 for different time period were stained with Giemsa stain and viewed under 100 x magnifications.

***5.4.11 In vivo Toxicity*:** There was no significant renal or hepatic toxicity in Swiss albino mice was observed with PS-203. Urea is produced as a waste product from the breakdown of proteins is usually excreted out in the urine. A high blood urea indicates the improper functioning of kidneys. Certainly, creatinine is a waste product produced by the muscles which is usually removed from the bloodstream by kidneys which is passed out in urine. A high blood creatinine level is an indication of improper functioning of kidneys. Hence, renal toxicity in treated mice was studied by measuring the level of urea and creatinine between the control and tested compound groups that is shown in Table 5.4. The simple way to detect the liver damage is by determining the certain liver enzymes in the blood, serum glutamic-pyruvic transaminase (SGPT) and serum glutamic-oxaloacetic transaminase (SGOT). These SGPT and SGOT levels in control and PS-203 treated cell were monitored which are shown in Table 5.4.

Table 5.4: In vivo toxicity studies in mice

Treatment	Urea	Creatinine	SGTP	SGOT
Control	32.0	0.54	47.33	54.33
AmB	31.08	0.39	40.58	60.91
PS-203	34.78	0.38	45.08	53.25

***5.4.12 Effect of PS-203 on In vivo Intracellular Amastigotes*:** The in vivo anti-leishmanial effect was studies in two sets of infected hamsters. The mean values obtained from the result of both experiment sets were taken for further analysis. The results are shown in Table 5.5. The in vivo anti-leishmanial activity studied in hamsters by calculating number of amastigotes per 500 nuclei × tissue weight which was found to be 53.3 ± 7.2 before treatment, 11.6 ± 1.6 after test compound PS-203 treatment and 16.1 ± 2.7 with control drug amphotericin B. The percentage of inhibition of parasite in spleen was 77.29 ± 3.0 and 70.93 ± 4.20 for test compound PS-203 and amphotericin B, respectively. Though there is not much difference in the efficacy of the test compound compared to amphotericin B, further research is needed to better understand PS-203 for its ability in treating leishmaniasis.

Table 5.5: In vivo efficacy studies in Syrian golden hamsters

Group	Before treatment, n=4	PS-203 (group 1) n=12	Am B (group 2) n=9	Control (group 4) n=6	P value [a]
Spleen Weight(gm) Mean±SD	1.04±0.10	1.00±0.097	1.09±0.11	1.4±0.12	
Parasite Burden(LDU[b]) (Post-treatment)($\times 10^4$)	53.3±7.2	11.6±1.64	16.1±2.76	89.3±6.85	
Percentage inhibition of splenic parasite load (PI) [PI=(PP – PT)/(PP) × 100]		77.29±3	70.93±4.20		0.002512
Percentage suppression of parasite replication (PS) [PS=(PC – PT)/(PC) × 100]		81.58±2.41	76.66±3.38		0.002424

[a]P Value between group 1 (Compound) and group 2 (Am B).
[b] Number of amastigotes per 500 nuclei × tissue weight (mg).
PP- Number of amastigotes per 500 nuclei in the spleen before treatment
PT- Number of amastigotes per 500 nuclei after treatment
PC- Number of amastigotes per 500 nuclei in the spleen after treatment in control group (Am B)

