In vitro and *in vivo* studies of Withanolides in experimental Visceral Leishmaniasis caused by *Leishmania donovani*

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy (Ph.D.)

By

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(09LAPH09)

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University of Hyderabad
(A Central University established in 1974 by act of parliament)
HYDERABAD– 500 046, INDIA

"DECLARATION"

I, **S. Chandrasekaran**, hereby declare that this thesis entitled "***In-vitro* and *in-vivo* studies of withanolides in experimental visceral leishmaniasis caused by *Leishmania donovani***" submitted by me is based on the results of the work done under the guidance and supervision of **Dr. Radheshyam Maurya** at Department of Animal Biology, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

S. Chandrasekaran
(Research Scholar)

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"CERTIFICATE"

This is to certify that this thesis entitled "***In-vitro* and *in-vivo* studies of Withanolides in experimental visceral leishmaniasis caused by *Leishmania donovani***" is a record of bonafide work done by **Mr. S. Chandrasekaran**, a research scholar for the Ph.D. programme in the Department of Animal Biology, School of Life Sciences, University of Hyderabad, under my guidance and supervision. The work presented in this thesis is original and plagiarism free. The thesis has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Dedicated to:

My Loving Parents

&

My Loving Wife

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S. Chandrasekaran

ABBREVIATIONS	
^{o}C	Degree centigrade/Degree celsius
µg	Micro gram
µl	Micro litre
µM	Micro molar
AmB	Amphotericin B
ALP	Alkaline phosphatase
ATCC	American Type Culture Collection
BCPIP/NBT	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/ Nitro-blue tetrazolium chloride.
BD	Becton Dickinson
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Cluster of differentiation
CIMAP	Central Institute for Medicinal and Aromatic Plants
CL	Cutaneaous leishmaniasis
CMI	Cell mediated immunity
DC	Dendritic cell
DCL	Diffuse Cutaneaous leishmaniasis
DHB	Dihydrobiopterin
DHF	7, 8-Dihydrofolate
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immunosorbent aasay
EDTA	Ethylene diamine tetraacetic acid
F5	Fraction 5
F6	Fraction 6
FACS	Fluorescent activated cell sorter
FITC	Fluorescein isothiocynate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte monocyte colony stimulating factor
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HBSS	Hanks balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPC	Hexadecyl phosho choline
IFN-γ	Interferon-γ
IL-2	Interleukin-2
IL-10	Interleukin-10
IL-4	Interleukin-4
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDNA	Kinetoplastid deoxyribonucleic acid
LAg	Leishmanial antigen

L-AmpB	Liposomal amphotericin B
LdPTR1	*Leishmania donovani* Pteridine reductase 1
LDU	Leishman donovan units
LN	Lymph node
LPA	Lysophospho lipids
M199	Medium 199
MCL	Mucocutaneous leishmaniasis
MFI	Mean fluorescent intensity
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidyl choline
PCR	Polymerase chain reaction
PI	Propidium iodide
PKDL	Post kala-azar dermal leishmaniasis
PMM	Mouse peritoneal macrophages
PMSF	Phenylmethanesulfonylfluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
TBS-T	Tris buffered saline tween 20
TGF-β	Transforming growth factor-β
Th	T-helper
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VL	Visceral leishmaniasis
WS	*Withania somnifera*
WSE	*Withania somnifera* Ethanol extract
WSEt	*Withania somnifera* Ethyl acetate extract
WSH	*Withania somnifera* Hexane extract
WSW	*Withania somnifera* Water extract

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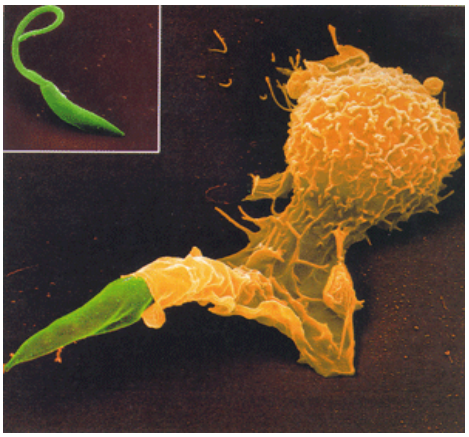
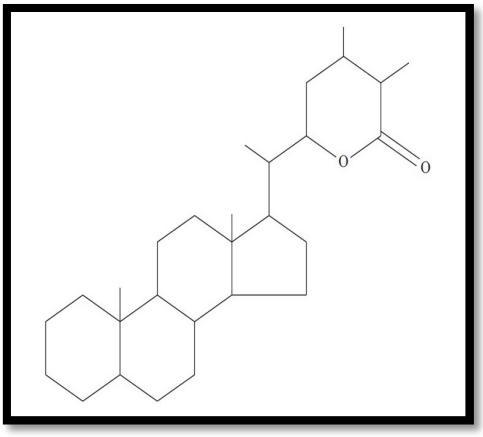
Chapter I:

Introduction





1.1 An overview

The leishmaniasis remains a severe public health problem, mostly in developing countries (Desjeaux 2001). The control measures are still highly dependent on research progress to obtain better tools and a more cost-effective strategy for case management and vector control. With valuable field tools available for step-by-step elimination, which should become feasible especially in anthroponotic foci where a man is a sole reservoir. Parasitic diseases pose a heavy burden for mankind. Diseases caused by protozoan parasites threaten the lives of nearly one-quarter of the human population worldwide. Leishmaniasis is ranked among the six major parasitic diseases identified by WHO (http://www.who.int/leishmaniasis/en). Visceral leishmaniasis (VL) causes more than 70,000 deaths per year and second in most deaths associated with the parasitic infection after malaria. Leishmaniasis is neglected tropical disease, which is commonly associated with poverty (Alvar et al., 2006). The disease is widespread in several areas, as a consequence of massive rural-urban migration and its association with AIDS. According to WHO, *Leishmania*-HIV co-infection is considered as a major threat in Spain and Mediterranean countries in south Western Europe (www.who.int/inffs/en/fact.html.factsheet-116, 2000).

Leishmania spp. is a digenetic obligatory intracellular protozoan parasite that thrives in hostile environments like the midgut of the sand fly and the phagolysosome of the macrophages (Pearson et al., 1996). Only about 30 different species of *Phlebotomine* sandflies transmits the disease to humans out of 200 species reported. This disease is considered as a major public health issue in tropical and subtropical regions of the world. Major characteristics of this disease are its diversity and complexity (Herwaldt, 1999) due to which in 88 countries around the world about 350 million men, women and children are affected. Every year about 12 million people are currently infected with 1-2 million new cases arise each year. (www.who.int/leishmaniasis). Leishmaniasis represents a complex disease with clinical and epidemiological diversity. VL is highly prioritized than cutaneous leishmaniasis (CL) because of its fatal nature with the absence of treatment.

1.2 Historical background

In the 10th century, Arab physicians including Avicenna described them as Balkh sore (Cox, 2002). In 1756, Alexander Russell gave the first detailed clinical description of the disease after examining a Turkish patient. As for the new world, evidence of a cutaneous form of the illness were depictied as skin lesions and deformed faces on pre-inca potteries

(http://www.who.intitdr/diseases/leish**).** Physicians in the Indian subcontinent would describe it as Kala-azar. In Jessore, India the Kala-azar first came to the attention of Western doctors in 1824. The name kala-azar for VL, which is the Hindi for "black fever", was given due to the darkening of the skin.

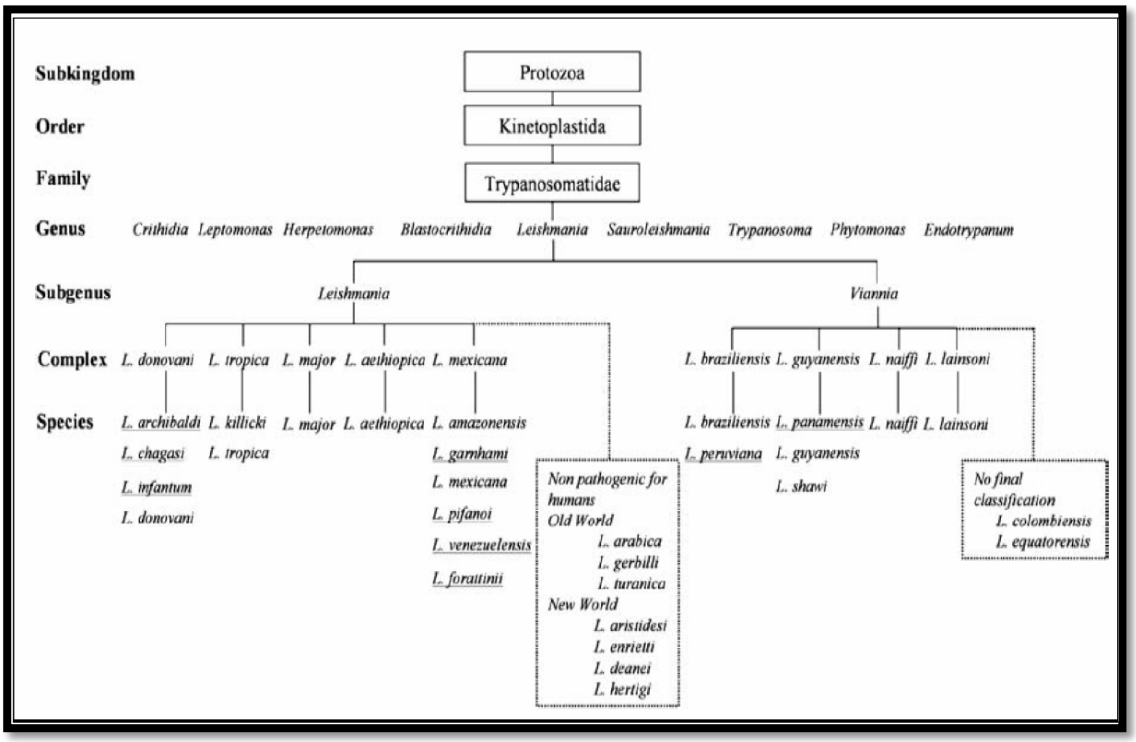


Figure 1.1: Taxonomy of Leishmania

Source: Based on the scheme published by the WHO, 1990

The genus *Leishmania* can be classified as:

Phylum: Sarcomastigophora

Sub. Phylum: Mastigophora

Class: Zoomastigophora

Order: Kinetoplastida

Sub-order: Trypanosomatina

Family: Trypanosomatidae

1.3 Geographical distribution

Till date, 350 million people are at risk and 20 million people are infected worldwide with leishmaniasis and an estimated of 1-2 million new cases occur each year (Leishmaniasis control, www.who.int/health-topics/leishmaniasis.htm, update 2007) with annual incidence of 1–1.5 million cases of CL and 500,000 cases of VL. In 88 countries,

Leishmaniasis is considered to be endemic (WHO, 2005) on five continents namely Asia, Africa, Europe, North and South America (22 in new world and 66 in old world) (Desjeaux 2001) (Figure1.2). This parasitic disease gained much importance due to the emergence of *Leishmania*/HIV co-infection in countries like Spain, Italy, France and Portugal (Berhe et al., 1999) (Figure 1.3).

Around 66 countries have reported the confirmed VL cases, of which Bangladesh, Brazil, India, Nepal and Sudan contribute to 90% of VL cases and some countries from Africa and South America contribute to 90% of CL cases (Desjeux, 2004; Modabber *et al.,* 2007). Climate change and other environmental changes also have an impact on expanding the geographic range of the vectors and transmission of leishmaniasis (Patz *et al.,* 2000).

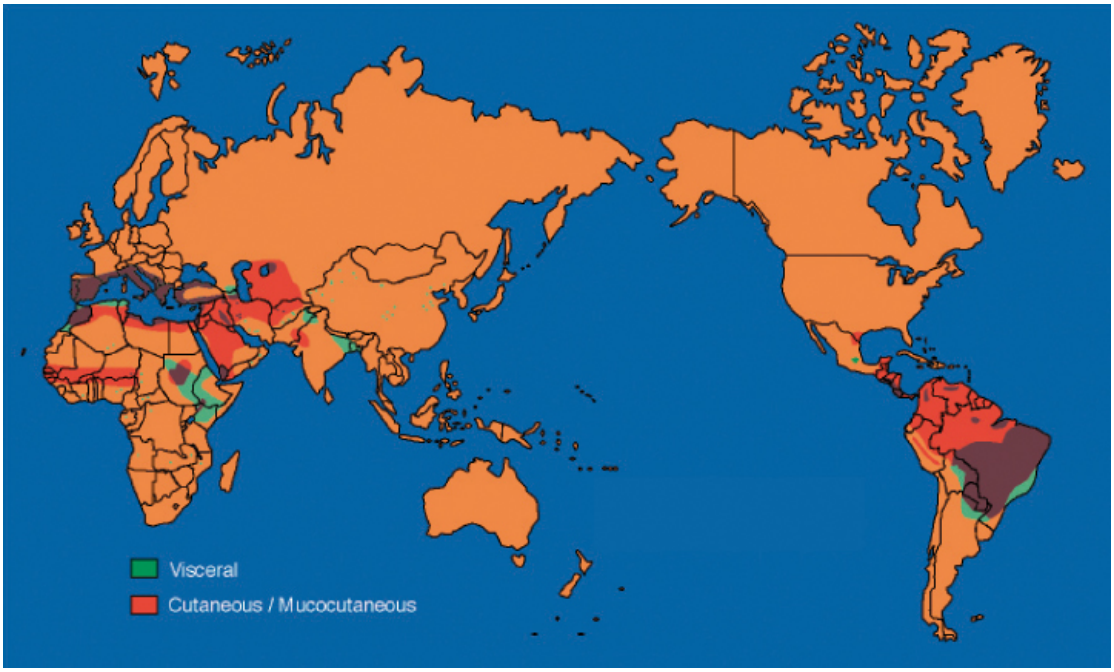


Figure 1.2: Status of leishmaniasis worldwide. (http://www.fp7-rapsodi.eu/150/)

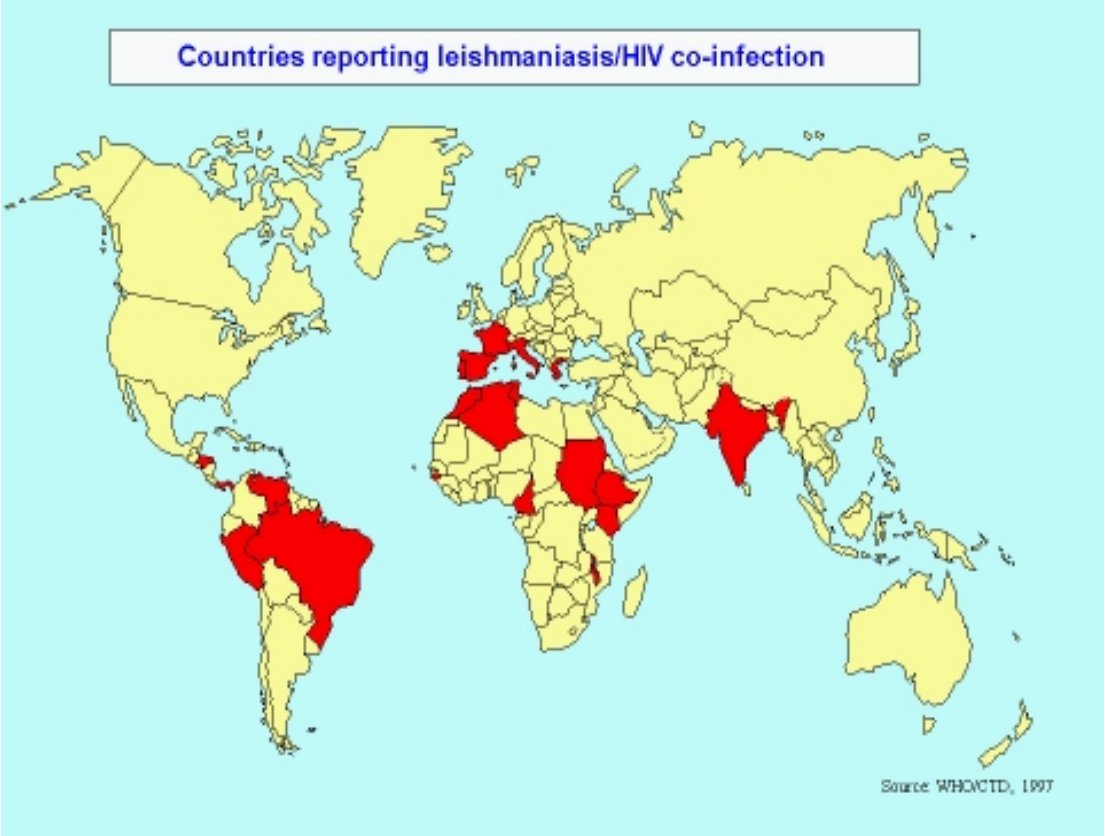


Figure 1.3: Countries reporting leishmania/HIV co-infection. (WHO/CTD, 1997)

1.4 Prevalence of leishmaniasis in India

In the Indian sub-continent, the eastern parts of the country viz. Bihar, West Bengal, eastern districts of Uttar Pradesh, are the endemic zone of the VL (Marin kelle, 1980). VL disease not only depends on the poverty but also on its adverse social impact (Muniaraj, 2014).

The situation is pathetic particularly in the state of Bihar, India, known as the "heartland of kala-azar" where 52 districts of Bihar state, 8 districts of West Bengal and 2 districts of eastern Uttar Pradesh are severly affected (Guerin et al., 2002). In Bihar, atleast 75% of VL cases live below the poverty threshold of less than one dollar a day (Figure1.4). Countries such as Nepal (12 districts) and Bangladesh (45 districts) are also affected (Sundar and Rai, 2002a; Alvar et al., 2006). Bhutan has recently joined the list of countries affected by VL (Bhattacharya et al., 2010).

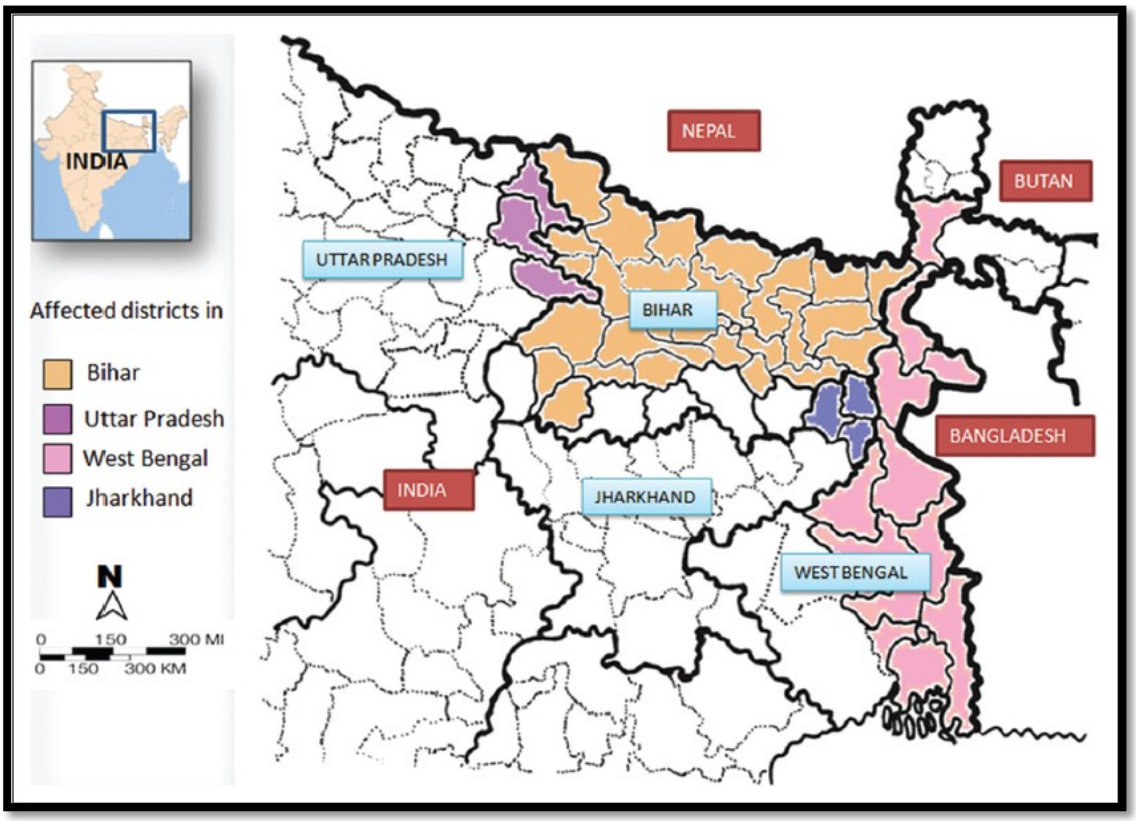


Figure 1.4: Areas affected with VL in india Source: Muniaraj, 2014

In a recent study, which shows that 68,358 VL cases between 1990 to 2008 were reported in Muzaffarpur district (Malaviya *et al.,* 2011).