5.5 Discussion

Inhibitors with two essential targets in *Leishmania*, invites to facilitate a feasibly different approach in the search for new drug. The PS-203 treated cells showed three-fold decrease in $T(SH)_2$ levels and nearly a three-fold increase in GSH (a substrate for TryS), indicating the action of inhibitor on the target. In contrast, increased levels of GSH, in PS-203 treated cell, is not good for the pathogen as *L. donovani* TryS shows high substrate inhibition which is observed in chapter III of this thesis. Hence, the increase in cellular GSH may eventually feedback to inhibit the TryS enzyme, under in vivo condition. In *T. brucei*, down-regulation of TryS (85%) caused fourfold increase of GSH and an 86% additional decrease in $T(SH)_2$ (*Comini et al., 2004; Ariyanayagam and Fairlamb, 2001*) compared to control. In a trypanothione metabolic modeling study it was shown that despite down regulation of TryS, an increased activity of TryR and γ-ECS (γ - glutamylcysteine synthetase) was observed. This is most likely to compensate for $T(SH)_2$ depletion, loss of parasite resistance to oxidative stress and cell viability compromise (*Olin-Sandoval et al., 2012, Krieger et al., 2000*). As TryS and TryR, two important redox enzymes controls $T(SH)_2$ levels are inhibited by PS-203, the reactive oxygen species (ROS) level in the PS-203 treated cells was measured. The ROS after PS-203 treatment was highest after 3 hours. Increase in time of treatment, led to decrease in the ROS level to some extent, hence indicating that the parasite had somehow managed to reduce the ROS level, which was induced due to PS-203 treatment. In NAC pre-treated promastigotes, there was significant decrease in ROS as well. At this point we had a conclusion that cell death was mediated by ROS in *L. donovani* treated with PS-203. Further, we found that the parasite death was through PS-203 induced apoptosis. The inhibition of TryS and TryR decreases the $T(SH)_2$ levels drastically in the cell, thereby increasing the ROS levels due to lack of required amount of $T(SH)_2$ for scavenging ROS. It appears that ROS generation induced by PS-203 lead to parasite apoptosis as NAC pre-treated cells exposed to PS-203 treatment stopped apoptosis to significant extent and only ~11% cells are in early apoptosis after 24 h (Figure 5.7B). This confirms that the production of ROS is leading to apoptosis. Further, $T(SH)_2$ is known to be involved in various metabolic processes, such as direct reduction of dehydriascorbate (DHA), by at least two times faster than GSH (*Krauth-Siegel and Lüdemann, 1996*) as well as reduction of ovithiol disulphide

and glutathione disulphide (GSSG) (*Ariyanayagam and Fairlamb, 2001; Steenkamp, 2002*). $T(SH)_2$ is also known to interact with nitric oxide (*Bocedi et al., 2010*), showing defense against toxic xenobiotics, involved in detoxification of ketoaldehydes and transferring its reducing equivalent to proteins such as ribonucleotide reductase (*Dormeyer et al., 2001*) and many more. Hence, the other metabolic functions which are $T(SH)_2$ dependent are also affected by the decrease in $T(SH)_2$ levels and may lead to immense stress.

Mitochondria are the power house of the cell where the cellular ATP is generated by oxidative phosphorylation process. Mitochondrion is an important organelle for cell's survival, therefore any damage or dysfunction to this organelle leads to cell death. This makes mitochondria a prime target for destruction in order to achieve programmed cell death or necrosis. PS-203 induces apoptosis by depolarization of mitochondrial membrane potential. It switches on the cascade of caspase like proteases, which causes genomic DNA fragmentation and further leads to death by means of apoptosis.

Apoptosis in kinetoplastid protozoan parasite is evident to have a role of caspase like proteases in cascading apoptosis process (*Das et al., 2001; Lee et al., 2002*). The essential role of caspase in apoptosis is well known in higher eukaryotic organisms which is absent in genome of *Leishmania* (*Uren et al., 2000*). In *Leishmania*, presences of metacaspases which are distinctly related to caspases are thought to be responsible for caspase like activity. But, recently metacaspases of *Leishmania* were reported to have trypsin like activity rather than caspase like activity (*Lee et al., 2007*), indicating no role of metacaspases in apoptotic cell death in *Leishmania* (*Castanys-Muñoz et al., 2012*). Even though activation of some nucleases (*Gannavaram and Vedvyas, 2008*) and metacaspases (*Moss et al., 2007*) during apoptosis in a cellular protozoan parasite is well known, the activation of protease like CED3/CPP32 and ICE family are also well studied in leishmanial cells (*Das et al., 2001; Sen et al 2007; Sen et al 2001*). The caspase 3/7-like proteases activity was teased in PS-203 treated parasite cells as tetra peptide; DEVD (Asp-Glu-Val-Asp) sequence is recognized by both caspase-3/7-like proteases. We have found caspase-like protease activity in PS-203 treated cell lysate. Hence, in *Leishmania* the presence of caspases like protease cannot be denied, suggesting that PS-203 induced apoptosis in leishmanial cells is elicited by the

activation of protease like CED3/CPP32, leading to fragmentation of DNA and finally cell death.

Down regulation of *TRYR* in *L. donovani*, by heterologous expression, affects intracellular survivability of the parasite (*Tovar et al., 1998*). The effect of PS-203 on the amastigote and infectivity was studied, suggesting that PS-203 have a significant role in intracellular parasite proliferation. It also showed an immense effect on the infectivity of pre-treated parasite cells with PS-203, as there was nearly 80% less infection observed compared to untreated control. Further there was no significant toxicity of PS-203 on macrophage cells was observed indicating the selective toxicity towards leishmanial cells. The available drugs in the market are known to have high toxicity. The most effective drug for leishmaniasis, amphotericin B is known to have high cytotoxicity in vitro in micromole concentrations. The use of pentavalent antimonials has been stopped due to their high toxicity and drug resistance problems. The only available oral drug, miltefosine is teratogenic in nature with high cytotoxicity. Hence, there is a need for an improved drug against leishmaniasis. The more effective drugs with specific targets such as TryS and TryR may evolve as a better treatment for leishmaniasis. There are many reports claiming that redox metabolism of the parasite plays an important role in host parasite interaction to overcome the oxidative burst in host cells. Hence, PS-203 has equal effect in both the forms of *Leishmania* i.e. promastigote and amastigote, which is a desired characteristic feature required by a drug in treating infection like leishmaniasis.

The in vivo studies with PS-203 conducted shown good anti-leishmanial activity with minimum toxicity. Though there was not much toxicity observed in PS-203, but there was a slightly increased level of SGOT observed in AmB treated mice, which indicates that PS-203 is better in terms of toxicity compared to AmB. The parasite burden in spleen, which showed no much difference in AmB treated and PS-203 studied in hamsters. For better assessment of PS-203 in treating leishmaniasis more studies has to be conducted as this is a very first preliminary study with compound of this kind.

5.6 Conclusion

The conclusion, this chapter reports a new class of compounds i.e. oxabicyclo[3.3.1]-nonanones as anti-leishmanial agents. The anti-leishmanial activity of these compounds is mediated through inhibition of two important redox enzymes of the parasite TryS and TryR which are necessary for the parasite survival. The inhibition of these enzymes further causes ROS accumulation in the cell causing oxidative stress that elicited apoptotic cascade in parasite. Although these compounds show modest IC_{50} and *Ki* values against the parasite and the target enzymes, respectively, they identify a chemical space to design and develop inhibitors based on these compounds for better anti-leishmanial agents. The anti-leishmanial activity in both in vitro and in vivo showed prominent effect with no significant toxicity.

Chapter VI

Summary

Leishmaniasis is a group of neglected tropical vector born diseases caused by protozoan parasites of genus *Leishmania*. The only vector that transmits *Leishmania* parasite to mammalian host is infected sandfly. The clinical outcome of leishmaniasis is mainly governed by *Leishmania* species involved, the host factors and immunoinflammatory responses which may result in any one form of infection cutaneous, mucosal or visceral. Visceral leishmaniasis is the most severe form of leishmaniasis and is fatal if left treated. This disease is widely spread around the globe with endemic in large areas of tropic, subtropic and Mediterranean regions particularly in the economically undeveloped areas of India, Nepal, Bangladesh, Sudan and Brazil. The control of leishmaniasis still primarily depends on chemotherapy due to unavailability of vaccine. The emergence of resistance to pentavalent antimonials had resulted in discontinuity of their use. Further, though the great efficacy is shown by new drug such as amphotericin B and miltefosine, their high cost and high toxicity limit their use. There is an urgent need for new improved chemotherapeutics for treating leishmaniasis. Thus, the search for new and improved anti-leishmanial drugs