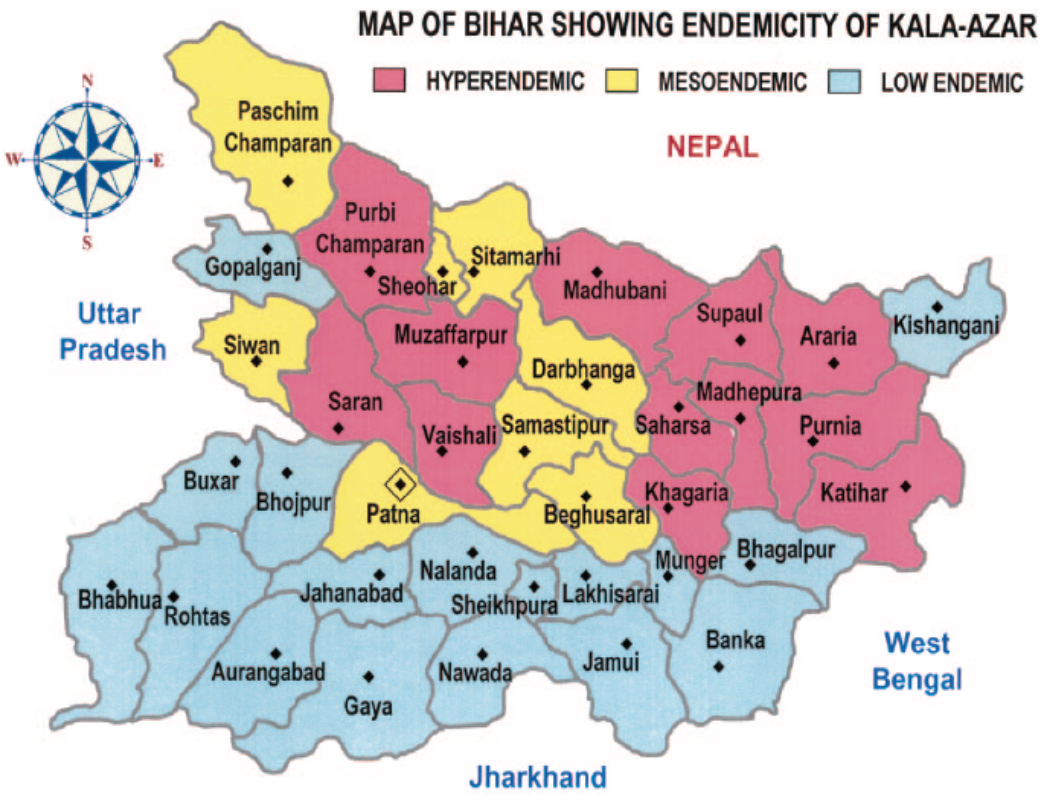


Figure 1.5: Distribution of resistance to pentavalent antimonials in leishmaniasis endemic areas of Bihar, India. (Simon et. al., 2006)

1.5 Disease manifestations

The leishmaniasis causes considerable morbidity and mortality. In man, the disease occurs in at least 5 major forms: Cutaneous, Diffuse cutaneous, Mucocutaneous, Visceral and Post kala-azar dermal leishmaniasis.

1.5.1 Cutaneous leishmaniasis (CL)

It is frequently self-healing but creates a aesthetic stigma when the lesions are multiple and disabling with a disfiguring scar. Recidivans leishmaniasis is the most severe form, which is long lasting, destructive, difficult to treat and disfiguring. The clinicopathological picture of cutaneous leishmaniasis is variable depending upon several host-parasite related factors.

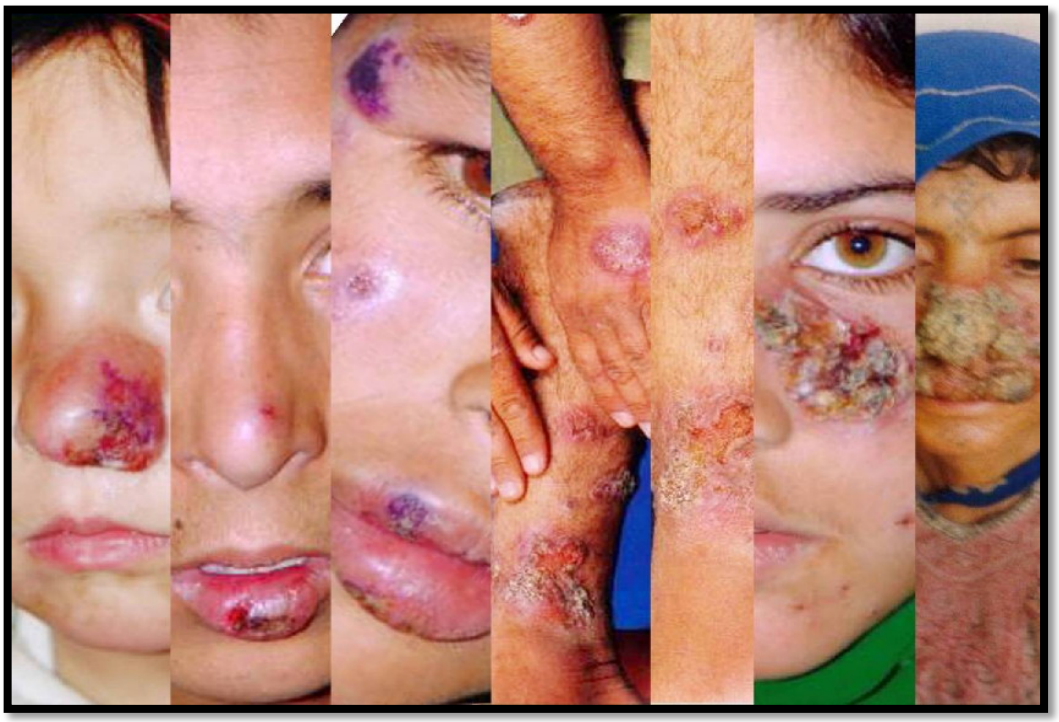


Figure 1.6: Lesions of cutaneous leishmaniasis which are mainly on the exposed parts of the body, particularly the face. (Kassi et al., 2008)

1.5.2 Diffuse cutaneous leishmaniasis (DCL)

This occurs in individuals with a defective cell-mediated immune response. This disease is characterised by disseminated lesions, which never heal spontaneously and cases of relapse are seen after treatment with any of the currently available drugs. Because of the devastating consequences to the patient, it is recognized as a serious public health problem. DCL is caused by *L. mexicana* species. Lesions like papules, nodules, or ulcers are characteristic of DCL and it responds well to the classic treatment.

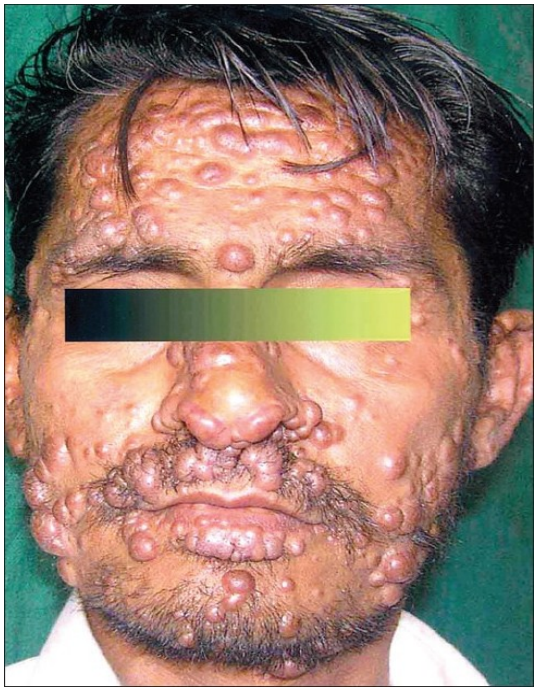


Figure 1.7: Diffuse cutaneous leishmaniasis showing asymptomatic papulonodular lesions on face. (Chaudhary et al., 2008)

1.5.3 Mucocutaneous leishmaniasis (MCL)

It is referred as 'espundia' in South America. It is caused by *L. braziliensis, L. panamensis* and *L. guyanensis* in the new world but *L. donovani, L. major* and *L. infantum* are also reported to cause mucosal lesions in immunosuppressed patients in old world (Desjeux, 1996). The parasite infects the mucocutaneous region and spread to the oronasal/pharyngeal mucosa, which leads to erosion of the soft tissues and cartilage of the oronasal/pharyngeal cavity. These lesions do not heal spontaneously when compared to cutaneous leishmaniasis. The patient undergoes severe suffering and mutilation and as a result, death occurs due to bronchopneumonia or malnutrition. A substantial part of the disfigurement is possibly due to immunological mechanisms.

Figure 1.8: Clinical features of mucocutaneaous leishmaniasis (www.itg.content-e.eu)

1.5.4 Visceral leishmaniasis (VL)

In Bihar, only one in eight cases is reported through official channels and approximately 20% of mortality cases are undiagnosed. It is caused by *L. donovani* complex i.e. *L. donovani donovani* (India, Africa), *L. d. infantum* (The Middle East and some parts of Asia) and *L. d. chagasi* (South America). These species are morphologically indistinguishable but have been identified by molecular methods, predominantly multilocus enzyme electrophoresis. The disease can be acute, sub-acute or chronic, but most cases are asymptomatic (Bittencourt *et al.,* 1995). The asymptomatic cases are characterized by positive serology to *Leishmania* and positive intradermal test.

It is characterized by fever, weight loss, splenomegaly, hepatomegaly and/or lymphadenopathies, anemia and blackness of skin, hence the name Kala-azar or black fever (Desjeaux 1996; WHO 1996). As the disease advances, an increase in splenomegaly, which is followed by concomitant hepatomegaly, severe anemia and cachexia. It causes large-scale epidemics with high fatality rate. After treatment and recovery, the patients may develop chronic cutaneous leishmaniasis that requires long and expensive treatment.

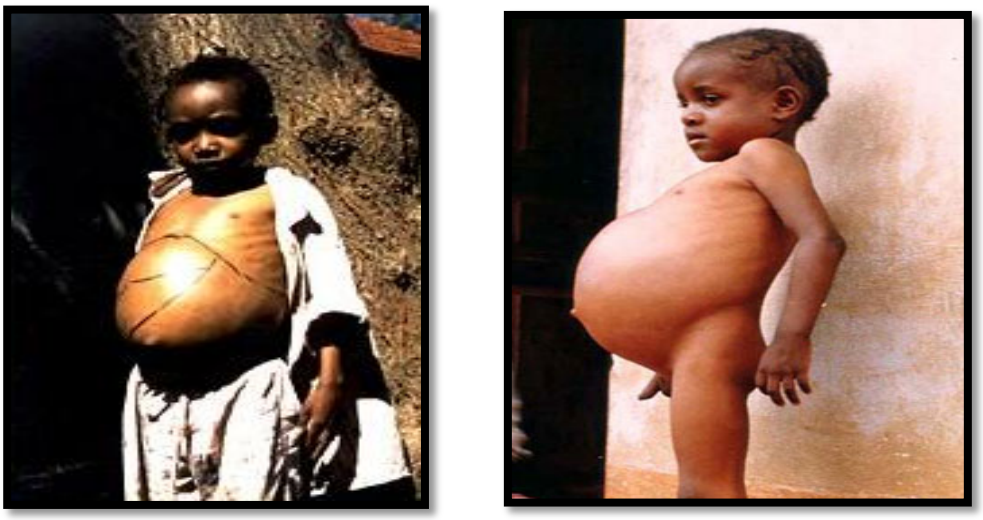


Figure 1.9: Clinical features of visceral leishmaniasis (www.pixshark.com)

1.5.5 Post kala-azar dermal leishmaniasis (PKDL)

Post kala-azar dermal Leishmaniasis is a consequence *L. donovani* infection. *L. infantum, L. donovani,* and *L. tropica* causes this disease in old world (rare; also may produce the atypical viscerotropic disease) and *L. chagasi* in the new world. The disease is characterized by skin lesions, nodules or papules, frequently on the face has been well characterized in India and Sudan. This disease develops after one or two years of antimonial treatment as non-ulcerative cutaneous lesion in about 10% of kala-azar patients (Zijlstra et al., 1991).

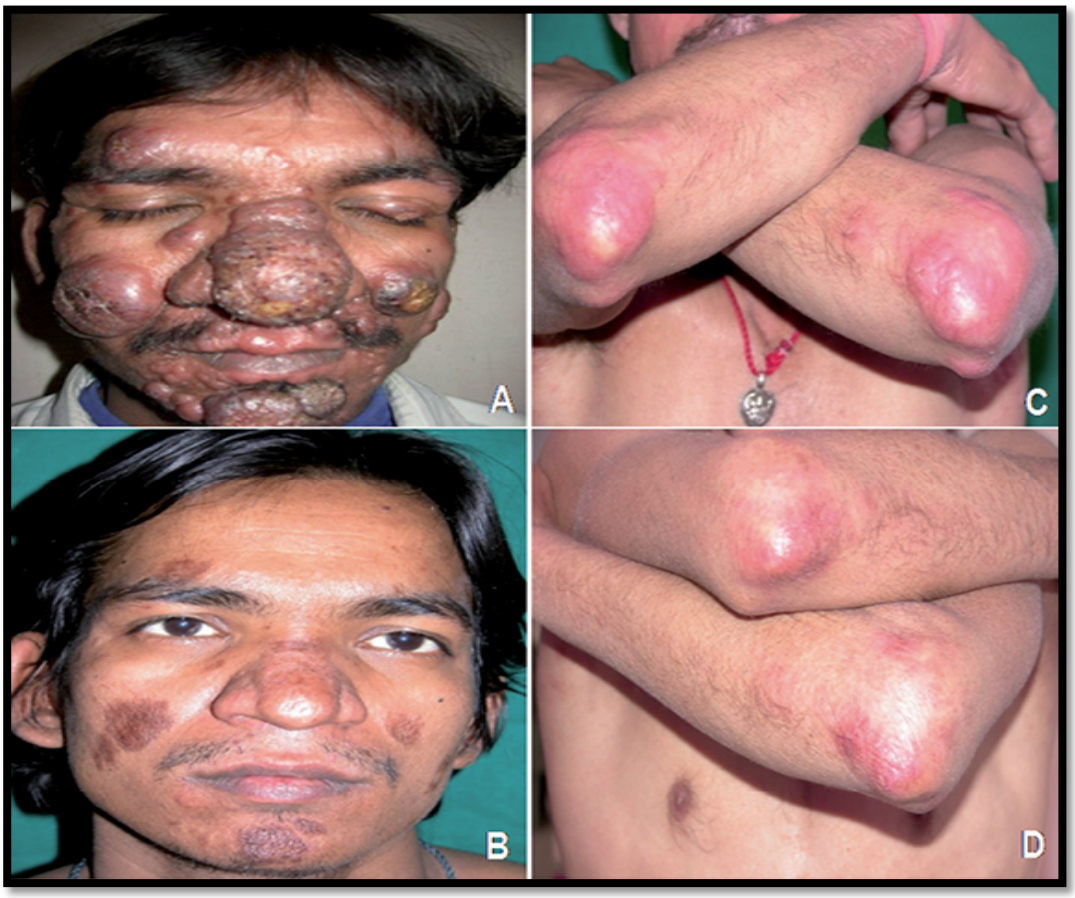


Figure 1.10: Patient with post kala-azar dermal leishmaniasis (Salotra et al., 2013)

1.6 Epidemiology

Leishmaniasis occurs in poorest segments of populations in Asia, Africa, South America and in lesser degree in Europe (Yamey & Torreele, 2002). Malnutrition, displacement, poor housing, illiteracy, gender discrimination, and weakness of the immune system and lack of resources are the major risk factors for this disease. The disease gets transmitted to humans only when they enter the sylvatic habitat. In domestic cycles, humans or dogs are the major infection reservoir. Female sand fly of genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World are the vectors for transmission of the disease (Berman, 1997). In India, *Phlebotomus argentipes* is the only proven vector for the disease. Infection occurs by blood meal taken up by female sand flies (*Phlebotomus* and *Lutzomyia* spp) from infected human beings or terrestrial mammals. Amastigotes from the blood meal enter into the sand fly gut, transform and replicate as promastigotes and reach the salivary glands where they mature into infective metacyclic promastigotes, which are regurgitated and injected into the skin of the host during the blood meal to complete the cycle (Rogers &

Titus, 2004). Vector competence is achieved by most species through parasite's ability to resist proteolytic enzymes during blood meal digestion and avoid excretion by binding to midgut epithelium. Binding to the gut wall is mediated by lipophosphoglycan present on the promastigote surface, and the phosphoglycan domains differ between species (Sacks, 2001). Sand fly saliva promotes experimental cutaneous infection by altering the local host immune responses (Sacks & Noben-Trauth, 2002). The major transmission of the infections are zoonotic, but some cases are reported for transmission of *L. donovani* from human to human.

1.7 *Leishmania* life cycle and morphology

Leishmania parasite leads digenetic life cycle between the invertebrate sand fly and vertebrate host and differentiates into insect-stage, which are flagellated promastigotes and mammalian-stage called as intracellular amastigotes.

1.7.1 Promastigote stage (Invertebrate host)

Promastigotes measure about 15-20 μm by 1.5-3.5 μm characterised by long and slender body with a 15-28 μm anterior flagellum which functions in locomotion and attachment to the insect gut wall (Herwaldt, 1999; Bogitsh & Cheng, 1990). The membrane has molecules such as glycoproteins and mannose receptors that are essential for the uptake of promastigotes by macrophages.

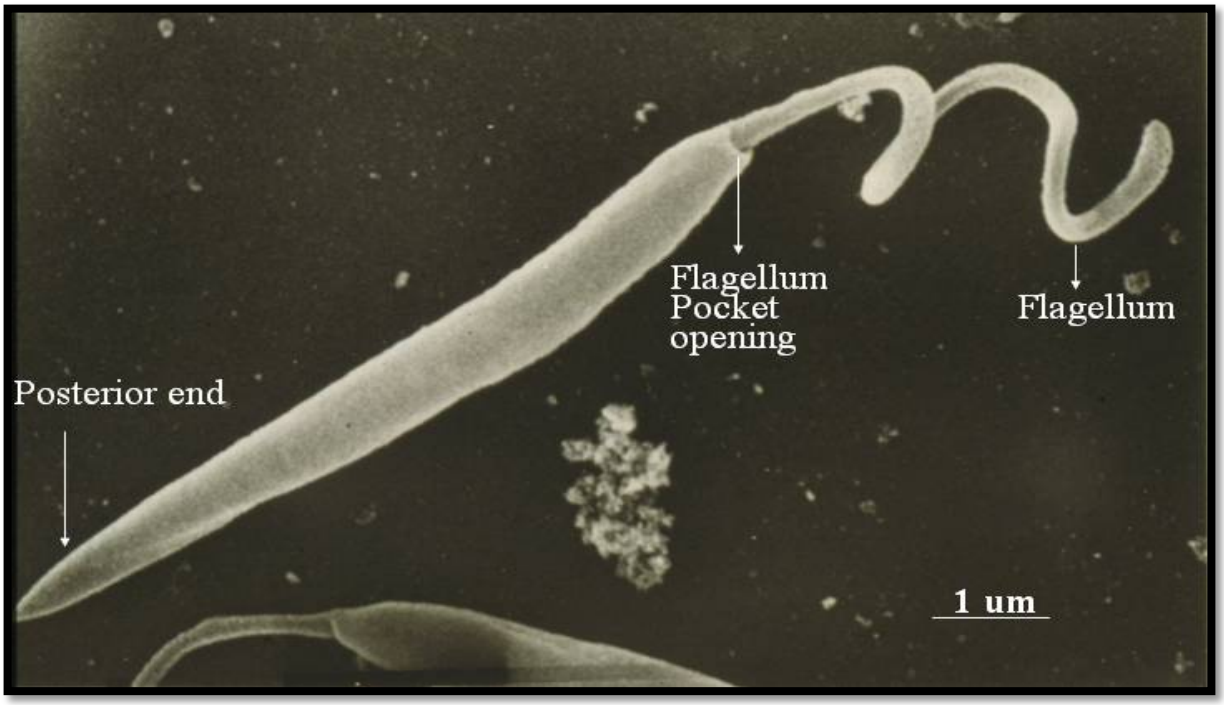


Figure 1.11: Ultrastructure representation of promastigote stage of *L. donovani* parasite (KP Chang, http://dna.kdna.ucla.edu)

1.7.2 Amastigote stage (vertebrate host)

Amastigotes are non motile, non-flagellated and smaller in size compared to promastigotes. They are ovoid in shape and measure approximately 2-4μm in diameter. This stage is found in parasitophorous vacuole of macrophages. The outer membrane has polysaccharide component, but there is no surface coat (Herwaldt, 1999).