continues. The specific drugs for leishmaniasis can be identified by targeting fundamental metabolic and/or biochemical diverse target enzymes of the parasite. For inhibitors of parasite target enzymes both synthetic and natural products identify a rich source for novel and potential drug candidates against leishmaniasis.

The enzymes of redox metabolism of the trypanosomatids parasites are validated drug targets due to their unique presence in trypanosomatids parasites like *Leishmania*. This study mainly focuses on identifying inhibitors of redox enzymes of *Leishmania* parasites, trypanothione synthetase (TryS) and trypanothione reductase (TryR), their anti-leishmanial affect and molecular mechanism of parasite death. Initially the search for inhibitors of redox enzyme TryS was started by virtual screening of natural product small molecular data set of 800 compounds. Due to unavailability of *L. donovani* TryS crystal structure, homology modelling of *L. donovani* TryS (UniProtDI: Q8IFU8) was performed using *L. major* TryS crystal structure as template (PDB ID: 2VOB). The top hits from virtual screening ware tabulated based on the larger cluster with lowest binding energy. To identify the inhibitors of *L. donovani* TryS, the commercially available compounds from top hits from *in silico* screening studies were chemically tested. The cloning, expression and characterization of *L. donovani* TryS enzyme was perform and inhibition studies ware done. We were able to identify four inhibitors of *L. donovani* TryS enzyme namely, tomatine, conessine, uvaol and betulin. These identified inhibitors also shod good anti-leishmanial activity. Further, the current study investigated the molecular mechanism of parasite death caused in response to treatment with one of the identified inhibitor of TryS, betulin. For better efficacy and targeted delivery to intracellular amastigotes, betulin attached to functionalized carbon nanotube was formulated.

As cost is one of the major drawbacks of the presently available drugs for leishmaniasis, our search for chemically synthesized inhibitors of redox enzymes of the *Leishmania* parasite resulted in identifying oxabicyclo derivatives. These compounds anti-leishmanial activity is mediated through inhibition of key redox enzymes TryS and TryR of *Leishmania* parasite. Further the detailed molecular mechanism of parasite death caused by one of the potent oxabicyclo derivatives (PS-203) was investigated. The in vitro and in vivo effect of PS-203 was also investigated.

6.1 Molecular modelling of trypanothione synthetase (TryS) of *L. donovani* and virtual screening of natural product dataset for inhibitors of trypanothione synthetase

The model of trypanothione synthetase (TryS) (UniProtDI: Q8IFU8) from *Leishmania donovani* was successfully generated through homology modelling method. The modelled TryS quality showed very close resemblance to that of the template crystal structure as analysed through PROCHECK and ProSA validation package. Virtual screening of a natural product data set of 800 diverse chemical entities resulted in identifying diverse set of molecules that may be the inhibitors of TryS. The top hits showed two major interactions at the active site region of TryS. The interactions were conserved; either they bind residues surrounding the ATP binding cleft which act as anchoring residues or to the residues surrounding the substrate binding site making them potential inhibitors of the enzyme.

6.2 Cloning, expression, characterization and inhibition studies on trypanothione synthetase, a drug target enzyme from *Leishmania donovani*

Leishmania donovani TRYS gene was successfully cloned into the *Nde*I/*Bam*HI sites of pET28a. Further, pET28a-*Ld*TRYS construct transformed into BL21 (DE3) produced soluble and enzymatically active protein. A good purification of *Ld*TryS was achieved with the typical yields of TryS between 4-6 mg L^{-1}. The *Ld*TryS has pH optima of 8.0 ± 0.1 and optimum temperature 25°C. The K_m was determined for all the three substrate: ATP, GSH and Spd. Tomatine, conessine, uvaol and betulin were identified as inhibitors of *Ld*TryS. These identified TryS inhibitors also showed anti-leishmanial activity against promastigotes. Treatment of TryS inhibitors led to dose dependent decrease in total free thiol in promastigotes.