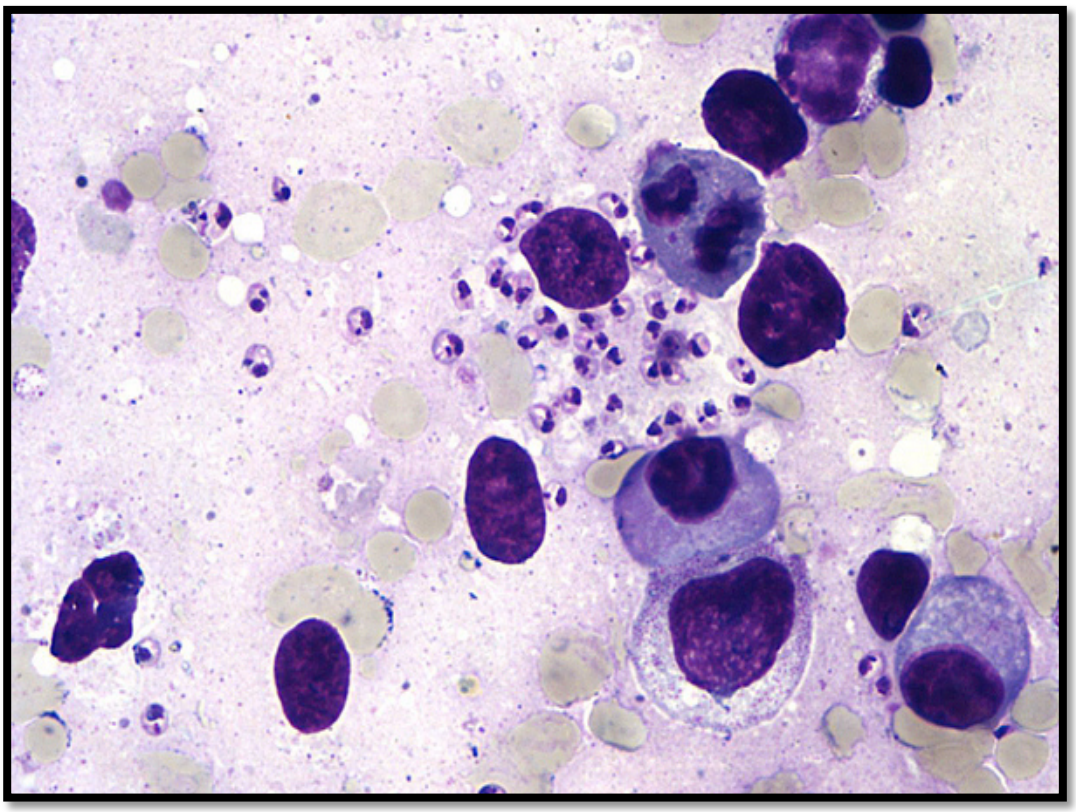


Figure 1.12: Bone marrow biopsy showing the *L. donovani* amastigote forms (http://www.idimages.org/atlas/organism)

1.7.3 Lifecycle

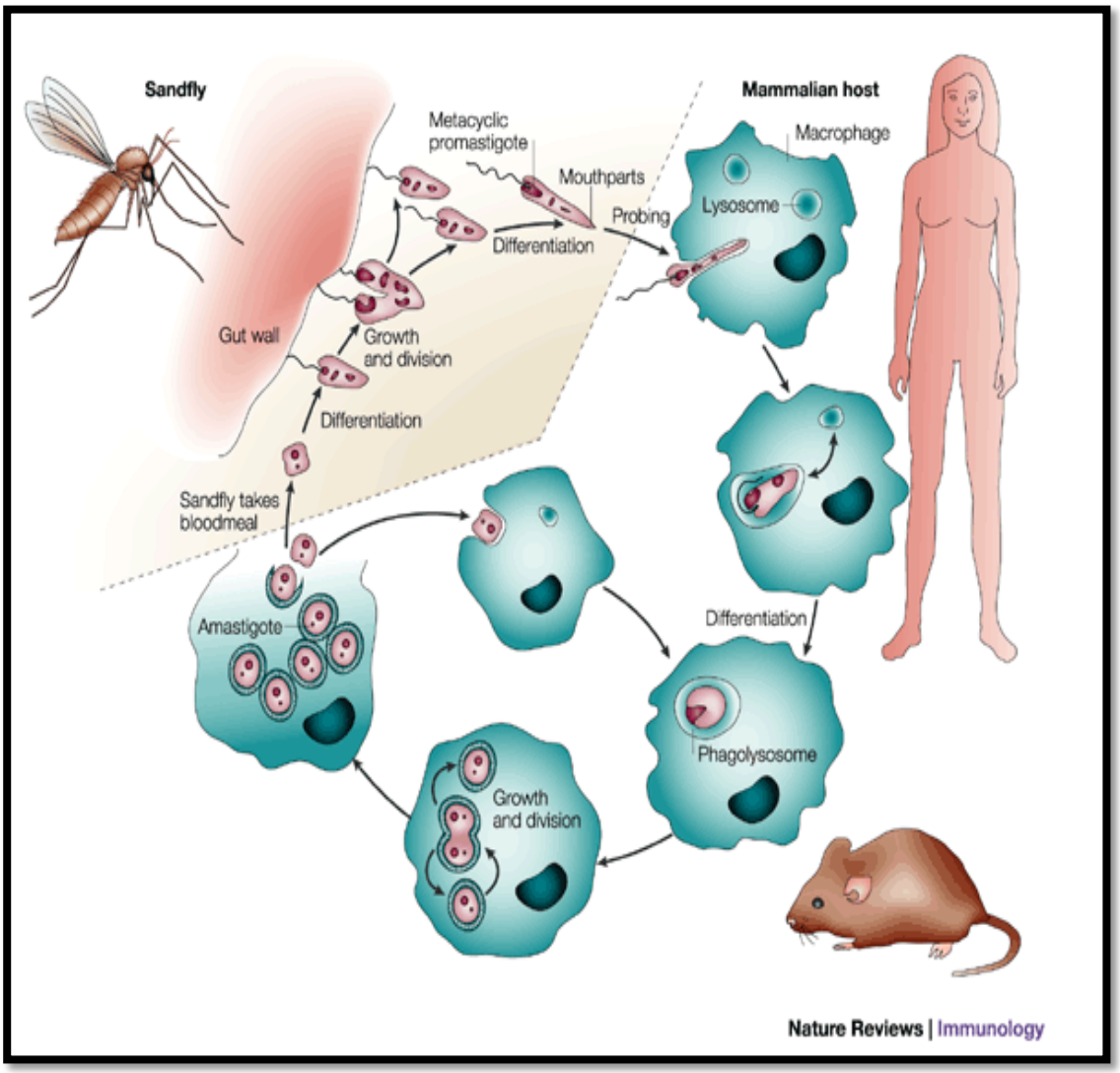


Figure 1.13: Life cycle of *Leishmania*. A schematic explaining the life cycle of *Leishmania* alternating between the promastigote stage in the sandfly and the amastigote stage in host macrophages. (Adapted from Sacks D *et al.* (2002) Nat. Rev. Immunol.)

1.7.4 Infection to host macrophages

After the sand fly blood meal, amastigotes reach the lumen of the sand fly where they differentiate into promastigotes and replicate by longitudinal binary fission. Approximately four days after ingestion, through metacyclogenesis the promastigotes convert into metacyclic promastigotes. These forms are characterised by being longer and narrower, have a longer flagellum, and display changes in surface coat proteins, which make the parasite adapting to infect macrophages of the host (Spath & Beverley, 2001). Metacyclic promastigotes migrate to the salivary glands and deposited on the skin of the host through the sandfly's proboscis during the next blood meal. The cells are then

phagocytosed by macrophages. The parasite undergoes transformation inside the endocytotic parasitophorous vacuole into amastigote form. These cells divide and increase in number by binary fission inside the macrophages, ultimately they rupture and release the amastigotes, which then infect the neighbouring macrophages. This cycle continues with the above steps (Bogitsh & Cheng, 1990).

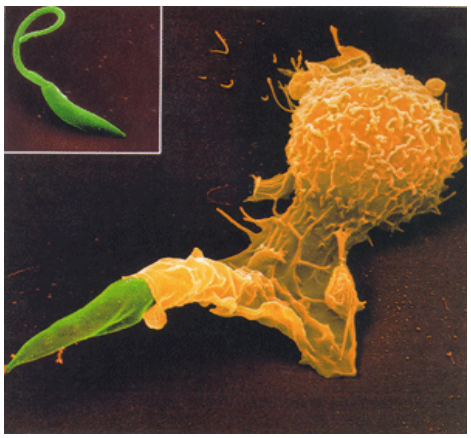
1.7.5 Diagnosis

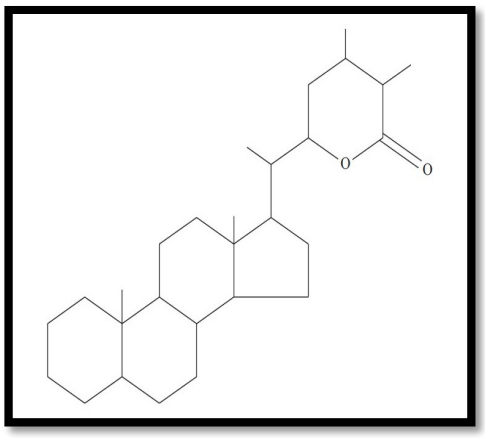
The clinical features in various forms of leishmaniasis are nonpathognomonic and these can mimic several other conditions.

- The serological diagnosis was based on IFAT and ELISA and had severe limitations for their use at the district level. DAT could be used at the periphery but needs cold chain (Sundar and Rai, 2002b; Oskam et. al., 1998).
- Molecular diagnosis: PCR needs to be more suitable for field use and less expensive (Maurya et al., 2005).
- Parasitological diagnosis existing tools (spleen, bone marrow or lymph nodes aspirates) are invasive methods, very difficult to decentralize and their average sensitivity varies from 95% (spleen), 70% (bone marrow) to 58% (lymph node). Spleen aspirate due to its high sensitivity (95%) and its specificity is considered as the gold standard for VL diagnosis. However, it requires the help of physician and the laboratory technician needs to be highly expertise, which is difficult to get outside a tertiary reference hospital or a specialized center. Splenic aspirate has some contraindications: severe anemia, pregnancy and bleeding tendency.
- A dipstick called K39, which is also commercially available (FDA approval). It is based on a recombinant surface antigen highly specific for VL. It is particularly relevant for field use (Sundar et al., 1998).
- A new latex agglutination test (KATEX), useful to detect antigens in urine. It is of great value for immuno-suppressed patients (Attar et al., 2001).

Chapter II:

Review Of Literature





2.1 Immune response in visceral leishmaniasis

Visceral leishmaniasis (VL) is a vector-borne disease transmitted by sandflies belonging to genus *Phlebotomus* in old world and the genus *Lutzomyia* in new world. Usually most human pathogenic *Leishmania* species reside in macrophages of the skin and skin-draining lymph nodes but *Leishmania donovani* and *Leishmania infantum* spread systemically to propagate in macrophage of visceral organs like liver, spleen, bone marrow, and lymph nodes. The symptoms of VL typically involves fever, enlarged spleen and liver, loss of weight, and hypergammaglobulinemia (Badaro et al., 1986). Other symptoms of VL include hypoalbuminemia, severe cachexia and bleeding, if left untreated. VL patients become immunosuppressed due to the loss of leucocytes and the common cause of death are due to bacterial infections in lethal cases of VL. Moreover, loss of leucocytes eventually makes VL patients generally immunosuppressed, and bacterial infections are a common cause of death in lethal cases of VL. Untreated VL will in most cases ultimately lead to death.

2.2 Various hosts for VL

The susceptibility and resistance to infection is dependent on the host genetic background and Th1/Th2 pattern of immunity. The best suited animal models for VL caused by *L. infantum* are dogs, which are also the natural reservoirs (Alvar et al., 2004). Strikingly, the infections caused by *L. donovani* complex are subclinical, but infection can result in severe life-threatening visceral disease (Alvar et al., 2004), while in canine VL it can also cause keratitis and skin pathology, which is not seen in human disease (Baneth et al., 2008). Usually, small rodents are preferred as models in experimental VL while dogs are used in limited extent due to high costs and ethical concerns.

2.2.1 Mouse/Rodent host

Mouse are preferred models to study both *L. donovani* and *L. infantum* infection. Murine VL infection has a genetic basis such as genetically resistant mouse (e.g., CBA) carries a functional *Slc11a1* gene that encodes a phagosomal component. It localizes to endosomes/lysosomes, solute carrier also known as Nramp1, confers the ability to control the early infection (Crocker et al., 1984; Blackwell et al., 1989). Susceptible strains (BALB/c) are not able to control the parasite multiplication in the liver during the initial phase of infection due to the mutant *Slc11a1* gene (Crocker et al., 1984; Vidal et al., 1995). The resolution rate of disease in these strains is determined by MHC class II haplotypes (H-2loci; Blackwell, 1983). Murine VL can be considered as a model for subclinical infection

as susceptible mice can control the disease as compared to the model of disseminated visceral disease and parallels to overt human VL disease. The parasite growth is controlled in the liver after first weeks of infection by the granulomatous response and cell-mediated immune response following intravenous or intracardial infection (primarily using amastigotes). Over a period of 2–3 months the infection is eventually cleared by mice from the liver and becomes resistant to re-infection (Murray et al., 1987). In spleen the parasites persist and infection slowly progress for longer periods as compared to liver infection. Eventually, the parasite numbers are controlled in spleen and parasites are maintained at a constant levels (Engwerda et al., 2004). The presence of parasites in spleen leads to splenomegaly and remodeling of splenic architecture with atrophy of lymphoid follicles (Engwerda et al., 2004). The immune response can be compared between the murine and human spleen, which is characterized by a mixed regulatory and inflammatory response. TNF-α, which is necessary for the protective immunity in liver is elevated in spleen along with IL-10 levels (Murray et al., 2000) and these elevated TNF-α levels cause destruction of the marginal zone macrophages and the gp38+ stromal cells (Carrión et al., 2006; Stanley and Engwerda, 2007). IL-10 is involved in immune regulation, like protecting the tissue from damage due to excessive inflammation, has suppressive effects on immune function. IL-10 is induced as a result of high levels of TNF-α (Ato et al., 2002), which prevent the tissue damage caused by TNF-α, but IL-10 has a role in the parasite persistence by inhibiting macrophage activation (Belkaid et al., 2001) by making it unresponsive to activation signals and also downregulates the CCR7 expression on dendritic cells (DCs), thereby loosing migratory capacity preventing them from proper accession to T-cell areas and efficient priming of T-cells responses (Ato et al., 2002). Enhanced granuloma formation in the liver and parasite killing is due to IL-10 receptor blockade, (Murray et al., 2002) which resulted in increased IFNγ production and enhanced expression of inducible nitric oxide synthase (iNOS) in infected tissue (Murray et al., 2003b). Given in combination with antileishmanial therapy (SbV), blockade of IL-10R was shown to enhance the effectiveness of SbV (Murray et al., 2005).

2.2.2 Hamster host

Similar to human VL, hamsters can develop progressive VL, eventually causing mortality (Ghosh and Ghosh, 1987; Requena et al., 2000; Melby et al., 2001). Hamsters show elevated expression of Th1-associated cytokine mRNA like IFN-γ, IL-2 and TNFα in

the spleen, but induction of IL-4 m-RNA is limited as seen in humans (Melby et al., 2001). In comparison to humans, hamsters are also poor producers of NO. In response to IFN-γ, the expression of NOS 2 mRNA is downregulated, which may be due to reduced NOS2 promoter activity in *L. donovani*-infected hamsters (Perez et al., 2006). This might lead to defective parasite killing seen in hamsters due to failure in the induction of iNOS (Melby et al., 2001; Wilson et al., 2005). Due to deposition of parasite immune complexes in kidneys, hamsters develop severe ascites and glomerulonephritis, which results in renal failure and nephritic syndrome ultimately causing death (Sartori et al., 1992). Hamsters are always an efficient model for testing the experimental drugs against VL, but the lack of reagents hamper this process.

2.2.3 Human host

Human VL symptoms range from fatal visceral disease to asymptomatic infection which is defined as the presence of *Leishmania*-specific antibodies or positive skin test to *Leishmania* antigen without any symptoms of the disease. However, factors responsible for an individual to precisely develop VL is still largely unclear. Peripheral blood mononuclear cells (PBMCs) from some but not all subclinical or asymptomatic patients produce IL-2, IFNγ, and IL-12 in response to stimulation with leishmanial antigen (LAg) (Carvalho et al., 1992). Neutralizing IL-12 cytokine abrogates both proliferation and IFN-γ production in the naturally exposed healthy individuals (Ghalib et al., 1995). Interestingly, recent studies have demonstrated that blood cells from whole blood (instead of purified PBMCs) from VL patient maintain capacity to produce IFNγ in response to soluble *Leishmania* antigen (Ansari et al., 2011; Gidwani et al., 2011a). Initially VL was associated with Th2 skewing response with elevated levels of IL-4 and/or IL-13, but most studies implicate that there is no clear Th2 skewing in human VL (Sundar et al., 1997; Nylen et al., 2007). During the acute phase of infection, IFN-γ mRNA is elevated in the spleen and bone marrow (Nylen and Sacks, 2007). These observations show that Th2 skewing is not involved in the pathogenesis of VL, instead there exist other mechanisms. Clinical studies demonstrate the role of IL-10 in the pathogenesis of VL (reviewed in Nylen and Sacks, 2007). High serum IL-10 and elevated IL-10 mRNA in the spleen, lymph nodes and bone marrow are found in patients with active VL (Nylen et al., 2007; Ansari et al., 2011). The presence of IL-10 can only be detected in whole blood cell cultures but not in PBMCs from VL patients after *Leishmania* antigen stimulation (Ansari et al., 2011).

IL-10 functions as conditioning the host macrophages for parasite persistence in VL.

It also down-regulates TNF-α and NO production, which in turn inhibits the killing of amastigotes. Apart from this IL-10 renders macrophages unresponsive to activation signals. In human VL, IL-10 inhibition in serum inhibit the parasite replicationin macrophages and it enhances IFN-γ response in antigen-stimulated PBMC (Ghalib et al., 1995; Nylen et al., 2007). Recent reports prove the host-protective effect of IL-10 neutralization in lesional tissue, which provides evidence that overproduction of IL-10 directly contributes to the pathogenesis of human VL (Gautam et al., 2011).

2.3 Immune mechanisms and regulation

2.3.1 Innate immunity

Immediately after the parasites are injected into the skin, a local inflammatory process is initiated, which involves the accumulation of neutrophilic and eosinophilic granulocytes to clear damaged tissue, followed by inflammatory macrophages and to initiate wound healing. IFN-γ production after activation by both parasite antigens and IL-12 from natural killer cells (NK cells) play an important role in parasite containment (Laskay et al., 1995). Humans also have NK cell-mediated mechanisms in the early defense to *Leishmania* infection similar to the murine model. A role of NK cells in the maintenance of the healing process in addition to initial response in naïve individuals is speculated (Maasho et al., 1998*)*. IFN-α/β cells and iNOS2 also play a role in dissemination and containment of the parasite. There is a dramatic change in the lymph nodes (LN) in the first 16-24 h of infection namely increase in LN cells with a relative decrease of CD4+ cells and increase of B cells. This increase in cell number is not due to *in situ* proliferation after parasite entry but rather the consequence of influxing cells from the peripheral blood.

2.3.2 Adaptive immunity

2.3.2.1 Antigen presentation

The prerequisite for protective *anti-Leishmania* immune response is presentation of appropriate antigens by antigen-presenting cells, the induction and expansion of $CD4^+$ Th1-T cells, and the activation of macrophages for efficient killing of the parasites. For immune evasion, *Leishmania* parasites interfere with the intracellular loading of MHC class II molecules with antigenic peptides. It is seen that during the transformation process of *Leishmania* from its promastigote form to intracellular amastigote stage, parasite antigens are sequestered from the MHC class II pathway of antigen presentation, which then can result in evasion of T cell activation.

2.3.2.2 Role of T cells

Understanding of the regulation of immune response involved in host resistance and susceptibility to *Leishmania* infection has improved by the usage of murine models (Mossman and Moore 1991). T-cell mediated macrophage activation is the major protective mechanism; resistance to infection is associated with a Th1 cytokine profile characterized principally by subsets of $CD4^+$ T cells producing IFN-γ, lL-2, TNF-α and IL-12 production, whereas susceptibility is dependent on Th2 cytokines with secretion of IL-4, IL-5, IL-6 and IL-10 (Kemp et al., 1996, Miralles et al., 1994, Heinzel et al., 1995). Later studies have shown that treating a BALB/C susceptible mouse with anti-IL-4 after infection with *Leishmania* resulted in recovery. Therefore, inhibiting IL-4 leads to clearing of the pathogen. Resistant C57BL/6 mice were treated with anti-IFN-y after infection with *Leishmania* resulted in their death (Sadick *et al.,* 1990), suggesting these cytokine profiles is related to the phenotype of disease expression. Conversely, IL-4 and IL-I0 equip the immune response towards the clearance of extracellular pathogens. Th1 cells control the activation of cytotoxic $CD8^+$ T cells, cytotoxic NK cells and macrophages while Th2 cells are responsible for the activation of eosinophils and the induction of B cells to produce antibody.

There are two subsets of CD8+ T-cells based on the cytokines that it produces. CD8+ cells are not solely the effector cells involved in target cell killing but are also involved in the cytokine control of the immune response. These findings were concluded by various experiments. For example, CBA mice resistant to *L. major* infection were found to have much more CD8+ T cells compared to susceptible BALB/C (Titus et al., 1987). The protective effect could be either due to direct lysis of infected macrophages or by the production of IFN-γ and other cytokines, which would in turn stimulate the macrophages to kill the parasite. The cytokines produced by CD8+ T cells (Tc1 and Tc2) are essentially the same as for Th1 and Th2 cells. As recent reports point out the role of both CD4+ and CD8+ T cells in immunity against the parasite (Liew and O'Donnell 1993). In humans too, levels of IL-10 and IFN-γ were found to be significantly elevated with active VL (Karp *et al.,* 1993, Ghalib *et al.,* 1993, Holaday *et al.,* 1993). Increased IL-10 production is associated with the pathology of VL as it is a potent inhibitor of macrophage activation and IFN-γ secretion in particular (Mosmann and Moore 1991; Howard and O'Gara 1992). It was also seen that at early stages of infection with *L. donovani,* lack of IFN-γ production by PBMC predicts progression of disease while individuals with high quantities of IFN-γ usually remain asymptomatic (Kemp et al., 1996*).* In the recent study they suggest that CD8+ T

cells are driven to anergy/exhaustion with elevated expression of CTLA-4 and PD-1 mRNAs in human VL which is affecting their inherent ability to contribute to protective immune response against parasite (Gautam et al., 2014).

2.3.2.3 Co-stimulatory molecules

In addition to the antigen-specific interaction between antigen presenting cells (APC) and T-cells, the latter require co-stimulatory molecules for activation. Infection of macrophages with *L. donovani* both *in vivo* and *in vitro* failed to trigger expression of CD80 molecule needed for the activation of T cells. Modulation of co-stimulatory signals leads to significant modification of antileishmanial T-cell responses (Kaye, 1995). Both CD80 (B7-1) and CD86 (B7-2) interact with CD28 and with the structural homolog CTLA-4 (CD152) expressed on T-cells. The binding affinity of CTLA-4 to B7 molecules is significantly higher than that of the CD28 molecule. In contrast to CD28, CTLA-4 was suggested to play a role in the negative regulation of T-cell activation (Krummel and Allison, 1995; Walnus et al., 1994). CTLA-4 engagement is likely to play a significant role in the T-cell unresponsiveness, characteristic for chronic visceral *Leishmania* infection.

Early studies established the role CD40: CD40L interactions in effective humoral response, but its importance in cell-mediated immunity is still being explored (Van Kooten and Banchereau, 2000; Banchereau et al., 1994; Danese et al., 2004). Apoptosis regulation in T cells (Blair et al., 2000), upregulation of co-stimulatory molecules like B7-1 and B7-2 in APCs and CD58 in DCs (Caux et al., 1994) is mediated by CD40 ligation and IL-12 production by DCs (Cella et al., 1996) is important during *Leishmania* infection (Heinzel et al., 1993; Sypek et al., 1993). Apart from the production of IL-12, CD40 signaling also mediates the production of IL-10 from the macrophages which favor the parasite persistence (Marovich et al., 2000; Foey et al., 2000). The amount of IL-10 and IL-12 produced by macrophages is dependent on the strength of CD40 as shown previously (Mathur et al., 2004), hence CD40 signaling maintains balance between IL-12 and IL-10 production, which determines the disease resolution or progression in a given infection model (Alexander et al., 1999). IL-10 production and Treg cell development is modulated by CD40 signaling (Martin et al., 2010).