6.3 Anti-leishmanial Property of Betulin: Depicting the molecular mechanism of parasite death and its targeted delivery

In *Leishmania* promastigotes, betulin exerted ROS generation at micro molar concentrations. Further, betulin induced apoptotic death in *Leishmania* promastigotes as evident by

externalization of Phosphatidylserine, mitochondrial membrane damage, activation of caspase-like proteases and DNA fragmentation which are hallmark events observed in the apoptosis process. Further, to increase the efficacy of betulin, carbon nanotube formulation of betulin (f-CNT-BET) was successfully produced. Studies on the cytotoxicity of BET, f-CNT and f-CNT-BET on macrophage cells demonstrated their biocompatibility nature at cellular level. The f-CNT-BET showed high anti-leishmanial activity with increased efficacy compared to BET on intracellular amastigote in vitro.

6.4 Molecular mechanism underlying anti-leishmanial effect of oxabicyclo derivatives: Inhibition of key redox enzymes of the parasite

The new class of molecules, oxabicyclo[3.3.1]nonanones derivatives are identified as novel anti-leishmanial agents that inhibits key redox enzymes, trypanothione synthetase (TryS) and trypanothione reductase (TryR) of *Leishmania* parasite. The PS-203 the most potent oxabicyclo derivative[4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester], induced ROS accumulation in the cell causing oxidative stress that elicited apoptotic cascade in parasite. As these compounds inhibit key redox enzymes (TryS and TryR), treatment with the PS-203 compound resulted in decreased levels of $T(SH)_2$ and increased reactive oxygen species (ROS) levels in promastigote cells. PS-203 elicited apoptotic death of the parasite as evident by externalization of Phosphatidylserine, mitochondrial membrane damage, activation of caspase-like proteases and DNA fragmentation which are hallmark events observed in the apoptosis process. Further, the in vitro and in vivo studies with PS-203 demonstrated its high activity against intracellular *Leishmania* amastigotes. The PS-203 treatment drastically reduced the parasite burden in infected macrophage cells studies in vitro and in vivo with no any significant toxicity.

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LIST OF PUBLICATIONS AND AWARDS

Journal Publications (*from Ph.D. Thesis work*)

1. **Prakash Saudagar** and Vikash K. Dubey. (2011) Cloning, expression, characterization, and inhibition studies on trypanothione synthetase, a drug target enzyme, from *Leishmania donovani*. Biological Chemistry. 392, 1113–1122.

2. Santhosh K. Venkatesan, **Prakash Saudagar** and Vikash K. Dubey. (2011) Identification of novel inhibitors of trypanothione synthetase from two *Leishmania* species: Comparative in silico analysis. Journal of Proteins and Proteomics. 2, 41–48.

3. **Prakash Saudagar** and Vikash K. Dubey. (2013) Mechanistic insights into anti-leishmanial Property of Betulin: molecular mechanism of parasite death. American Journal of Tropical Medicine and Hygiene. DOI: 10.4269/ajtmh.13-0320**.**

4. **Prakash Saudagar**, Saha P, Anil K. Saikia and Vikash K. Dubey. (2013) Molecular mechanism underlying antileishmanial effect of oxabicyclo[3.3.1]nonanones: Inhibition of key redox enzymes of the pathogen. European Journal of Pharmaceutics and Biopharmaceutics, 85, 569–577.

5. **Prakash Saudagar** and Vikash K. Dubey. (2013) Carbon Nanotube Based Betulin Formulation shows better efficacy against *Leishmania* parasite (Communicated to Colloids and Surfaces B: Biointerfaces).