2.3.2.4 Role of nitric oxide

Analysis of murine model has shown that recovery from *Leishmania* infection relies on induction of a Th1 response (Holaday et al., 1991; Belosevic et al., 1989), with production of IFN-γ, which in turn activates macrophages to a microbicidal state. This leads

to intracellular death of the parasite as a result of enhanced expression of nitric oxide synthase and ensuing production of NO (Liew et al., 1991; MacMicking et al., 1997), a highly toxic molecule for *Leishmania* (Mauel et al., 1997; Zangger et al., 2002). The NO-pathway appears to be a common mechanism for *Leishmania* killing since not only murine but also human monocytes/macrophages were able to control *L. major* in an NO-dependent manner (Vouldoukis et al., 1995*)*. NO is formed by the oxidative deamination of the amino acid L-arginine by NOS. There are two isoforms of NO synthase: constitutive and inducible forms. Constitutive NO synthase is found in a variety of cells, but iNOS occurs only in activated macrophages. NO produced by activated macrophages is a potent antimicrobial compound and is able to kill a range of organisms from bacteria to helminth worms by covalently reacting on iron-sulphur clusters in macromolecules. This represses DNA synthesis and inhibits iron-containing enzymes including those involved in respiration. NO can also react to form other reactive free radicals such as $N0^{2-}$ and hydroxyl radical (OR), which in turn might be responsible for killing the parasite (Liew and O'Donnell 1993). While IFN-γ, TNF-α, MIF lead to induction of NOS in infected macrophages, IL-3, IL-4, TGF-β, and IL-I0 inhibit its expression (Liew 1991). NO is, therefore, a key molecule in the immune response, whose production and activity is favored by Th1 responses and inhibited by Th2 responses.

2.4 Th1/Th2 paradigm

Th1 and Th2 cells exist in a feedback relationship with each other. When an antigen stimulates an immune response, it does not activate both the humoral and cell-mediated responses equally. One response usually predominates and influences the potency of the secondary response. In the murine model, the cytokine pattern present in the animal at the time of infection can determine whether a Th1 or a Th2 response will develop. In other words, the polarization of the response towards one or the other T-cell subset is itself conditioned by the early cytokine environment. Studies from human and murine *in vitro* cells hace established the role of certain cytokines, (e.g. IFN-γ, IL-12) which favor the maturation of Th1 responses whereas others (e.g. IL-4, IL-10) support Th2 development. The polarization of Th1 or Th2 subsets in CL takes place very early in infection (3 days in mice). The immune response is arrested in either Th1 or Th2 after its generation, even when it is harmful to the host (Kemp et al., 1996*)*. Th1 cells activate cell-mediated immunity, primarily by inducing macrophages and dendritic cells to secrete IL-12, a potent immune activating cytokine. Th2 cells, on the other hand, secrete immune cytokines that help B-

lymphocytes to synthesize antibodies. Th3 cells are a recently discovered population that secretes transforming growth factor beta (TGF-β), an immune cytokine with both immune activating and inhibiting properties. Both Th1 and Th2 cells can reciprocally inhibit the activity of the other. Th3 cells can inhibit the activity of both cells, although TGF-β is primarily known for inducing the release of IgA (mucosal) antibodies, a Th2 response, and the inhibition of Th1 cell activity (Weiner 2001). TGF -β and IL-10 both deactivate macrophages and inhibit the release of IL-12 (Kemp et al., 1996; Karp et al., 1993). IFN-γ, on the other hand, inhibits the release of IL-10. In humans, the balance of cytokines is also influenced by the regulator, autoantibodies (Bendtzen *et al.,* 1990), steroid, hormones and prostaglandins (Rook et al., 1993). The clear Th1/Th2 pattern of disease development demonstrated for *L. major* and *L. mexicana* has not been observed in VL caused by *L. donovani* in mice and humans (Kaye et al., 1991, Kemp et al., 1996, Miralles et al., 1994). It has been suggested that it is the lack of a Th1 response rather than the presence of a Th2 response, which determines disease susceptibility in VL (Engwerda et al., 1998; Kaye et al., 1991). Several studies have shown that IL-4 can prime for IL-12 production (Cua et al., 1997; D'Andrea et al., 1995; Takenaka et al., 2002; De Waal Malefyt et al., 1993). In addition to its effects on innate IL- 12 production, small amounts of IL-4 may be needed not only to drive Th1 differentiation (Kamogawa et al., 1993, Nakumura et al., 1997) but also to maintain IFN-γ production by activated T cells (Noble et al., 1995; Platzer et al., 1992a). It is also shown that IL-4 plays a crucial role in maintaining IFN-γ production following drug therapy (Alexander et al., 2000). Thus, IL-4 plays no counter protective role in *L. donovani* infection.

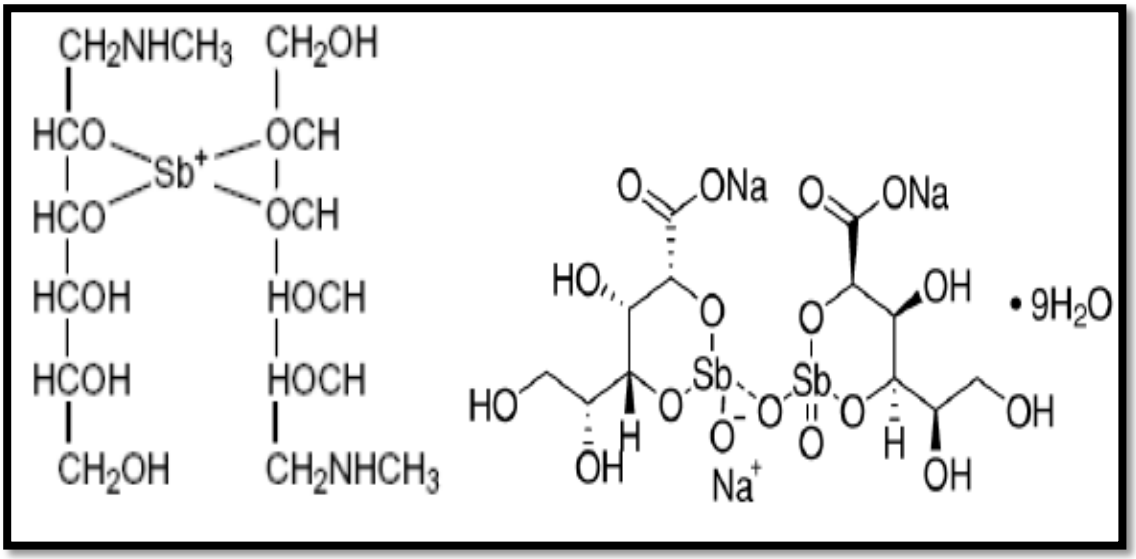
2.5 Chemotherapy against visceral leishmaniasis

Conventional therapies against Leishmaniasis which include pentavalent antimonials, amphotericin B, and pentamidine continue to play an important role, but it is evident that new drugs or strategies must thwart the limitations, such as a long-term usage, high toxicity, high cost and resistance to these drugs. One of the most promising new oral drug miltefosine was approved for treatment of visceral leishmaniasis with only slight adverse effects. The nature of drugs, their mode of action is discussed below.

2.5.1 Pentavalent antimonials

The first line of treatment for VL include N-methylglucamine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) since the 1940s. Antimony still remains as therapeutic regimen in all regions except Bihar State, India, and Southern

Europe. In Bihar only 35% cure rate was observed and in southern Europe secondary resistance developed in patients who had relapse has ended the usage of antimony as first line of treatment (Sundar et al., 2000). Sodium stibogluconate and meglumine antimoniate at 20 mg/kg/day to a maximum of 1275mg over 20 or 30 days were given intramuscularly. The active intracellular reduced trivalent form alterates the parasite bioenergetic pathways and trypanothione inhibition (Ephros et al., 1999; Wyllie et al., 2004). Antimonials have severe side effects like cardiac arrhythmia and acute pancreatitis, which are life threatning. (Chappuis et al., 2007).



A B

Fig 2.1. Chemical structure of Glucantime (A) and Pentostam (B)

Source www.aventis.com and www.sigmaaldrich.com

2.5.2 Pentamidine Isothionate

Pentamidine, an aromatic diamidine is the second line of treatment for VL, but its mode of action on the parasite is unknown. It is predicted that its leishmanicidal activity is through its effect on polyamine biosynthesis and mitochondrial membrane potential since it is a competitive inhibitor of arginine transport and noncompetitively inhibits putrescine and spermidine. In Sb^{v} resistant kala-azar cases in India, Pentamidine was initially proven to be effective (kala-azar unresponsive to antimonial were treated with pentamidine in a dose of 4 mg/kg body weight alternatively for 20 days) but due to high cost and severe toxicity which ranges from irreversible insulin dependent diabetes mellitus, death and it's declining efficacy (as only about 70% patients could be cured), has led to it's being totally abandoned

in India (Sundar & Chatterjee, 2006).

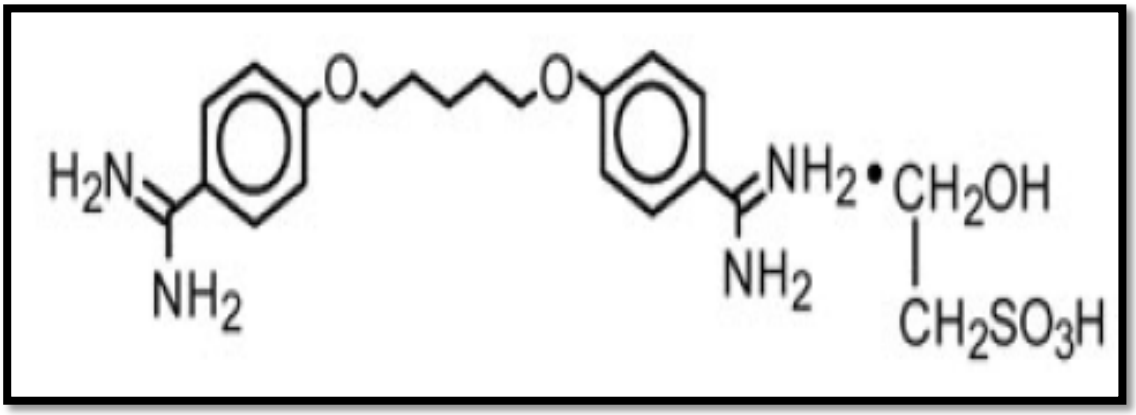


Fig 2.2. Chemical structure of pentamidine isothionate

Source www.scielo.br

2.5.3 Amphotericin-B and its Formulations

Amphotericin B (AmB) is routinely used as a fungal antibiotic. The mode of action is through interaction with ergosterol in the cell membrane, altering the ion balance through pore formation and ultimately cell death (Roberts et al., 2003). *Leishmania* parasites have abundant ergosterol in their cell wall, which makes AmB an extremely effective drug. In the endemic areas in India where resistance to pentavalent antimonials is high, AmB is being used as first line of treatment for VL in spite of its high cost. The recommended dosage consists of 15 doses of 1 mg/kg on alternate days (Pape, 2008). Oral dosage amphotericin B is currently in Phase I trials and also undergoing (animal) efficacy studies for VL (Den Boer et al., 2009). There have been some reports on the emergence of amphotericin B resistance in *L. infantum*/HIV-infected cases in France, as it is prefered choice of drug for treating VL (Di Giorgio et al., 1999). A micellar formulation of AmB showed 100 fold less ED_{50} than the normal AmB formulation against an AmB *L. donovani*-resistant line (Espuelas et al., 2000). The resistance factor cannot be overlooked by the increasing use of amphotericin B in lipid formulations that have longer half-lives. AmBisome® has a longer half life as it accumulates in tissues and is slowly released and excreted (Bekersky et al., 2000). Till date, no cases of no *in vivo* resistance has been reported despite the extensive use in VL. This might be due to the rapid killing of parasites by AmBisome®, and thus they get little opportunity to develop resistance. The main purpose of using AmBisome® is to increase solubility and thermal stability and decrease the systemic toxicity of amphotericin B.

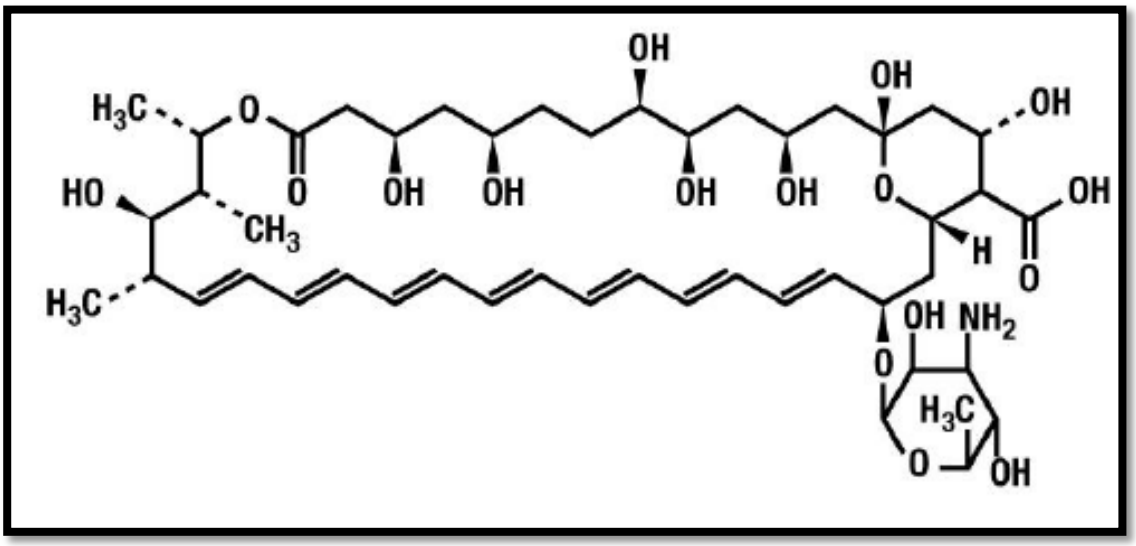


Fig 2.3. Chemical structure of Amphotericin B

Source www.ambisome.com

2.5.3.1 Liposomal Amphotericin B

The development of lipid formulations of AmB (L-AmB) has significantly enhanced the efficacy of AmB. The liposomal amphotericin B formulation, AmBisome®, is registered treatment for visceral leishmaniasis (Meyerhoff, 1999). The advantages like higher cure rate, lesser relapse cases, better convenience to the patients, it has over the conventional AmB and it has increased its efficacy but its use is hindered by high cost in the VL endemic areas. However, the cost has been reduced by now 10% of its original price by WHO under the national elimination program. AmBisome® is given at a dosage of 20mg/kg (total dose) in different parts of the world for treating VL patients but lower doses may be sufficient for the Indian subcontinent. A study from India have showed 91% cure rate when patients were given a single dose of 5 mg/kg of AmBisome® (Den Boer et al., 2009). With the recent decrease in pricing offered by the manufacturers, it is possible that AmBisome® could become economically feasible for treatment, even in resource-poor countries (DNDi Annual report 2007- 2008).

2.5.3.2 Other commercial Amphotericin B

Several other lipid forms such as Abelcet® (an amphotericin B lipid complex) and Amphocil® (amphotericin B colloidal dispersion) have been evaluated against VL at the dosage of 1-5 mg/kg epr day and 1 mg/kg per day respectively. But no studies has compared the efficacy these lipid formulations with AmBisome® in a clinical trial. Berman et al., (1998) also reported the effectiveness of unilamellar liposome formulation of AmBisome®,

against VL in immunocompetent adults and childrens in Europe, Sudan, Kenya, and India. Solid nanoparticles of amphotericin B deoxycholate at a dose of 5mg/kg/day given for 5 days have shown cure rates upto 99% after being injected intraperitoneally into *L. donovani* infected hamsters (Manadhar et al., 2008). Oral administration of novel lipid-based AmB at doses of 10 and 20 mg/kg twice daily for 5 days has reduced *L. donovani* parasite burden by 99.5% and 99.8% respectively in mice models (Wasan et al., 2009). N-(2-hydroxypropyl) methacrylamide- GFLG-amphotericin B copolymer conjugates showed cure rates up to 94% in the liver of *L. donovani* infected BALB/c mice (Nicoletti et al., 2009). This approach was extended to investigate poly (HPMA)-GFLG-amphotericin B-alendronic acid conjugates for the treatment of VL in models (Nicoletti et al., 2010).

2.5.4 Paromomycin

PM or aminosidine is an aminoglycoside is a broad-spectrum antibiotic against bacteria and an oral agent against intestinal protozoa. The first successful usage was in the 1980s against human VL in Kenya (Chunge et al., 1990). This drug went for the development in India, which was sponsored by WHO, but later its has been retracted due to the lack of funding (Thakur et al., 1992; Davidson et al., 2009). After the adoption by iOWH, this drug showed an excellent safety profile in phase III trial in India at a dose of 15 mg/kg intramuscular injections daily for 21 days after the International Dispensary Association (The Netherlands) brought it back into production (Sundar et al., 2007b). PM should be used in combination with other drugs, as the monotherapy in Sudanese VL showed low efficacy rates but compartively the Ethiopian VL responded well. (El-On J et al., 2007; Fong et al., 1994; Maarouf et al., 1998). In cutaneous leishmaniasis, secondary resistance were reported after 60days of PM injections (Teklemariam et al., 1994). PMs efficacy and safety in co-infected patients are still unknown.

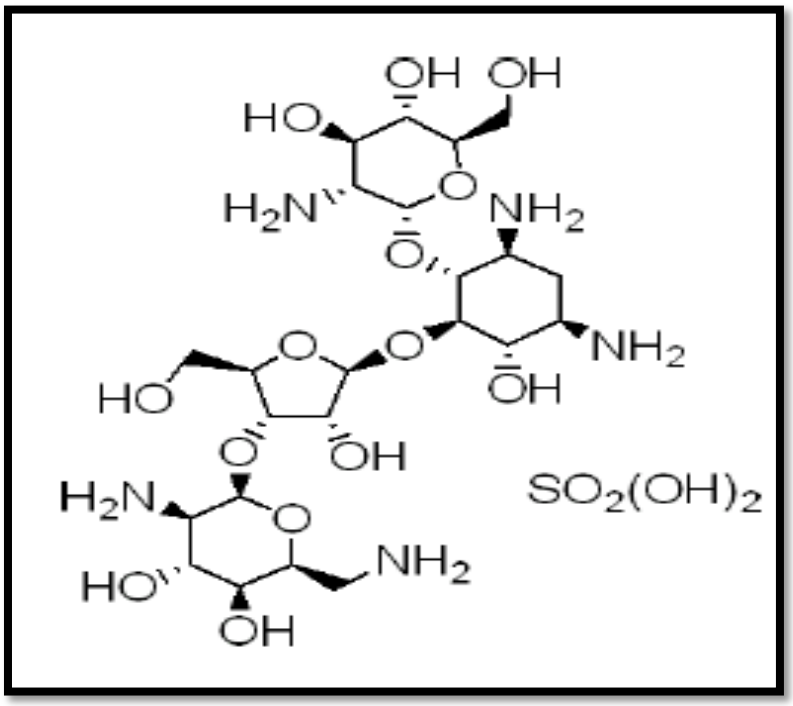


Fig 2.4. Chemical structure of Paromomycin

Source www.sigmaaldrich.com

2.5.5 Miltefosine

Hexadecylphosphocholine (HPC), an analog of phosphatidylcholine (PC) is also called as Miltefosine. In the late 1980s, Croft and their group had demonstrated that miltefosine was effective against *Leishmania* promastigotes from culture, which was initially developed as an anticancer agent. Attention to this compound led to preclinical and clinical studies conducted for leishmaniasis (Croft et al., 1987). In 2002, miltefosine was registered in India for the treatment of VL, in Germany in 2004 and in 2004 in Colombia for both cutaneous and visceral leishmaniasis (Croft et al., 2006a). In 1982, the treatment of VL using miltefosine was started with the focus on the metabolism of phospholipids in *L. donovani* promastigotes (Hermann et al., 1982), where it was concluded that ethers of lysophospholipids (LPAs) completely eliminated the parasites after less than 5 h of exposure to 25 μM. *In vivo* studies in BALB/c mice infected with *L. donovani* and *L. infantum*, oral administration of miltefosine at 20mg/kg bodyweight dosage reduced the parasite burden by 95% (Kuhlencord et al., 1992). The results led to the completion of first phase I/II clinical program in India for VL in 1997. (Sundar et al., 1998; Murray, 2000; Escobar et al., 2001).

In mammalian cells, LPA inhibits phosphocholine biosynthesis by the induction of programmed cell death (Wieder et al., 1999). Miltefosine inhibits alkyl specific acyl-CoA acyltransferase which is an important enzyme for ether lipid remodeling which may affect the cellular growth of *L. mexicana* (Lux et al., 2000). The mode of action of miltefosine in the parasites is through programmed cell death, which explains the selective antiparasitic effects of this compound *in vivo* (Paris et al., 2004).

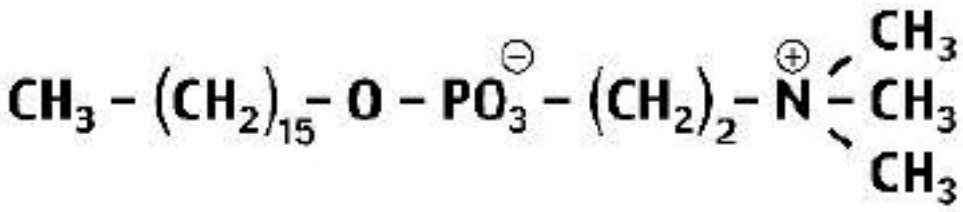


Fig 2.5. Chemical structure of Miltefosine

Source www.mpibpc.mpg.de

2.5.6 Sitamaquine (WR6026)

Sitamaquine (WR6026) belongs to 8-aminoquinoline family, which is currently in clinical trails by Glaxo Smith Kline for oral treatment of VL (Yeates, 2002). Synthetic chemistry department at the Walter Reed Army Institute for Research (WRAIR) discovered Sitamaquine having antileishmanial effects (Tekwani & Walker, 2006). Phase II dose-ranging studies in India and Kenya showed an 83% and 87% cure rates Kenyan patients (Wasunna et al., 2005) and Indian patients respectively after 180 days of treatment. The side effects being abdominal pain and headache in the Kenyan study and vomiting, dyspepsia and cyanosis by the Indian investigators. Methemoglobinemia was only reported in Indian patients (Jha et al., 2005). Cytochrome P-450 metabolizes sitamquine into having desethyl and 4- CH_2OH derivatives, studied using rat and hamster liver microsomes (Theoharides et al., 1985; Yeates, 2002). 8-aminoquinolines metabolism involves important steps such as side chain oxidation and 5-hydroxylation (Idowu et al., 1995; Yeates, 2002). Presystemic elimination of sitamaquine in the liver with low systemic availability was observed in Beagle dogs (Taylor et al., 1991). In humans, the half-life was estimated to be 26.1 hours. Desethyl species being the minor metabolite and 4- CH_2OH derivatives is the major urinary metabolite in humans. (Yeates, 2002). This drug induced morphological changes both in macrophages and *L. tropica* amastigotes (Langreth et al., 1983). The collapse of mitochondrial membrane potential in *L. donovani* promastigotes has also been shown

(Vercesi et al., 1992) as well as alkalization of acidocalcisomes (Vercesi et al., 2000). Recently antileishmanial activity has been demonstrated as unrelated to sitamaquine accumulation in this organelle (Lopez-Martin et al., 2008) The interaction of sitamaquine with membrane lipids of *L. donovani* promastigotes has been assessed and described as a two-step process (Duenas-Romero et al., 2007).