6. **Prakash Saudagar**, Shyam Lal M, Pipas Saha, Anil K. Saikia, Shyam Sundar and Vikash K. Dubey. (2013) *In vivo* assessment of antileishmanial property of 4-(4,4,8-Trimethyl-7-oxo-3-oxabicyclo[3.3.1]non-2-yl)-benzoic acid methyl ester, an oxabicyclo[3.3.1]nonanones, in hamster model. (Communicated to European Journal of Medicinal Chemistry).

Journal Publications (*from other collaborated project works*)

1. **Prakash Saudagar** [↕], Mousumi Das[↕], Shyam Sunder and Vikash K. Dubey. (2013) Miltefosine unresponsive *Leishmania donovani* has better ability of resist reactive oxygen species, FEBS Journal; 280, 4807–4815 ([↕] Equal contribution).

2. Santhosh K. Venkatesan, **Prakash Saudagar**, Anil K. Shukla and Vikash K. Dubey. (2011) Screening natural products database for identification of potential anti-leishmanial chemotherapeutic agents. Interdiscip. Sci. Comput. Life Sci. 3, 1–15.

3. Ruchika Bhardwaj, **Prakash Saudagar** and Vikash K. Dubey. (2012) Nanobiosciences: A contemporary line for Antiparasitic Drug. Molecular and Cellular Pharmacology. 4, 97–103.

4. Hazara S, Ghosh S, Sharma MD, Das M, **Saudagar P**, Prajapati V.K, Dubey V.K, Sundar S, Hazra B. (2013) Evaluation of a diospyrin derivative as antileishmanial agent

and potential modulators of ornithine decarboxylase of *Leishmania donovani*. Experimental Parasitology, 135, 407–413.

5. Abhinav Grover, Sushil K. Shakyawar, **Prakash Saudagar**, Vikash K. Dubey and Sundar Durai. (2012) Epitopic analysis of potential vaccine candidate in *Leishmania infantum* for development of human vaccine. Letters in Drug Design & Discovery. 9, 698–705.

Conference proceedings

1. **Prakash Saudagar** and Vikash K. Dubey. Oxabicyclo derivatives as a new class of anti-leishmanial agents: target identification and understanding molecular mechanism of parasite death. International Meeting on Emerging Diseases and Surveillance (IMED 2013). Vienna, Austria. February 15–18, 2013. (**Poster)**

2. **Prakash Saudagar** and Vikash K. Dubey. Trypanothion synthetase: A validated drug target enzyme from *Leishmania donovani*. The 18th International Conference (POST ISCBC-2012) IASST Guwahati, Assam, India, January 28-30, 2012. (**Poster)**

3. **Prakash Saudagar** and Vikash K. Dubey. Trypanothione Synthetase from *L. donovani*: Cloning, expression, characterization, and inhibition studies. 80th Annual Meeting of Society of Biological Chemists, held at Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, India, November 12-15, 2011. (**Poster)**

4. **Prakash Saudagar**, Santhosh K. Venkatesan and Vikash K. Dubey. Dubey. Molecular modeling, virtual screening and comparative analysis of Trypanothione Synthetase from *L. donovani* and *L. major* for the identification of new inhibitors as a drug. International Conference on Frontiers on Biological Sciences. NIT, Rourkela. October 1-3, 2010 (**Oral Presentation)**

Patent

1. **Prakash Saudagar**, Vikash Kumar Dubey, Anil Kumar Saikia and Pipas Saha. Oxabicyclo derivatives as anti-leishmanial agents. Application No. **863/KOL/2013.**

Awards

1. **B.S Narasinga Rao Best Poster presentation Award** during 80th Annual Meeting of Society of Biological Chemists, India held at CMAP, Lucknow, November 12-15, 2011.

2. **International travel award** by Department of Science and Technology, Government of India, for attending "International Meeting on Emerging Diseases and Surveillance" (IMED 2013). Vienna, Austria. February 15–18, 2013.