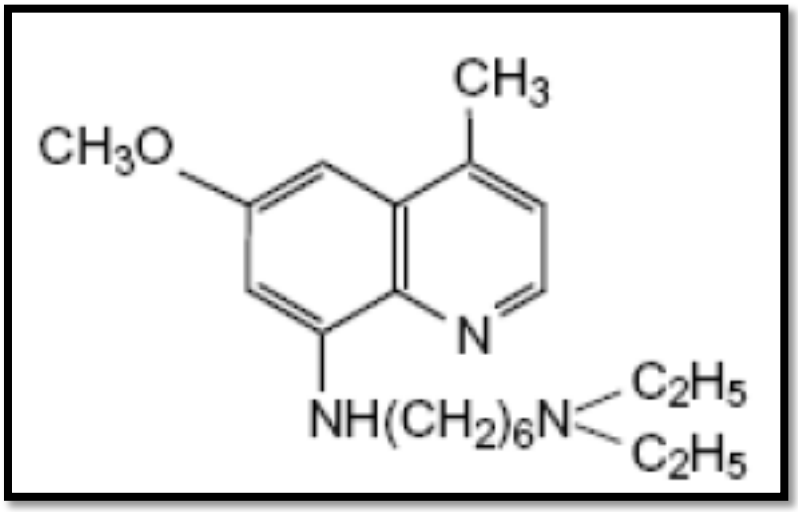


Fig 2.6. Chemical structure of Sitamaquine

Source www.parasite-trends.com

2.6 Plants as a source of drugs for leishmaniasis

In the search for new antiprotozoal drugs, plants provide a repertoire for starting material and most of the biological activity from plants has been attributed to compounds belonging to diverse chemical groups including alkaloids, flavonoids, phenylpropanoids, steroids, and terpenoids (Iwu et al., 1994; Rocha et al., 2005; Wang et al., 2008). Different strategies are employed to obtain a herbal medicine or an isolated active compound, among them, investigation of the traditional use, the chemical composition, the toxicity of the plants, or the combination of several criteria (Rates, 2001). The extraction of the compounds is done using different plant parts and different solvents. There is always scope for the improvement in the extraction methodologies for screening for biological activity of the plant extracts as there exist a wide variety of techniques to prepare extracts (Eloff, 1998; Beutler, 2009). Solvent polarity gradients are widely employed for the plant extractions. The pure compound can be sequentially extracted into fractions from the parent extracts using bioassay guided fractionation, which further will be tested for biological activity and toxicity. This strategy is simple, reproducible, rapid, and low-cost (Beutler, 2009; Sereno et al., 2007).

All the forms of *Leishmania* can be used to screen for biologically active plant substances. *In vitro* grown axenic amastigotes represent a useful model for testing of drugs as these are easy to manipulate and quantify (Sereno et al., 2007; Hodgkinson et al., 1996; Croft et al., 2006a). The methods for quantification of amastigotes in the laboratory include cell counter, a colorimetric method with Alamar blue or acid phosphatase activity, MTT-based method for viability and using a fluorescent dye such as propidium iodide (Sereno et al., 2007; Callahan et al., 1997; Mikus and Steverding, 2000; Ganguly et al., 2006). Interestingly, green fluorescent protein (GFP) or luciferase expressing transgenic *Leishmania* strains has opened up new alternatives for the development of drug-screening tests (Chan et al., 2003; Monte-Alegre et al., 2006). Screening of new antileishmanial drugs in the clinical isolates can be done using colorimetric-lactamase assay (Mandal et al., 2009). **Table 1** compares some antileishmanial activities that have been reported in the last 5 years.

In vitro screenings represent the first step in evaluating the efficacy and safety of medicinal plants for application in the treatment of leishmaniasis. In addition, variation in the effectiveness of drugs in treating leishmaniasis may often result from differences in the drug sensitivity of *Leishmania* species, the immune status of the patient, or the pharmacokinetic properties of the drug (Croft et al., 2006b).

Family/Plant species	Extracts or Compounds	*Leishmania* species	IC_{50}(µg/ml) Pm	Am	References
Aloe nyeriensis	Methanolic extract Aqueous extract	*L. major* *L. major*	68.4 53.3	ND ND	Kigondu et al., 2009
Annona coriacea	Total alkaloids extract	*L. chagasi*	41.6	ND	Tempone et al., 2005
Annona crassiflora	Total alkaloids extract	*L. chagasi*	24.9	ND	Tempone et al., 2005
Annona muricata	Ethyl acetate extract	*L. amazonensis*	25.0	NT	Osorio et al., 2007
Guatteria australis	Total alkaloids extract	*L. chagasi*	37.9	ND	Tempone et al., 2005
Polyalthia suaveolens	Methanolic extract	*L. infantum*	1.8	8.6	Lamidi et al., 2005
Pseudomalmea boyacana	Ethyl acetate extract	*L. amazonensis*	48.9	NT	Osorio et al., 2007
Rollinia exsucca	Hexane extract	*L. amazonensis*	20.8	NT	Osorio et al., 2007
Rollinia pittieri	Hexane extract	*L. amazonensis*	12.6	NT	Osorio et al., 2007
Xylopia aromatica	Methanolic extract	*L. amazonensis*	20.8	NT	Osorio et al., 2007
Himatanthus sucuuba	Ethanolic extract	*L. amazonensis*	20.0	5.0	Castillo et al., 2007
Pagiantha cerifera	Dichloromethane extract	*L. amazonensis*	25.0	12.5	Billo et al., 2005
Achillea millefolium	Essential oil	*L. amazonensis*	7.8	6.5	Santos et al., 2008
Anthemis auriculata	Anthecotulide 4-Hydroxyanthecotulide 4-Acetoxyanthecotulide	*L. donovani* *L. donovani* *L. donovani*	NT NT NT	8.18 3.27 12.5	Karioti et al., 2009
Baccharis dracunculifolia	Crude extract Hautriwaic acid lactone Ursolic acid Uvaol 2a-Hydroxy-ursolic acid	*L. donovani* *L. donovani* *L. donovani* *L. donovani* *L. donovani*	45.0 7.0 3.7 15.0 19.9	NT NT NT NT NT	Silva Filho et al., 2009
Calea montana	Ethanolic extract	*L. amazonensis*	NT	10.0	Valadeau et al., 2009
Elephantopus mollis	Dichloromethane extract	*L. donovani*	NT	0.6	Gachet et al., 2010
Tanacetum parthenium	Plant powder Dichloromethane extract Parthenolide Guaianolide	*L. amazonensis* *L. amazonensis* *L. amazonensis* *L. amazonensis*	490 3.6 0.37 2.6	74.8 2.7 0.81 ND	Tiuman et al., 2005a Tiuman et al., 2005b Silva et al., 2010
Vernonia polyanthes	Methanolic extract	*L. amazonensis*	4.0	NT	Braga et al., 2007
Carica papaya	Ethanolic extract	*L. amazonensis*	NT	11.0	Valadeau et al., 2009
Maytenus putterlickoides	Methanolic extract	*L. major*	60.0	ND	Kigondu et al., 2009
Calophyllum brasiliense	(-) Mammea A/BB	*L. amazonensis*	3.0	0.88	Brenzan et al., 2007
Kalanchoe pinnata	Quercetin diglycoside	*L. amazonensis*	NT	45.0	Muzitano et al., 2006

Acacia tortilis	Aqueous extract	*L. major*	52.9	ND	Kigondu et al., 2009
Albizia coriaria	Aqueous extract	*L. major*	66.7	ND	Kigondu et al., 2009
Copaifera reticulata	Oleoresin	*L. amazonensis*	5.0	15.0	Santos et al., 2008
Laetia procera	Casearlucine A Caseamembrol A Laetiaprocerine A Laetiaprocerine D Butanolide	*L. amazonensis* *L. amazonensis* *L. amazonensis* *L. amazonensis* *L. amazonensis*	11.1 11.0 10.9 50.9 111.0	5.98 10.5 47.4 30.3 129.0	Jullian et al., 2005
Ginkgo biloba	Isoginkgetin	*L. amazonensis*	NT	1.9	Weniger et al., 2006
Scaevola balansae	Dichloromethane extract	*L. amazonensis*	8.7	NT	Desrivot et al., 2007
Hyptis lacustris	Ethanolic extract	*L. amazonensis*	NT	10.0	Valadeau et al., 2009
Ocimum gratissimum	Essential oil Eugenol Methanolic extract	*L. amazonensis* *L. amazonensis* *L. chagasi*	135.0 80.0 71.0	100.0 NT NT	Ueda-Nakamura et al., 2006 Braga et al., 2007
Premna serratifolia	Dichloromethane extract	*L. amazonensis*	4.4	NT	Desrivot et al., 2007
Careya arborea	Arborenin	*L. donovani*	15.0	12.5	Mandal et al., 2006
Asparagus racemosus	Methanolic extract Aqueous extract	*L. major* *L. major*	58.8 56.8	ND ND	Kigondu et al., 2009
Lophanthera lactescens	LLD3	*L. amazonensis*	NT	0.41	Danelli et al., 2009
Dysoxylum binectariferum	Chloroform fraction Rohitukine	*L. donovani* *L. donovani*	50.0 100.0	ND ND	Lakshmi et al., 2007
Cissampelos ovalifolia	Total alkaloids extract	*L. chagasi*	63.9	ND	Tempone et al., 2005
Minquartia guianensis	Dichloromethane extract	*L. donovani*	NT	2.8	Gachet et al., 2010
Bocconia integrifolia	n-Hexane extract Dichloromethane extract Methanol extract	*L. donovani* *L. donovani* *L. donovani*	NT NT NT	1.8 0.5 0.7	Gachet et al., 2010
Piper auritum	Essential oil	*L. donovani*	12.8	22.3	Monzote et al., 2010
Piper dennisii	Ethanolic extract	*L. amazonensis*	NT	10.0	Valadeau et al., 2009
Piper hispidum	Ethanolic extract	*L. amazonensis*	69.0	5.0	Estevez et al., 2007
Piper regnellii	Eupomatenoid-5	*L. amazonensis*	9.0	5.0	Vendrametto et al., 2010
Piper strigosum	Ethanolic extract	*L. amazonensis*	>100	7.8	Estevez et al., 2007
Piper sp	Dichloromethane extract	*L. donovani*	NT	2.2	Gachet et al., 2010
Cymbopogon citratus	Essential oil Citral	*L. amazonensis* *L. amazonensis*	1.7 8.0	3.2 25	Santin et al., 2009
Gouania lupuloides	Dichloromethane extract	*L. donovani* *L. donovani*	NT NT	1.9 2.9	Gachet et al., 2010

Gouania lupuloides	Dichloromethane extract Methanol extract	*L. donovani* *L. donovani*	NT NT	1.9 2.9	Gachet et al., 2010
Galipea panamensis	Coumarin compound 1 Coumarin compound 2 Phebalosin Artifact murralongin Murrangatin acetonide	*L. panamensis* *L. panamensis* *L. panamensis* *L. panamensis* *L. panamensis*	NT NT NT NT NT	9.9 10.5 14.1 >100 NT	Arnago et al., 2010
Scoparia dulcis	Dichloromethane extract	*L. donovani*	NT	1.8	Gachet et al., 2010
Scrophularia cryptophila	Crypthophilic acid A Crypthophilic acid C Harpagide Acetylharpagide Buddlejasaponin III	*L. donovani* *L. donovani* *L. donovani* *L. donovani* *L. donovani*	NT NT NT NT NT	12.8 5.8 2.0 6.9 6.2	Tasdemir et al., 2008
Brugmansia sp	Dichloromethane extract	*L. donovani*	NT	3.0	Gachet et al., 2010
Ferula szowitsiana	Auraptene Umbelliprenin	*L. major* *L. major*	5.1 4.9	NT NT	Iranshahi et al., 2007
Lantana sp	Ethanolic extract	*L. amazonensis*	NT	10.0	Valadeau et al., 2009

NT: Not tested; ND: Not determined; IC_{50}: Concentration in mg/ml that inhibits growth of 50% of the cells; Pm: Promastigote; Am: Amastigote Ref: Tiuman et al., 2011

Table 2.1: Plant crude extracts, fractions, isolated compounds, and essential oils evaluated against the *Leishmania* genus

2.7 Immunomodulators

Immunomodulators are agents that may augment the immune responses or restore the effector mechanism of host leading to the establishment of a balance between therapeutically desirable and undesirable host reactions. Immunomodulators act in a biphasic manner, either by stimulating or inhibiting the immune response. Immunomodulators of microbial origin include methanol extractable residue of BCG, glucose from saccharomyces, lentinan from Basidiomycetes, krestins, and endotoxins. Immunomodulators, which are synthetically originated, involve levamisole, polyribonucleotides, maleic anhydride, vinyl ether and tuftsin. Immunomodulators of cytokine origin include interferons, interleukins, TNF, and GM-CSF. Immunomodulators physiologically originated include perforins, antibodies, complement, etc. Immunorestorative agents possess no direct cytotoxic effects. They restore or stimulate the depressed antigen response, and cellular immune functions e.g. are thymopoietin, thymosin, etc. Immunosuppressive agents include cyclosporine A, Tacrolirnus (FK506), Adrenocortical steroids, cytotoxic drugs and antibody reagents.

2.7.1 Immunomodulators of plant origin

Plant secondary metabolites belonging to different groups like flavonoids, isoflavonoids, alkaloids, etc and various herbal formulations are known to be good immunomodulatory agents in different diseases (Thatte and Dahanukar 1988; Shukla et al., 2011). Plants as a natural reservoir for many compounds have been extensively explored for bioactive leishmanicidal and immunomodulatory compounds. Plant extracts being a storehouse for many biomolecules which can perform the dual action of simultaneously killing the *Leishmania* parasites and also exerting immunostimulatory properties, on otherwise depressed immune system during the diseased state. Immunomodulatory effects of plant extracts or compounds usually have a skewing effect on the immune system from Th2 (diseased state) to Th1 (cure) causing the up or downregulation of proinflammatory and anti-inflammatory cytokines respectively. The immunomodulatory effect in the leishmaniasis is production NO, which being the principle effector molecule in killing *Leishmania* amastigotes.

For example in the case of *Viscum album* the active peptide component is responsible for various immunomodulatory functions like macrophage activation, antibody production, natural killer cell activity and antibody dependent cellular cytotoxicity (Kuttan and Kuttan 1992). Similarly, an extract from the plant *Picrorhiza kurroas* could enhance phagocytosis, lymphocyte proliferation complement, and neutrophil activation. (Thatte and Dahanukar 1986). Polysaccharides and saponin from *Pannx ginseng* are responsible for antibody production, an increase in serum complement content and IgG levels. *Asparagus racemosus, Azadirachta indica, Ocimum sanctum, Tinospora cardiflora* are plants with promising immunomodulatory activity (Thatte and Dahanukar 1988). Curcumin from the plant *Curcuma longa* has been shown to be a good immunostimulant in normal as well as tumor bearing mice.

Ayurveda being a traditional system of medicine from the ancient times (Hersch 1982) lays emphasis on the promotion of health, a concept to strengthening host defense against different diseases. Several plants are labeled as rasayanas such as *Tinospora cardiflora, Asparagus racemosus, Piper longum and Withania somnifera* etc. *Withania somnifera* is the major component of Ashwagandha Rasayana used in cancer therapy. It performs various functions like improving health and used as an immunostimulant. Roots *of Asparagus racemosus* also known as Shatavari is used in the treatment of cancer, dysentery, inflammation and diseases of the eye. *Tinosporn cordifolia* and Ashwagandha Rasayana

have been found to stimulate macrophages as evidenced by an increase in phagocytosis. It was also found to produce leucocytosis with a predominant neutrophilia and prevented varying degrees of leukopenia induced by cyclophosphamide (Thatte and Dahanukar 1988). *Curcumin longa* has been found to stimulate the immune system through the increase in total WBC count. *Piper longum* and *Zingiber officinalis* have been found to effective against gastric ulceration. Oral administration of Brahma rasayana was found to enhance total WBC count, bone marrow cellularity and a-esterase positive cells, as well as cytokines such as IFN-γ, lL-2 and GM-CSF in normal and radiation, treated mice. It is used as an immunostimulant in ayurvedic formulations.

2.8 *Withania somnifera*

In India and Southeast Asian countries, Ayurveda is still prevalent as one of the ancient medicinal systems. This system was in full practice from 6th century B.C. The Ayurvedic texts describe a set of rejuvenating measures to impart biological sustenance to the bodily tissues. These remedies are called `rasayana' which are organ and tissue specific. Those specific to brain tissue are called `medhya rasayana', which retard brain aging and help in regeneration of neural tissues besides producing anti-stress, adaptogenic and memory enhancing effects.

Ashwagandha **(***Withania somnifera* Dunal) (WS) has been safely used for centuries for the treatment of different ailments is one of the examples for medhya rasayana**.** In Sanskrit "ashwa" means horse and "gandha" means odor and the relieving stress avtivity comes from the Latin name "*somnifera"* which means "sleep-inducer" (Singh et al., 2008; Ven Murthy et al., 2010).

W. somnifera is an erect, evergreen shrub with long tuberous roots. Different chemotypes of the herb are widely cultivated in India (Kaul et al., 2005). The fruit is orange colored when ripen, usually has many seeds and also has a membranous covering. Genus *Withania* is readily distinguishable as *Withania somnifera* and *Withania coagulans* (Singh and Kumar, 1998). WS is also known as winter cherry, or Indian ginseng. It belongs to the Solanaceae family, its various parts (berries, leaves, and roots) are being in the folk remedies. Currently, WS isa part of health supplements, tonics, to promote health and quality of life. In modern days, many of the medicinal herbs which are used traditionally have received considerable scientific attention, and WS is one such herb, which is being validated for its traditional medicinal usage (Singh et al., 2010). A total of 62 and 48 major

and minor primary and secondary metabolites has been identified from leaves and roots respectively using metabolic profiling of crude extracts. Structural elucidation of ten withanolides (steroidal lactones) from *Withania somnifera* extract were done by ESI-QqTOF-MS/MS technique (Musharraf et al., 2011).

Fig 2.7: *Withania somnifera* plant with leaves and dried roots (Source: Internet)

Withania somnifera leaves consists of C_{28}-steroidal lactone triterpenoids compounds called as withanolides as the major chemical constituents, having an intact or rearranged ergostane backbone, functionalized at carbons 1, 22 and 26, and the lactone ring is formed from the oxidation of C_{22} and C_{26} (Chatterji et al., 2010; Namdeo et al., 2011; Srivastava et al., 2008; Pretorius et al., 2009). From the roots of WS a bioactive dimeric thiowithanolide, Ashwagandhanolide, was isolated (Subbaraju et al., 2006). Withanolides are synthesized de novo possess various biological and pharmacological activities (Sangwan et al., 2008; Mirjalili et al., 2009; Chen et al., 2011).

The comprehensive report on the diverse activities of WS was published **(**Winters 2006**).** The whole plant possesses anti-inflammatory, immunomodulatory and anti-stress activities. Out of all activities, the anti-tumor activity of WS is most promising. Antitumor mechanisms of WS reveal its potential to upregulate phase II liver enzymes, cell cycle proliferation, tumor apoptosis, inhibition of angiogenesis; NF-KB suppression enhanced the immune system. WS has been used in different forms, such as dried powder of root and leaf. In the majority of studies, water or a solvent derived extract, or one of its withanolide

constituents have been used.

2.8.1 Neuroprotective activity

Neuroprotection is one prominent effects of WS (Kumar and Kumar, 2009; Kumar et al., 2010; Rajasankar et al., 2009; Jeyanthi and Subramanian 2009), which include activities against Parkinson's and Huntington's syndromes (Kumar and Kumar, 2009; Rajasankar et al., 2009; Kasture et al., 2009), and anxiety disorder (Andrade, 2009). The other pharmacological properties include cognitive improvement (Banerjee, 2010), anti-cholinesterase (Vinutha et al., 2007), GABA-mimetic (Bhattarai et al., 2010), anti-fibriloid against β-amyloid (Jayaprakasam et al., 2010), and anticonvulsant (Kulkarini et al., 2008; Akula et al., 2009). WS also has protective role in attenuation of morphine, alcohol and benzodiazepines addiction and in the management of ethanol withdrawal reactions, and in stress induced neurological disorders (Kasture et al., 2009; Gupta and Rana, 2008; Lu et al., 2009; Bhatnagar et al., 2009). WS has sleep-promoting effect in sleep disturbed state with the Involvement of GABAergic mechanism (Kumar and Kalonia, 2008). WS possess antidepressant and mood stabilizer effect (Gupta and Rana, 2007). The anti-stress activity of WS in both acute as well as chronic stress models is well documented (Tuli et al., 2009).

2.8.2 Immunomodulatory activity

WS is also an excellent anti-stress and immunomodulatory agent (Yadav et al., 2010; Patil et al., 2010; Yamada et al., 2011). WS controlled azoxymethane-induced colon cancer by altering the level of leucocytes, lymphocytes, neutrophils, immune complexes and immunoglobulin's (Ig) A, G and M (Muralikrishnan et al., 2010b). The immunologic effects of WS were observed in the expression of CD4 on CD3+ T cells, activation of CD56+ NK cells by increasing the expression of the CD69 receptor, inducing immune cell activation (Mikolai et al., 2009). WS also enhanced total WBC count, inhibited delayed type hypersensitivity reaction and increased phagocytic activity of macrophages (Gupta and Rana, 2007).

Bani et al., (2006) showed potential immunoprotective and immunoadjuvant activities of WS aqueous root extract using flow cytometry. WS upregulated the Th1 response by increasing the number of CD4 and CD8 cells compared to cyclosporin A, with a faster recovery of CD4+ T cells in immune suppressed animals. Cellular and humoral responses were stimulated by the WS extracts which were comparable to levamisole under immune suppressed conditions. This study proved that WS had selective Th1 up-regulating

activity and suggested use for selective Th1/Th2 modulation. Khan et al., (2009) identified WS components which activate the immune system, demonstrating a significant role. *In-vitro* Con-A primed splenocytes treated with WS showed enhanced Th1 cytokine IFN-γ expression. WS caused proliferation of T cells in dose-dependent manner in mice sensitized with ovalbumin with Freund's adjuvant and also increased the secretion of IL-2 and IFN-γ, but had no effect/very less Th2 cytokine IL-4 secretion. Further, WS upregulated the expression of beta-integrins LFA (CD11a) and Mac-1 (CD11b) in immunized mice splenocytes.

2.8.3 Anticancer activity

Anticancer potential of WS has been underrated by numerous studies, but among WS and its constituents the most promising being withaferin A for its anti-tumor properties. WS exhibited growth-inhibitory activity on primary cells from patients with lymphoblastic and myeloid leukemia showing (Mandal et al., 2008a), human lung cancers (NCI-H460) (Choudhary et al., 2010), U937 cells (Oh et al., 2008; Kalthur and Pathirissery, 2010; Mayola et al., 2011), human breast cancer (Stan et al., 2008; Thaiparambil et al., 2011; Hahm et al., 2011; Lee et al., 2010), colon cancer (Koduru et al., 2010) and cervical cancer (Munagala et al., 2011; Pretorius et al., 2009). WS was found to prevent azoxymethane-induced colon cancer in mice (Muralikrishnan et al., 2010; Choi et al., 2011; Yang et al., 2011). WS showed the apoptotic effect on both myeloid and lymphoid cells and also on primary cells derived from leukemia patients (Mandal et al., 2010). Existence of antiproliferative, differentiation-inducing and anti-migratory/anti-metastasis activities has been reported in neural cells (Kataria et al., 2009; Shah et al., 2009) and in cell lines from pancreas (Yu et al., 2010). WS showed its effect on adipocytes by reducing cell viability and induced apoptosis (Park et al., 2008). One report has shown its anti-tumor and anti aging effects (Widodo et al., 2009). WS also affect on benzo(a)pyrene-induced lung cancer in mice (Senthilnathan et al., 2006). WS was active against active against acute lymphoblastic leukemia through L-asparaginase enzyme (Oza et al., 2009; Oza et al., 2010; Mulabagal et al., 2009). It has been speculated that WS or its bioactive constituents kill cancer cells (Widodo et al., 2008).

2.8.4 Other activities

WS is also attributed for its antibacterial, antimalarial, anti-fungal, anti-stress etc (Owais et al., 2005; Heyman et al., 2009; Krishnamurthy et al., 2008; Geeta and Murugan, 2008; Muregi et al., 2007; Kirira et al., 2006; Ali and Meitei, 2011; Nakajima et al., 2011;

Ganesan et al., 2011; Kumar et al., 2011; Malviya et al., 2011; Hahm et al., 2011; Sinha et al., 2011; Modak et al., 2007; Anwer et al., 2008; Udaykumar et al., 2009; Jatwa and Kar, 2009; Udayakumar et al., 2010a; Das et al., 2010) and antileishmanial activities (Pramanick et al., 2008; Sharma et al., 2009).

It also shows effects on male reproductive system (Mahdi et al., 2009; Ahmad et al., 2010; Shukla et al., 2011), and gentamicin-induced nephrotoxicity (Jeyanthi and Subramanian, 2010). Many other effects of WS are also documented (Maitra et al., 2009; Sumantran et al., 2008; Oh and Kwon, 2009a; Pretorius et al., 2009; Pawar *et al.,* 2011; Sandhu et al., 2010; Panjamurthy et al., 2008; Mohanty et al., 2008; Priyandoko et al., 2011).

2.9 Objective of the study

Leishmaniasis is a serious public health issue in parts of India, and the development of resistant parasites to existing drugs has created a need to understand the host-parasite relationship and to develop new combating strategies. Impairment of cell-mediated immunity in *L. donovani* infected macrophages is one of the mechanisms by which parasite evades intracellular killing. Thus, altering the immune status represents an attractive target to restore the macrophage functions. Altering the immune status of the cell using immunomodulators represent a new strategy for treating *Leishmania* infection.

In *L. donovani*, which is the cause of VL in India subcontinent, protection is thought to be T-cell mediated with both CD4+ and CD8+ T cells required to resolve *Leishmania* infections after immunization. Resistance against *Leishmania* infection is dependent on the development of a Th1 response. Thus in order to confer better protection against *L. donovani* infection, the cell-mediated response has to be improved. Th1 immune modulation helps in the resolving the infection by the production of IFN-γ, iNOS, which are main players of resistance to *Leishmania* infection. Immunomodulators modulate the immune response of the host according to the requirement of the cell. Plant-based products are gaining much importance in the present scenario due it's fewer side effects and more potency.

Withania somnifera is a herb, which is widely used by practitioners in the folk medicines as immune boosting agent. Several parts like root, leaves and fruits are commonly utilized in the tonics and health supplements. The various medicinal activities from plants have been attributed due to the presence of steroidal lactones called as

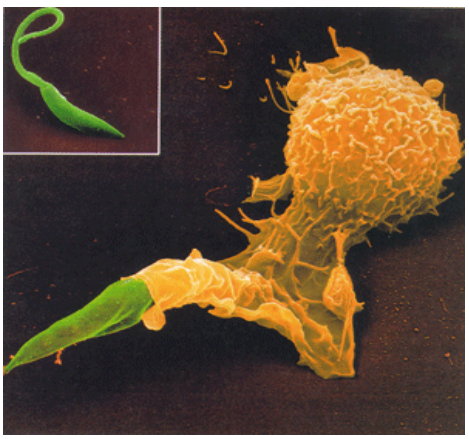
withanolides. Bani et al., (2006) showed potential immunoprotective and immunoadjuvant activities of WS aqueous root extract using flow cytometry. WS upregulated the Th1 response by increasing the number of CD4 and CD8 cells compared to cyclosporin A, with a faster recovery of CD4+ T cells in immune suppressed animals. Cellular and humoral responses were stimulated by the WS extracts which were comparable to levamisole under immune suppressed conditions. This study proved that WS had selective Th1 up-regulating activity and suggested use for selective Th1/Th2 modulation. Khan et al., (2009) identified WS components which activate the immune system, demonstrating a significant role. *In-vitro* Con-A primed splenocytes treated with WS showed enhanced Th1 cytokine IFN-γ expression. WS caused proliferation of T cells in dose-dependent manner in mice sensitized with ovalbumin with Freund's adjuvant and also increased the secretion of IL-2 and IFN-γ, but had no effect/very less Th2 cytokine IL-4 secretion. These results pointed out the Th1 immune modulating capacity of WS leaf extract.

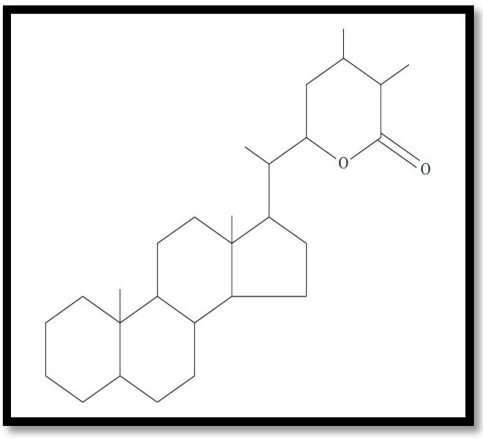
So we postulated our hypothesis by addressing the immunomodulatory activity of WS leaf extract in case of *L. donovani* infection both *in vitro* and *in vivo*. In addition to this we also evaluate the potential of WS leaf extract to possess a potent antileishmanial activity against both the stages of the parasites. By the above literature we framed the following objectives:

1. Isolation and identification of withanolides from *Withania somnifera* leaf extracts and antileishmanial effect *in vitro*.
2. Antileishmanial and immunodulatory effects of withanolides *ex vivo*.
3. *In vivo* antileishmanial and immunomodulatory activities of withanolides.
4. Evaluation of mode of action of withaferin A on *L. donovani* parasites.

Chapter III:

Materials And Methods





3.1 Parasites, cell lines and Animals

L. donovani strain LEM138 (MH0M/IN/0000/DEVI) was kindly provided by Prof. Shyam Sundar, Infectious Disease Research Laboratory, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India, and DD8 (MH0M/IN/80/DD8) was procured from ATCC, USA. J774A.1 mouse macrophages were obtained from National Centre for Cell Science, Pune, India.

Inbred female BALB/C 4-6 weeks old used for the study were obtained from National Centre for Laboratory Animal Sciences, Hyderabad, India and were maintained in animal house of School of Life Sciences, University of Hyderabad, India.

3.2 General chemicals and reagents

All the biochemicals and immunochemical used for the study were of analytical grade with highest purity. RPMI-1640, M-199 media, Penicillin/Streptomycin antibiotic mixture, $CaCl_2$, D-glucose, FBS, sodium bicarbonate, IPTG, Anti-mouse IgG ALP conjugate produced in goat, betaine, Imidazole, BCIP/NBT solution premixed, propidium iodide, 6-biopterin, sodium acetate buffer solution, Withaferin-A, protease cocktail inhibitor were procured from Sigma chemicals, USA. Sodium pyruvate, skimmed milk broth, thioglycollate broth, Tris buffer, glycine, SDS, triton X-100, glycerol, hydroxyurea, NaCl, tween-20, acrylamide, kanamycin sulphate, agar agar, peptone, yeast extract powder, Giemsa stain, ammonium bicarbonate, NADPH, MTT, sodium lauroyl sarcosine, proteinase K, DNase I were procured from Hi-Media, Mumbai, India. Dream Taq polymerase, Phusion high fidelity polymerase, protein molecular weight markers, DNA ladders, ligase, restriction enzymes were procured from Thermo Scientific inc. Ethidium bromide, ponceau S, coomassie Blue G-500, immersion oil for microscopy, β-mercaptoethanol, PMSF, EDTA, TEMED, BSA, bis-acrylamide, tris-saturated phenol, isopropyl alcohol, isobutanol, glacial acetic acid disodium hydrogen orthophosphate anhydrous, sodium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium dihydrogen orthophosphate, bromophenol blue were procured from SRL, India. Plasmid purification kit, PCR, and gel cleanup kit, RNA isolation kits were purchased from Machery-Nagel. DMSO, chloroform, silica gel mesh 100-200 were purchased from Merck (India). JC-1 dye, Annexin-V apoptosis kit was obtained from e-Bioscience, USA. PCR primers were purchased from Integrated DNA Technologies, USA. HPLC grade methanol, ethyl acetate and water, hydrochloric acid, sulphuric acid, paraformaldehyde was purchased from Qualigens. Seakem LE agarose for gel electrophoresis was purchased from Lonza, USA.

pET 28a expression was obtained from Novagen, USA. Ni-sepharose 6 fast flow procured from GE healthcare. Sybr green and cDNA synthesis was procured Takara. H_2DCFDA and anti-his antibody were procured from life technologies, USA. Cell culture flasks, dishes, serological pipettes were procured from Corning India.

3.3. Culture media

Before use, all the media prepared were sterilized and handled under aseptic conditions.

3.3.1 Growth media for *Leishmania donovani* promastigotes

10.6 g of M-199 and 0.35 gm of sodium bicarbonate was dissolved in 800 ml of autoclaved double distilled water. The pH was adjusted to 7.4. The final volume was adjusted to 1liter by adding autoclaved double distilled H_20 and labeled as an incomplete medium. To make 1liter complete medium 150 ml of heat inactivated FBS and 10 ml of penicillin/streptomycin antibiotic mixture was added and the rest of the volume was adjusted with the incomplete medium. The resulting media was then sterilized by filtering it through a membrane filter of 0.22μm pore size and stored at 4^oC prior to use.

3.3.2 Growth media for culturing macrophages

16.4 g of RPMI 1640 and 2 g of $NaHC0_3$ were dissolved in 800 ml of double autoclaved distilled water. The pH of the solution was adjusted to 7.5 and volume was adjusted to 1 liter with double distilled water and labeled as an incomplete medium. To make 1-liter complete medium 100 ml of heat inactivated FBS and 10 ml of penicillin/streptomycin antibiotic mixture was added and the rest of the volume was adjusted with the incomplete medium. The resulting media was then sterilized by filtering it through a membrane filter of 0.22μm pore size and stored at 4^oC prior to use.

3.3.3 Growth media for bacterial culture

3.3.3.1 Luria Bertani broth (LB)

10 g of peptone, 5 g of yeast extract and 5 g of NaCI were dissolved in 800 ml of double distilled water, and the pH was adjusted to 7.4 with 10N NaOH. The volume was adjusted to 1000ml, and the medium was sterilized by autoclaving for 15 min at 15lbs pressure.

3.3.3.2 LB agar plate

To each liter of LB, 15 gm of agar-agar was added and sterilized by autoclaving. The media was cooled to 50°C, kanamycin antibiotic solution at a final concentration of 50μg/ml was added and was poured into 100mm plates (25-30 ml per plate). The plates were kept for

at least 30 min under the laminar flow for solidification and drying (Sambrook, et al., 1989).

3.4 Culturing of *Leishmania*

3.4.1 *In vitro*

Promastigotes were maintained in M-199 with 15% FBS at 25°C. Subculturing was done every third day when the promastigotes attained log phase of growth. The amount of inoculum for DD8 strain of *Leishmania donovani* during sub-culturing varied from 10-20% of the total volume. For various *in vitro* and *in vivo* experiments, late log phase or stationary phase promastigotes were harvested by centrifugation at 5000rpm for 20 min. Determination of the cell number was done by immobiling the promastigotes with 4% formalin and counted in a hemocytometer.

3.4.2 *In vivo*

The virulent strain of *Leishmania* DD8 was maintained in BALB/c mice by passaging every 3-4 weeks. Each mouse received a tail vein dose of 1 x 10^8 promastigotes in phosphate buffered saline (PBS). After 1-month post infection, parasites were isolated from the lesions from the infected spleen of BALB/c mice. The spleen was removed aseptically, hand homogenized under sterile conditions and suspended in M-199 with 10% FBS. This suspension was incubated at 25°C for 48 to 72 h. Freshly transformed promastigotes were checked under the microscope and counted. To remove the splenic debris, the suspension was centrifuged at 100 x g for 10 min at 4°C and the cells were pelleted down at 800 x g for 15 min at 4°C. The pellet was resuspended at a concentration of at a 10^8 cells/ml in PBS (PH 7.4). 100µl of this freshly transformed promastigotes (10^8cells/ml) were injected into the tail vein of 2-4 weeks old mice.

3.5 Maintenance of macrophages

3.5.1 Isolation of mouse peritoneal macrophages (PMM)

Mice were given an intraperitoneal injection of 4% sterile thioglycollate broth before 48hrs of harvesting. After the time interval mice were dissected to open the abdomen without puncturing the peritoneal cavity. Into this cavity 2ml of ice-cold RPMI 1640 media with 10% FBS was injected and massaged for sometime for the cells to detach from peritoneal cavity walls, and then the injected media was collected into a new tube. The cell suspension was centrifuged at 400g for 10min at 4°C, and 10^6 cells were plated in 100mm culture dish and incubated at 37°C and 5% CO_2 for 24hrs for macrophage adhering. These macrophages were used for *L. donovani* infection studies.

3.5.2 Culturing J774A.1 macrophages

The J774A.1 macrophages were maintained at 37°C in RPMI-1640 medium with10% FBS in a CO_2 incubator with 5% CO_2. The macrophages were seeded at a density of 1 x 10^6 cells/plate in tissue culture plates (100 mm or 60 mm) and incubated for 24 h before being used in the infection studies.

3.6 Extraction of withanolides

The *Withania somnifera* plant leaves were collected from Central Institute for Medicinal and Aromatic Plants (CIMAP), Hyderabad, India. The leaves were collected once in the spring season of the year 2010.

3.6.1 Preparation of plant extract

The leaves of *Withania somnifera* under study were collected and shade dried at room temperature for 5-10 days. After shade drying, plant materials were coarsely powdered, separately using a mechanical grinder. All the dried materials were stored at room temperature until required. The powdered plant material (30g) was placed in a Soxhlet apparatus, which is on top of a collecting flask beneath a reflux condenser. The hot extraction was carried out successively with organic solvents from non-polar to polar, which were added to the flask and refluxed. The steam of the solvent, which comes into the contact with material dissolves metabolites and brings them back to the flask. Solvents used were n-hexane, ethyl acetate, ethanol, and water successively. Continuous extraction was carried out for a period of 8-10 h at a temperature not exceeding the boiling point of the solvent with about twenty refluxes or till the solvent in siphon tube of soxhlet apparatus become colourless (Lin et al., 1999)**.** Each time before extracting with the next successive solvent, it was ensured that the plant material being extracted was free from previous organic solvent. The solvents in the round-bottomed flask were collected, filtered using Whatman filter paper (No.1) and concentrated to dryness in a flash evaporator (Buchii, USA) under reduced pressure and controlled temperature [35-40°C]. Water extracts were prepared by soaking 10g powder of respective plant material in 100 mL of distilled water for overnight at room temperature. The extracts were centrifuged at 2000 x g for 10-20 min at room temperature. The supernatant volumes were collected and subsequently concentrated by lyophilisation, and the water was removed by freeze-drying (Tilak et al., 2004). On concentration, it yielded respective solvent extracts and weights of the different extracts obtained were recorded and used for percentage yield calculations. The extracts were named as WSH, WSEt, WSE and WSW for *W. somnifera* hexane, ethyl acetate, ethanol and water extracts respectively.

3.6.2 Column chromatography

Column chromatography was used as a fractionation step for crude extracts, which provided a partial separation of chemical compounds from mixtures of different compounds. A sintered glass column of internal diameter 40 mm and length 100 cm was packed with 200g of silica gel G (100-200 mesh) as stationary phase prepared as a slurry in hexane. The clear solvent from the column was drained after 24h of stabilization of the silica gel. The active crude extracts were adsorbed on silica gel by preparing the slurry in ethanol, and the solvent was recovered under reduced pressure. This slurry was loaded on the stationary phase of the column. Further, varying solvent combinations of increasing polarity were used as the mobile phase for the separation of the fractions from the active crude extracts.

3.6.3 Column chromatography separation of ethanol extract of *W. somnifera*

The WSE crude extract (~5 g) was subjected to column chromatography to separate the extract into its component fractions. The elution was carried out with systems of gradually increasing polarity using hexane (Hex), ethyl acetate (EA) and ethanol (EtOH). The elution process was done using the sequential ratios of solvent combinations; Hex:EA; 100:0, 80:20, 50:50; 0:100 EA:EtoH; 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100 (v/v). A measured volume (500ml) of each solvent combination was collected gradually poured into column each time. Eluents were collected in portions of 50 mL volume were collected in numbered conical flasks. A total of 58 fractions of 25 ml each was collected and all the eluents were pooled into one flask according to their chemical composition detected on pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} plates. The eluents were pooled and excess solvent was evaporated. A total of 7 fractions were obtained and designated as F1, F2, F3, F4, F5, F6, and F7. The fractions were weighed and stored at 4°C.

3.6.4 Analytical thin layer chromatography

The phytochemical profiles of each of the aforesaid fractions were monitored by pre-coated thin layer chromatography (TLC) silica gel 60 F_{254} aluminum strips by spotting 10 µL. The strip was lowered into a small chromatographic jar containing the developing solvent system. The plates were developed with ethyl acetate/ethanol at different ratios to create eluting solvent of various polarities. The combinations within the parenthesis were used:

EA:EtOH (90:10; 80:20;70:30; 60:40 v/v)

The jars were covered with a glass lid to prevent the evaporation of developing solvents. The solvent was allowed to till ¾ of the length of the strip. The strip was removed and dried by a hot air dryer and sprayed with a p-anisaldehyde sulphuric acid solution and heated at 100^{o}C for 5-10 min for visualizing the withanolides in purple color. All the major subfractions obtained were further subjected to MTT assay against the promastigote stage of the parasite. Fractions F5 and F6 were most active against the promastigotes and concentrated stock solution of active fractions was prepared in DMSO and stored at -20^{o}C until required.

3.7 LC-MS/MS analysis of the fractions

High performance liquid chromatography coupled with mass spectrometry (LC-MS/MS) is a key enabling technology for the detection and characterization of metabolites of biological samples, providing the researchers with one of the most powerful analytical tools of modern times (Ho et al., 2003).

3.7.1 Instrument

LC-MS/MS analysis was performed at the Metabolic facility of School of Life Sciences, University of Hyderabad, Hyderabad on 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC equipped with UV–VIS detector. Instrument control, data acquisition and processing were performed using Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent). A Zorbax Eclipse XDB-C 18, 4.6×50 mm, 1.8 µ was used.

3.7.2 Sample preparation

The powdered material of F5 and F6 (1mg) were dissolved in HPLC-grade methanol and filtered with 0.2 µm membrane filters. The obtained extract was used for LC–MS/MS analysis.

3.8. *In vitro* antileishmanial activity

3.8.1 Antileishmanial effects of the fractions using propidium iodide dye

Exponentially growing (1x10^{6} parasites/ml) *L. donovani* promastigotes were treated with F5 and F6 in increasing concentrations for 72 hrs and the percentage of dead parasites was analyzed by PI staining (1µg/ml) by flow cytometry. The percentage of PI-positive parasites gives an indication for cytotoxicity of the above compounds. The IC_{50} concentrations for each fraction were calculated from the graph (Chandrasekaran et al., 2013).

3.8.2 Morphological alterations using scanning electron microscopy

L. donovani parasites were treated with IC_{50} concentrations of F5 (60µg/ml) and F6 (15µg/ml) for different time intervals, i.e., 24, 48 and 72 hrs. Parasites were then harvested and resuspended in phosphate buffer saline (PBS), pH 7.4 and fixed with 2.5% (v/v) glutaraldehyde in PBS for 3 hrs at 4°C. After incubation, the cells were post-fixed in 1% (w/v) osmium tetroxide for 30 min, and then processed in ethanol propylene oxide series. Finally, the samples were point dried by utilizing liquid CO_2 and coated with gold particles. These samples were observed and imaged using scanning electron microscope (Philips XL30 ESEM).

3.8.3 *In situ* visualization of DNA fragmentation

In situ detection of DNA fragments by terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using the DNA fragmentation assay kit (ApoAlert DNA fragmentation assay kit, Clontech). The treated parasite samples were counterstained with 0.5µg/ml PI and visualized under a fluorescence microscope. At least 5 microscopic fields were observed for each sample.

3.8.4 Flow cytometric analysis of sub G_0/G_1 phase of cell cycle

The cell cycle of *L. donovani* parasites was synchronized with 5 mM hydroxyurea for 12 hrs. Thus synchronized parasites were then washed once with PBS and resuspended in media lacking hydroxyurea and were allowed to progress through the cell cycle. Both treated (F5 and F6), and untreated parasites were harvested after different time intervals, washed twice with PBS and fixed in ice-cold 70% ethanol overnight at 4°C. The parasites were again washed twice with PBS and resuspended in 25µl of PI (1mg/ml in PBS) containing RNase A (500µg/ml) and the mixture was incubated for 45 min at 25°C in dark. The fluorescence intensity was analyzed using BD™ FACS Calibur and CellQuest Pro software.

3.8.5 Detection of phosphatidylserine externalization

Double staining for annexin V-fluorescein isothiocyanate (FITC)-PI was performed using annexin-V apoptosis detection kit according to the manufacturer instructions. Briefly, untreated and F5, F6 treated parasites were washed twice with PBS and centrifuged at 2000 x g for 20 min. The pellets were resuspended in 195µl of 1X binding buffer with 5µl of annexin V-FITC and incubated for 10 min at room temperature in the dark. Cells were then washed once with 1X binding buffer and resuspended in 190µl of 1X binding buffer with

10µl of PI. The intensity of annexin V-FITC was analyzed using BD^{TM} FACS Calibur and CellQuest Pro software.

3.8.6 Measurement of reactive oxygen species (ROS)

Cell permeable dye, H_2DCFDA, was used to monitor the ROS levels in the *L. donovani* parasites. Both untreated and F5, F6 treated parasites were washed once with PBS and then incubated with 10µM H_2DCFDA in PBS for 15 min in dark at room temperature. Green fluorescence of H_2DCFDA was measured using flow cytometry and Mean Fluorescence Intensity (MFI) was represented as a bar graph.

3.8.7 Measurement of mitochondrial membrane potential

Ψ_m was estimated using a cell permeable dye, JC-1. The lipophilic dye, JC-1, exists either in a monomeric state or in aggregates depending on Ψ_m of the cell. In case of normal cells, where the mitochondrial membrane is intact with high Ψ_m, JC-1 forms aggregates, which emit red fluorescence at 590 nm. With the onset of apoptosis, the Ψ_m decreases and JC-1 remains as a monomer, which emits green fluorescence at 530 nm. The ratio 590/530 nm represents the relative Ψ_m. Briefly, parasites were harvested after treatment with F5 and F6, resuspended in Hank's balanced salt solution (HBSS) with 10µg JC-1 and incubated for 10 min in dark at 37^{o}C. Cells were analyzed by flow cytometry and MFIs of red and green fluorescence were recorded. The ratio of MFIs was represented as a bar graph, which indicates Ψ_m.

3.8.8 Antileishmanial activity of Withaferin-A

To assess the antileishmanial activity of Withaferin-A against *L. donovani* parasites, MTT assay was performed. Briefly, exponentially growing *L. donovani* ($1x10^6$) parasites were treated with varied concentrations of Withaferin-A (1µM to 10µM) in triplicates for 48 hrs at 25^{o}C. After the incubation the plate was spun at 1000 x g for 5 mins, the media was carefully aspirated and 200µl of new media was added. 20µl of 5mg/ml MTT solution was added to each well and further incubated for 4hrs at 37^{o}C with 5% CO_2. Then the plate was spun after the incubation of 4hrs at 1000 x g for 5mins, and the media was carefully aspirated and discarded. To this 100µl of DMSO was added to dissolve the purple formazan crystals and absorbance was taken at 540nm. The percentage viability was calculated using the formula:

Absorbance of treated/absorbance of control x 100

3.9 Cytotoxicity assay of the withanolides on mouse peritoneal macrophages

The toxic effects of the F5 and F6 fractions on PMM were evaluated using MTT assay. Briefly, $1x10^5$ macrophages isolated from mouse peritoneal cavity was seeded in 200µl volume in 96 well plates for overnight for macrophage adhering and the remaining cells were washed twice with 1x PBS. The withanolides (F5, F6 and Withaferin-A) were added in different concentrations, and the plate was incubated for 72hrs at 37°C with 5% CO_2. After the incubation the plate was spun at 1000g for 5 mins, the media was carefully aspirated and 200µl of new media was added. 20µl of 5mg/ml MTT solution was added to each well and further incubated for 4hrs at 37°C with 5% CO_2. Then the plate was spun after the incubation of 4hrs at 1000g for 5mins and the media was carefully aspirated and discarded. To this 100µl of DMSO was added to dissolve the purple formazan crystals and absorbance was taken at 540nm. The percentage viability was calculated using the formula:

Absorbance of treated/absorbance of control x 100

3.10 *Ex vivo* infection of peritoneal macrophages

Ex vivo assessment of antileishmanial activity was done with PMM. After 24hrs of incubation, unadhered cells were washed, and parasites were added at 1:10 ratio. Infection was done for 16hrs, and uningested parasites were washed three times with serum-free RPMI1640 medium. F5 (15µg/ml), F6 (10µg/ml) and Withaferin-A was added at different concentrations (0.5, 1.0 and 1.5µM) and incubated for additional 72 hrs. The coverslips were removed, fixed with methanol and Giemsa staining was done after 72 hrs of incubation. The amastigotes were counted per 100 macrophages and compared with control macrophages.

3.11 Nitric oxide assay

Peritoneal macrophages were isolated from peritoneal cavity lavage and seeded in 96 well plates ($5x10^5$) and allowed for 24hrs for macrophage adhering at 37°C at 5% CO2. The incubation was followed by treatment with F5 (15µg/ml), F6 (10µg/ml), Withaferin-A (1.5µM) and LPS (1 µg/ml) and incubated at 37°C for another 48hrs. The supernatants were collected for nitrite concentration after 48hrs and were assayed using an automated procedure based on Griess reagent as described by (Stuehr and Nathan 1989). In brief, 100µl of culture supernatants were mixed with 100µl of Griess reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at

room temperature for 30 min. The absorbance was measured at 540nm (Green et al., 1982). These results are representative of triplicate samples. $NaNO_2$ was used as standard to determine nitrite levels.

3.12 Estimation of ROS in peritoneal macrophages

PMM were treated with F5 (15μg/ml), F6 (10μg/ml), Withaferin-A (1.5μM), miltefosine (3.2μM) and LPS (100ng/ml) and incubated at 37°C for 6hrs. They were washed in PBS once and transferred to FACS tube. Cells were incubated with H_2DCFDA at a final concentration of 10μM for 15 min at room temperature, washed once with PBS, acquired and analysed by measuring their mean fluorescence intensity on BD LSR fortessa flow cytometer.

3.13 Real-time PCR

3.13.1 RNA isolation

Total RNA was extracted from $5x10^6$ peritoneal macrophages and 25mg of spleen tissue using a Nucleospin RNA extraction kit (Machery-Nagel) following the instructions of the supplier, and the total RNA content was measured in a spectrophotometer at 260 nm.

3.13.2 cDNA preparation

900ng of total RNA was then transformed into cDNA by reverse transcription using a First Strand cDNA synthesis kit (Takara). The cDNA samples were stored at -80°C until use.

3.13.3 Program and data analysis

The resulting cDNA was then used for real-time PCR for iNOS and mouse cytokines (IL-12, TNF-α, IL-10, IFN-γ, IL-4 and TGF-β) using an ABI 7500 real-time PCR system (Applied Biosystems, UK) with the DNA binding SYBR green dye. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference. The forward and reverse specific primer sequences used were as follows. iNOS, forward 5'-CAGCTGGGCTGTACAAACCTT-3' and reverse 5'-CATTGGAAGTGAAGCGTTTCG-3'; IL-10, forward 5'-GGTTGCCAAGCCTTATCGGA-3' and reverse 5'-ACCTGCTCCACTGCCTTGCT-3'; IL-12p40, forward 5'-GGAAGCACGGCAGCAGAATA-3' and reverse 5'-AACTTGAGGGAGAAGTAGGAATCG-3'; TNF-a, forward 5'-CATCTTCTCAAAATTCGAGTGACAA-3'; and reverse 5'-TGGGAGTAGACAAGGTACAACCC-3'; IFN-γ, forward 5'-TCAAGTGGCATAGATGTGGAAGAA-3' and reverse 5'-

TGGCTCTGCAGGATTTTCATG-3'; IL-4, forward 5'-ACAGGAGAAGGGACGCCAT-3' and reverse 5'-GAAGCCCTACAGACGAGCTCA-3'; TGF-b, forward 5'-TGACGTCACTGGAGTTGTACGG-3' and reverse 5'-GGTTCATGTCATGGATGGTGC-3'; and GAPDH, forward 5'-CAAGGCTGTGGGCAAGGTCA-3' and reverse 5'-AGGTGGAAGAGTGGGAGTTGCTG-3'. For real-time quantitative PCR, each reaction contained 1X SYBR Green PCR master mixture (SYBR Green PCR Master Mix; Takara), 10 pmol of each primer and 1.0μl of cDNA in a final volume of 20μl. The reaction conditions used were: initial activation step (10 mins at 94^{o}C) and cycling step (denaturation for 15s at 94^{o}C, annealing for 1min at 60^{o}C for 40 cycles) followed by melt curve analysis. Detection of the dequenched probe, calculation of threshold cycles (Ct values) and further analysis of these data were performed by the Sequence Detector software. Relative changes in iNOS and cytokine (IL-12, TNF-α, IL-10, IFN-γ, IL-4 and TGF-β) mRNA expression were compared with unstimulated control, normalized to GAPDH and were quantified by the $\Delta 2^{-ddCt}$ method. Thus, all the values for experimental samples were expressed as fold differences between the sample mRNA and the calibrator (GAPDH) mRNA. The data are represented as the means ± SD of data from three independent experiments that yielded similar results.

3.14 Assay of IFN-γ and TNF-α using cytometric bead array

To evaluate the immune alterations, we have estimated the amount of cytokines in culture supernatants of peritoneal macrophages. Briefly, after the various treatments the culture supernatants were used to measure the amount of IFN-γ and TNF-α using mouse Th1/Th2 cytometric bead array (BD Biosciences) in BD LSR fortessa flow cytometer. The amount of cytokines was represented as pg/ml by comparing with the standards of the respective cytokines.

3.15 Cloning, expression and purification of *L. donovani* PTR1 gene.

3.15.1 PCR amplification of PTR1

PCR amplification was carried out using Phusion high fidelity Taq (Thermo Scientific). Reactions were carried out in a Veriti 96 well thermal cycler, applied biosystems with nuclear DNA as a template. The primers was designed based on the published *L. donovani* PTR1 sequence (GenBank Accession No. XM_003860884) with EcoRI and XhoI restriction sites at the 5′end of the forward and reverse primers, respectively. Primers included: PTRF 5'-ATTGGAATTCATGGCTGCTCCGACC-3' and PTRR 5'-AATTCTCGAGTCAGGCCCGGGTAA-3'. The PTR1 coding region was amplified using

the genomic DNA. The PCR mix contained 0.5 µg of DNA, 0.1mM dNTP, 10 picomoles each of PTR1 specific primers, 1 unit of Taq DNA polymerase in a final volume of 25µl. The PCR reaction was carried out using 35 cycles of 10s at 98^{o}C, 30s at 56^{o}C, and 30s at 72^{o}C. The reaction solution was incubated for 30s at 98^{o}C and 7min at 72^{o}C before and after PCR cycling, respectively. The PCR product was separated using a 1% agarose gel and then stained with ethidium bromide. The PCR product was purified using nucleospin PCR clean up/Gel extraction kit (Machery-Nagel) as per manufacturer's instructions. The purified PCR products were then used for ligation in pET28a+ cloning/expression vector (Novagen).

3.15.2 Ligation

The PCR amplified 867 bps product using primers having EcoRI and Xhol sites was cloned into pET28a+ vector. Initially, the PCR product was double-digested with the above enzymes for 4hrs at 37^{o}C. The reaction mixture of the 10µl final volume contained: 2X Ligation Buffer, pET28a+ vector, Insert DNA (PTR1 PCR product) T4 DNA ligase and MilliQ The reaction was carried out at 22°C for 16hrs.

3.15.3 Preparation of Competent cells

Competent cells (DH5α and BL21DE3) were prepared for the transformation of the desired plasmid. A single colony was inoculated into 5 ml LB medium and grown overnight at 37^{o}C with vigorous shaking in the absence of antibiotics. 50 ml of fresh LB medium without antibiotics was inoculated with 200µl of overnight grown culture and incubated at 37^{o}C till the $O.D_{600}$ of the culture reaches to 0.4-0.5. The culture was kept on ice to arrest the growth, harvested at 2000 x g for 5 min at 4^{o}C. Bacterial pellet thus obtained was thoroughly suspended in 20ml of chilled 0.1M $CaCl_2$ with 15% glycerol solution and kept on ice for 20 min. The cells were centrifuged at 2000 x g for 5 min at 4^{o}C. Obtained pellet was resuspended in 2ml of chilled 0.1M $CaCl_2$ with 15% glycerol solution. The resulting competent cells were dispensed in 100µl aliquots and stored at -80°C until further use.

3.15.4 Determination of transformation efficiency

10ng of vector plasmid (e.g., pET28a) was added to 100 µl of competent cells and heat shock was given at 42^{o}C for 90s to which 400µl of LB broth was added. Different aliquots of LB competent cell mix were taken and streaked onto LB-agar plates containing kanamycin. The transformation efficiency was determined by a number of transformants obtained/µg of DNA used. Usually, ~10^7 transformants/µg of DNA is considered as a good transformation efficiency.

3.15.5 Transformation of ligated product

For transformation, an aliquot of frozen competent cells (100µl) was thawed on ice for 10min, mixed with 20ng of plasmid DNA and kept on ice for 30min. These cells were then given a heat shock at 42^{o}C for 90s. The tubes were then quickly transferred on ice for 5 min. Finally, 600µl of LB medium was added to the reaction tube followed by incubation at 37^{o}C for 1hr with gentle shaking to allow the bacteria to express the antibiotic resistance marker. The transformed cells were plated on LB plates containing the kanamycin antibiotic.

3.15.6 Plasmid preparation and restriction digestion

Into 5ml of LB medium, selected clones were inoculated and allowed to grow in the presence of 50µg/ml kanamycin at 37^{o}C, 250 rpm, for overnight. Plasmid isolation was done using nucleospin plasmid isolation kit (Machery-Nagel) according to the manufacturer's instructions. The plasmid DNA was checked for quality and quantity on 1 % agarose gel electrophoresis. Around 1µg of plasmid DNA was digested with 1 unit of EcoRI and XhoI restriction enzymes in a final volume of 20µl at 37^{o}C for 4 hrs. Digested samples were checked on 1 % agarose gel. The tested positive plasmids were mass prepared by the nucleospin plasmid isolation kit (as per the manufactures protocol).

3.15.7 Gene sequencing

The cloned gene in the plasmid was then sequenced to match the identity with *L. donovani* PTR 1 sequenced using T7 promoter primers.

3.15.8 Recombinant protein expression

The *E. coli* strain BL21 (DE3) was transformed with PTR1+pET28a+ plasmid and selected on LB agar plate containing 50µg/ml of kanamycin. The transformed culture was inoculated into 5 ml test tube and allowed to grow at 37^{o}C in a shaker at 220 rpm. Cultures in logarithmic phase (at OD_{600} of ~0.5-0.6) were induced for different time intervals with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, cells were lysed in 2x sample buffer (100 mM Tris-HCl pH8, 20% glycerol, 4% SDS, 2% 2-ME 0.2% bromophenol blue) and analyzed by 12% SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250. Uninduced control culture was analyzed in parallel.

3.15.9 SDS–PAGE and Western blotting

Uninduced and induced protein samples were separated by 12% SDS–PAGE and transferred to a nitrocellulose membrane. The nitrocellulose membrane (Pall life sciences) was blocked in blocking buffer (1X TBS, 5% (w/v) skimmed milk) for 2h at room temperature. The blot was washed three times with 1X TBS-T (TBS having 0.05% tween

20) for 10 min each and incubated for 16h at 4°C with the anti-his antibody (1:2000) in 1X TBS-T. Later, the blot was again washed three times with 1X TBS-T (10 min each) and incubated for 1h with secondary antibody conjugated to ALP (Rabbit anti-mouse IgG (Sigma), 1:10,000) diluted in 1X TBS-T followed by three-time washing with 1X TBS-T. Bands were visualized by using BCPIP/NBT pre-mixed (Sigma) as a substrate.

3.15.10 Determination of protein solubility

To test the solubility of the recombinant protein, log phase culture was induced with 1.0 mM IPTG for 4 hrs at 37°C. The cells were harvested by centrifugation at 3500 x g for 10 min at 4°C. The cell pellet was resuspended in 5 ml of lysis buffer (50 mM Tris pH8.0, 0.5M NaCl, 5mM β-mercaptoethanol, 1% Triton X-100, 10% glycerol). The cell suspension was sonicated (5x30 sec) with 40% duty cycle on the ice. The resulting cell lysate was centrifuged at 8500 x g for 20 min at 4°C. The clear supernatant (soluble fraction) was collected and remaining pellet (insoluble fraction), after centrifugation, which contains inclusion bodies, was also resuspended in 1 ml of lysis buffer. Soluble and insoluble fractions were analyzed on 12% SDS-PAGE gel.

3.15.11 Protein purification

Colonies from LB agar plates were used for preparing the pre-inoculum in LB media containing 50μg/ml kanamycin. The pre-inoculum (~1 %) was used to grow 1 litre culture of cells in LB media with 50μg/ml kanamycin at 37°C to OD_{600} of 0.6-0.8, followed by IPTG induction (final concentration 1 mM) for 4hrs at 37°C. The cells were harvested by centrifugation at 3500 x g for 10 min at 4°C. The cell pellet was resuspended in 5ml lysis buffer containing protease inhibitor cocktail. The cell suspension was sonicated (5x30 sec) with 40% duty cycle on the ice. The resulting cell lysate was centrifuged at 8500 x g for 20 min at 4°C and supernatant was stored at -20°C till protein purification The clear supernatant was collected and used for protein purification. The recombinant protein was purified based on its N-terminal His_6 tag by affinity chromatography using a Ni-sepharose fast flow beads (GE healthcare) with imidazole gradient.

3.15.12 Protein estimation

Protein concentration was measured by Bradford assay (Bradford, 1976). 100μl of standards of bovine serum albumin (ranging from 2-10μg) and samples were taken and 900μl of Bradford reagent was added to each. Bradford reagent was prepared by mixing 100 mg of coomassie brilliant blue G-250 in 50 ml of 95% ethanol, to which 100 ml of 85% phosphoric acid was mixed, and the volume was made up to one litre with double distilled

water. After mixing the Bradford reagent to the samples and standards, the tubes were kept at room temperature for 5 min and the absorbance was read at 595 nm. The standard curve was plotted, and the protein concentration of the samples was computed (Murray, 1990).

3.16 Docking Analysis

3.16.1 Software

Python 2.7, Cygwin c:\program, Python 2.5, Molecular graphics laboratory (MGL), AutoDock4.2 and Discovery studio visualizer 2.5.5 was retrieved from online sources. The docking of Withaferin-A into the binding site of the Pteridine reductase 1 (PTR1) protein was explored using Autodock software. The docking accuracy of Withaferin-A into the binding site was validated using molecular modeling programs.

3.16.2 Protein Preparation for Docking

The 3D structure of *L. donovani* PTR1 (PDB ID: 2XOX) was retrieved from Protein Data Bank (PDB) (http: //www.pdb.org/pdb/home/home.do). Before the initiation of docking simulations, all non-protein molecules were removed from PTR1. Using Autodock 4.0 all the docking calculations were performed. PTR1 docking was done by adding polar hydrogen's and by keeping rigid throughout the process, whereas all the torsional bonds of ligands were set free by ligand module in Autodock Tools.

3.16.3 Ligand Preparation for Docking

The ligand Withaferin-A was built using Chemsketch and optimized using "Prepare Ligands" in the AutoDock 4.2 for docking studies. The conformation of binding site was constructed manually to accommodate Withaferin-A. The validation accuracy was done using docking of Withaferin-A into the binding site of PTR1. AutoDock binding affinities of the Withaferin-A into PTR1 and the binding free energies (kcal/ mol), inhibition constants (Ki), hydrogen bonds, and RMSD values were calculated.

3.17 PTR1 enzyme activity

3.17.1 *In vitro* inhibition assay of recombinant enzyme (*Ld*PTR1)

Reductase activity (*Ld*PTR1) was assayed in 1ml quartz cuvettes, and the volume of each reaction was 500µl. The reaction was started with the addition of substrate after incubation at 30^{o}C for 45mins at time zero, and the rate were measured for 1min intervals for 3 min. At 340nm, NADPH oxidation was monitored. *Ld*PTR1 inhibition was performed at 30^{o}C in the presence of NADPH (100µM) and several fixed concentrations (0 to 120µM) of substrate biopterin at pH 4.8 in 20 mM sodium acetate buffer (Sigma). Pteridines exhibit

absorbance changes when reduced and the extinction coefficient 7,230 $M^{-1}cm^{-1}$ was used for the coupled oxidation/reduction of NADPH/biopterin (Bello et al., 1994). The kinetic parameters *Km* and *V*max for the Pteridine substrates were evaluated by fitting the Michaelis-Menten equation. For inhibition studies, recombinant *Ld*PTR1 was incubated with Withaferin-A, and NADPH and substrate biopterin addition was used to initiate the reaction. Lineweaver-Burk plots were used to determine the mode of inhibition by the inhibitor, examining ligand competition under conditions where [inhibitor] << [enzyme] and [biopterin].

3.17.2 PTR1 enzyme assay in treated crude lysates of *L. donovani* parasites

L. donovani promastigotes ($1x10^6$) were treated with Withaferin-A at IC_{50} concentration for different time intervals and total protein was isolated from the parasites. Briefly, the parasites were resuspended in 180µl of milliQ water and six freeze-thaw cycles were repeated at -80°C. Further to this lysate, 20µl of proteinase inhibitor cocktail (1X) was added and sonication at 20% amplitude was performed. The total protein concentration was estimated using Bradford method. The PTR1 enzyme assay was performed as described above.

3.18 *In vivo* studies

3.18.1 BALB/C mice infection with *L. donovani* and treatment

Female BALB/c mice (20–30 g) were injected via the tail vein with $1x10^8$ *L. donovani* stationary phase promastigotes. F5 (25, 50 mg/kg/day, for 10 days), F6 (25, 50 mg/kg/day, for 10 days), Miltefosine (5mg/kg/day for 10 days) was administered orally, Withaferin-A (2mg/kg/day for 10 days) was administered intraperitoneally starting after 28days of infection. Removing liver and spleen from 6-week post infection/ 2-weeks post treatment mice were assessed for visceral infection using multiple impression smears and stained with Giemsa. Blood was collected to separate the serum and stored at -80°C.

3.18.2 Preparation of soluble antigen from promastigotes

L donovani promastigotes strain DD8 (MH0M/IN/80/DD8) were harvested and washed thrice with PBS, pH 7.2 at 4°C followed by six sonications at 40% amplitude for 30s each with 45s interval using an ultrasonicator. The lysate was centrifuged at 400 x g at 4°C for 5 min. The supernatant was collected, and centrifuged at 8500 x g at 4°C for 20mins. The supernatant was collected and the protein was estimated by Bradford method as described above. This soluble leishmanial antigen (SLA) was used in ELISA and splenocyte proliferation assay.

3.18.3 Determining organ parasite load

After 6 weeks of infection and 2 weeks of treatment post infection, mice were sacrificed and liver and spleen touch biopsies were microscopically examined after fixing and staining the slides with Giemsa. In order to quantitate levels of infection, Leishman-Donovan units (LDU) were calculated as: (number of amastigotes/number of organ nuclei) X weight of organ in grams (Das et.al 2001).

3.18.4 Splenocyte proliferation assay

Spleens were aspectically removed from mice in a sterile dish containing RPMI 1640 media. Single cell suspensions were prepared by grinding the spleen with disk bottom of the plunger of 10 ml syringe. 5-10 ml of RPMI 1640 media was added to it, and the dish was kept undisturbed for two minutes and the clear supernatant was pipetted out slowly. Cells were pelleted by centrifugation at 4°C at 400 x g for 10 min. The pellet containing erythrocytes and splenocytes were collected. The RBC was lysed using 1x erythrocyte lysis buffer. The remaining cells were resuspended to a density of 2.5 x 10^6 cells/ml in RPMI 1640 containing 10% FBS and 0.05mM 2-mercaptoethanol. After addition of SA (50μg/ml) and ConA (10μg/ml) the plates were incubated for 72hrs for the cytokine secretion. The supernatants were collected and stored at -80^{o}C till further analysis.

3.18.5 ELISA for determining the IgG levels

The level of IgG antibody and its isotypes in sera samples of mice of different experimental groups was measured (Samant et.al 2009). Briefly, 96-well ELISA plates were coated with SLD (0.2μg/100μl/well) overnight at 4^{o}C and blocked with 1.5% bovine serum albumin at room temperature for 2hrs. Mouse sera were used as primary antibody at a dilution of 1:100 for IgG1 and IgG2a and kept for 1hr at 37^{o}C. HRP conjugated rat anti-mouse IgG1 and IgG2a were added at a dilution of 1:10,000 for 1hr at 37^{o}C (AbCam) for 1hr. Finally, the substrate TMB/H_2O_2 was added, kept in dark for 30mins and 50μl stop solution was added and the plate was read at 450 nm.

3.18.6 Histopathological examination

Livers from control and treated groups were isolated and fixed in 10% formal saline. Wax blocks were made, and sections were stained with hematoxylin/eosin stain to study the histology.

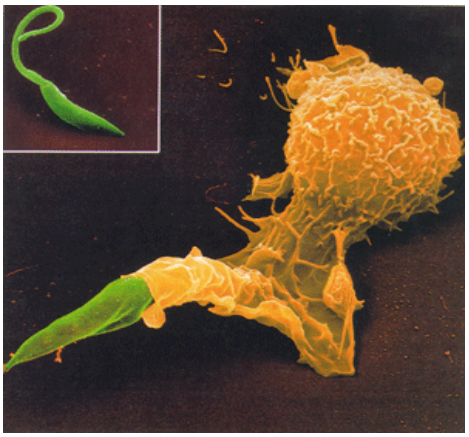
3.18.7 Real-time PCR analysis of cytokines genes from spleen tissue

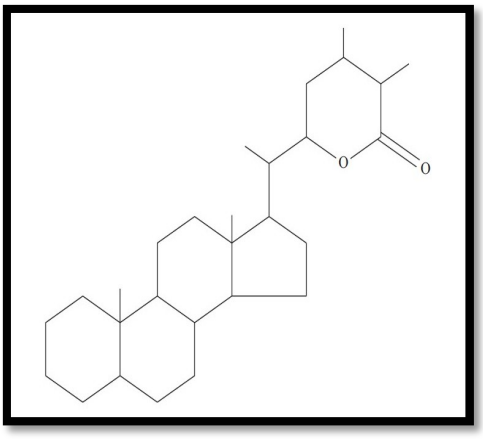
Quantitative real-time PCR (qRT-PCR) was performed to assess the expression of mRNAs for various cytokines and iNOS in splenic cells. Total RNA from splenic tissues

was isolated using Nucleospin RNA kit (Machery-Nagel) and cDNA was synthesized using a first-strand cDNA synthesis kit (Takara). The real-time PCR analysis was carried as described above.

Chapter IV:

Results





4.1 Elucidation of withanolides

The withanolides from *W. somnifera* leaves were extracted using soxhlet method (**Figure 4.1 A**), which utilizes the sequential extractions of the compounds using different polar solvents. For the extraction of withanolides, we used solvents namely n-Hexane, ethyl acetate, ethanol and water in the increasing order of their polarity (**Figure 4.1 B**). We found that there was the absence of withanolides in all the extracts except ethanolic extract as previously reported (Khan et al., 2009). We determined the antileishmanial effect of all these extracts on the promastigote stage of the parasite using MTT assay and found that only ethanolic extract was showing profound inhibitory activity with half maximal inhibitory concentration (IC_{50}) in the range of 100-200µg/ml (**Table 4.1**). We further enhanced the antileishmanial activity of this extract by bioassay-guided fractionation using silica gel chromatography.

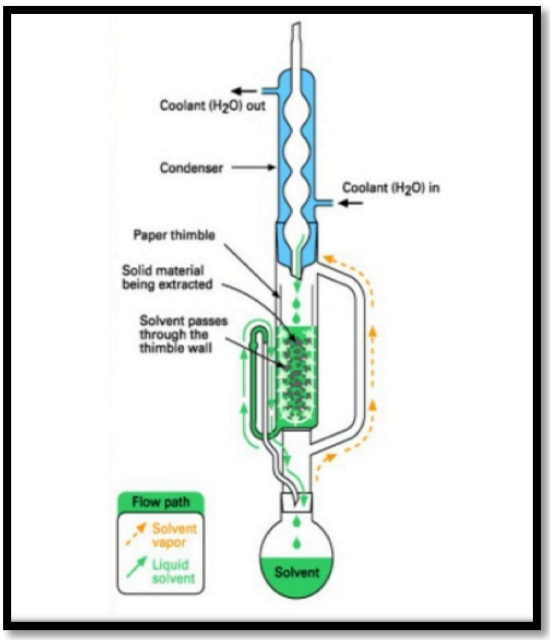


Figure 4.1 (A): Pictorial representation of soxhlet apparatus used for the extraction procedure

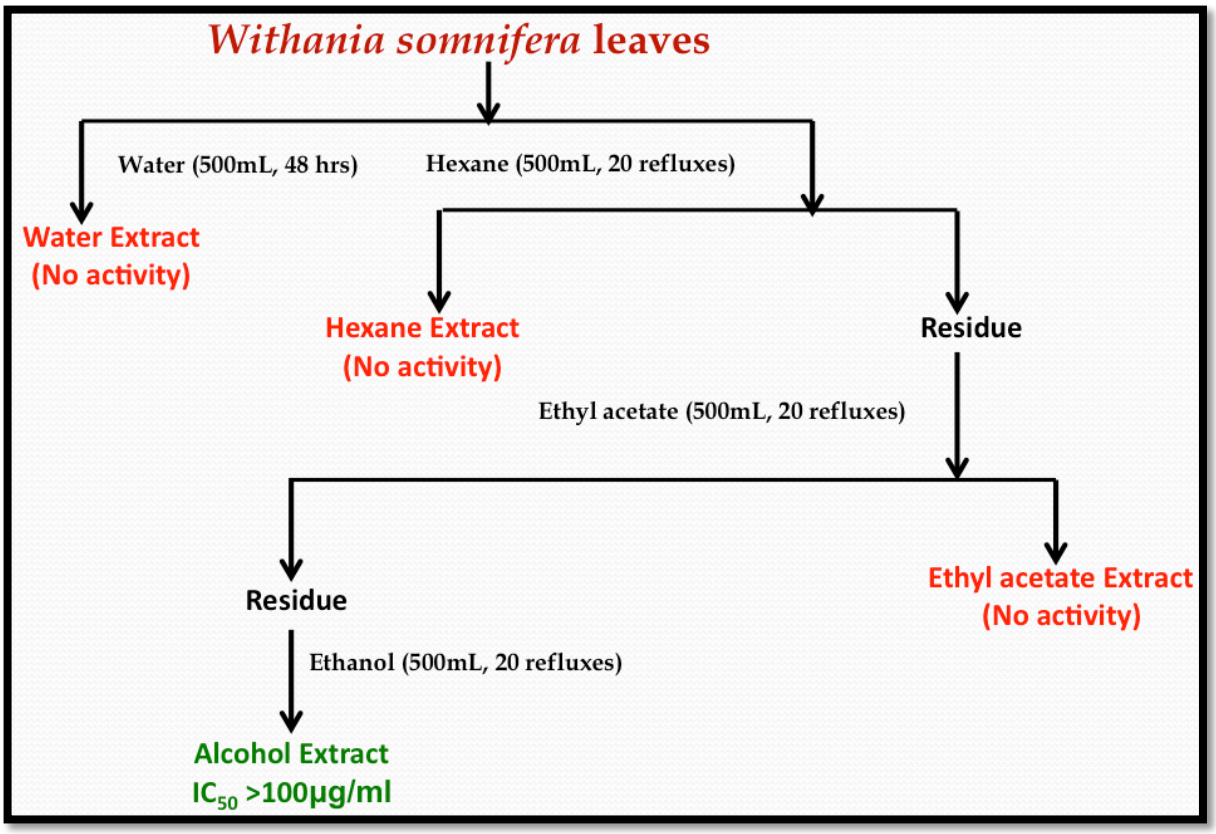


Figure 4.1 (B): Schematic representation of sequential extraction procedure for the leaves using the solvents with increasing polarity

Extract	Activity against *L. donovani* parasites
Hexane	No activity
Ethyl acetate	No activity
Ethanol	Active (IC_{50}= >100µg/ml)
Water	No activity

Table 4.1: Activity of crude leaf extracts of *W. somnifera* against *L. donovani* promastigotes

This method gave an added advantage of separating the withanolides from other entities in this extract using the solvent gradient. We adsorbed the extract onto the column with silica gel and loaded it on the silica gel column. Using different gradients of ethyl

acetate: ethanol (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60), we collected different fractions, which we subsequently pooled into seven major fractions based on the separation pattern on thin layer chromatography (TLC). The presence of withanolides in these seven fractions was identified using separation on TLC and spraying with p-anisaldehyde sulphuric acid solution specific for withanolides and was found that only Fraction 5 & 6 (F5 & F6) were having the withanolides which were compared with pure compound (Withaferin-A) (**Figure 4.2**).

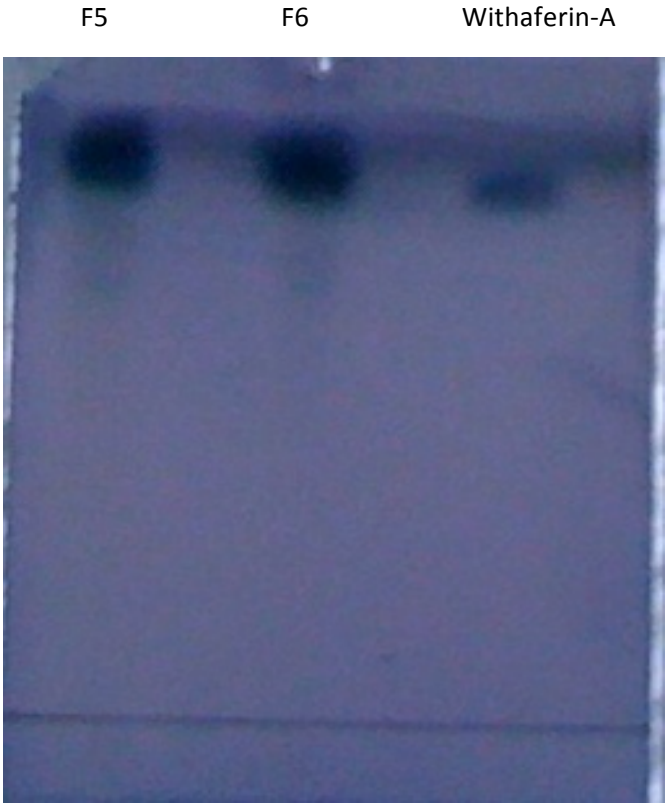


Figure 4.2: Thin layer chromatography of F5, F6 and Withaferin-A. The TLC plates are sprayed with p-anisaldhehyde sulphuric acid to detect the withanolides

In order to determine the antileishmanial effect of all the seven fractions obtained, we carried out the MTT assay and determined that only F5 and F6 were potent against the parasites, while rest of the fractions didn't show any effect (**Table 4.2**).

Fraction number	Incubation time (Hrs)	IC_{50} (ug/ml)
1	24	-
	48	-
	72	-
2	24	-
	48	-
	72	-
3	24	-
	48	-
	72	-
4	24	-
	48	-
	72	-
5	24	-
	48	74
	72	52
6	24	-
	48	13.5
	72	13.5
7	24	-
	48	-
	72	-

Table 4.2: Determination of antileishmanial activity of the fractions against the promastigote stage of the parasite using MTT assay

To identify the withanolides present in F5 and F6, we did an LC-MS/MS analysis of these fractions and found seven and five compounds in F5 and F6 respectively (**Table 4.3, 4.4**). The compounds obtained in LC-MS/MS analysis were compared using the MS fragments with compounds present PubChem library and also with the available literature (Musharraf et al., 2011).

Compound Name	Retention Time	Mass	Molecular formula	MS/MS fragments
Withanolide E	7.815	486.2631	$C_{28}H_{38}O_7$	105,131,143,145,155,169,179, 183,197
Somniferanolide	8.399	468.2521	$C_{28}H_{36}O_6$	105,131
6,7-Epoxy-17-hydroxy-1-oxo witha-4,24-dienolide	8.833	454.2731	$C_{28}H_{38}O_5$	105,115,117,129,131,141,143, 145,155,169,181,183,195,197, 239
27-Dehydroxylated withanolide	9.051	452.2572	$C_{28}H_{36}O_5$	105,129,131,141,143,145,155, 165,167,169,171,179,181,183
Withanolide D/Withanolide A/Withaferin A	9.093	470.2676	$C_{28}H_{38}O_6$	105,121,129,131,143,155,171, 183
Withanolide F	9.43	488.2793	$C_{28}H_{40}O_7$	105,107,117,119,121,129,131, 133,141,143,147,155,159,165, 179,181,183
Withanolide G2	11.824	436.2623	$C_{28}H_{36}O_4$	105,115,119,127,129,131,141, 143,153,155,165,171,179,193

Table 4.3: Compounds obtained in fraction 5 through LC-MS/MS analysis. Fraction 5 was subjected to LC-MS/MS analysis using the methanol: water gradient in 0.1% acetic acid. The above table lists the retention times, monoisotopic masses, molecular formulae and MS/MS fragments of the respective compounds. The monoisotopic masses were used to identify the compounds from the PubChem library.

Compound Name	Retention Time	Mass	Molecular formula	MS/MS fragments
Withanolide F	9.474	488.2782	$C_{28}H_{40}O_7$	105,143
27-Dehydroxylated withanolide	10.191	452.2575	$C_{28}H_{36}O_5$	105,119,121,129,131,141,143, 153,155,165,167,169,179,181, 191,193,205,215,233,237
Withanolide D/Withanolide A	10.426	470.2686	$C_{28}H_{38}O_6$	105,107,121,129,131,143,165, 171,179,181,193
6,7-Epoxy-17-hydroxy-1-oxo with a-4,24-dienolide	12.736	454.2735	$C_{28}H_{38}O_5$	105,107,109,115,119,129,131, 141,143,153,155,165,167,179, 193
Withanolide G2	16.607	436.2624	$C_{28}H_{36}O_4$	105,117,119,121,129,147,171

Table 4.4: Compounds obtained in fraction 6 through LC-MS/MS analysis. Fraction 6 was subjected to LC-MS/MS analysis using the methanol: water gradient in 0.1% acetic acid. The above table lists the retention times, monoisotopic masses, molecular formulae and MS/MS fragments of the respective compounds. The monoisotopic masses were used to identify the compounds from the PubChem library.

4.2 Antileishmanial effects of the fractions on the promastigote stage of the parasite

In order to investigate the mode of inhibition of these fractions on the *in vitro* form of the parasite i.e. promastigotes, we examined several marker features of the apoptosis. As *Leishmania* belongs to eukaryotes, but it lacks the caspase enzymes for the initiation of the apoptosis, so we investigated the other features, which are induced in the apoptosis mechanism.

4.2.1 Determination of IC_{50} of the fractions *in vitro*

To determine the IC_{50} of F5 and F6 fractions, PI dye exclusion test was performed. An increase in PI fluorescence (red color) indicates the percentage of death in the promastigotes as PI permeates only dead cells due to the disrupted plasma membrane. Treatment of promastigotes with F5 shows a dose-dependent inhibition of parasite growth with 52µg/ml of IC_{50} and 90% death was observed at 70µg/ml concentration. Similarly,

treatment with F6 exhibited a rapid and dose-dependent inhibition of the range of 0-10µg, reaching 90% at 30µg/ml (**Figure 4.3**) with an IC_{50} concentration 13µg/ml calculated from the graph. The graph represents the values of three independent experiments with standard deviation. Thus, this data reveals the IC_{50} concentration of the above fractions.

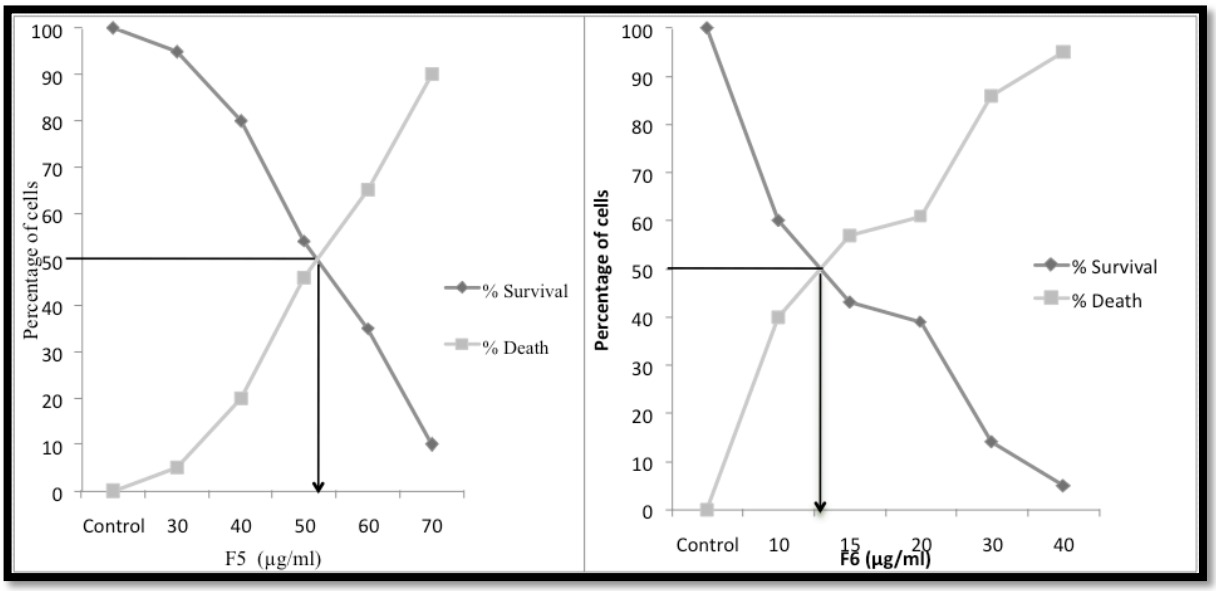


Figure 4.3: Estimation of IC_{50} of F5 and F6 using PI dye exclusion test.

4.2.2 Morphological alterations in parasites with F5 and F6 treatment

Scanning electron microscopic studies of untreated and F5, F6 treated promastigotes showed certain morphological changes, which represent the characteristics of apoptotic-like death. Promastigotes treated with an IC_{50} concentration of F5 showed a drastic change in the morphology with no significant change in flagella size. On the other hand, F6 treated parasites with IC_{50} concentration at 24, 48 and 72 hrs exhibited size reduction from slender to more ovoid shape and also the loss of flagella (**Figure 4.4**). Cell size shrinkage is a hallmark of apoptotic death. The control parasites retained their normal features like slender shape and long flagella at all time points. This observation reveals that treatment with withanolide fractions results in morphological changes of *L. donovani*.

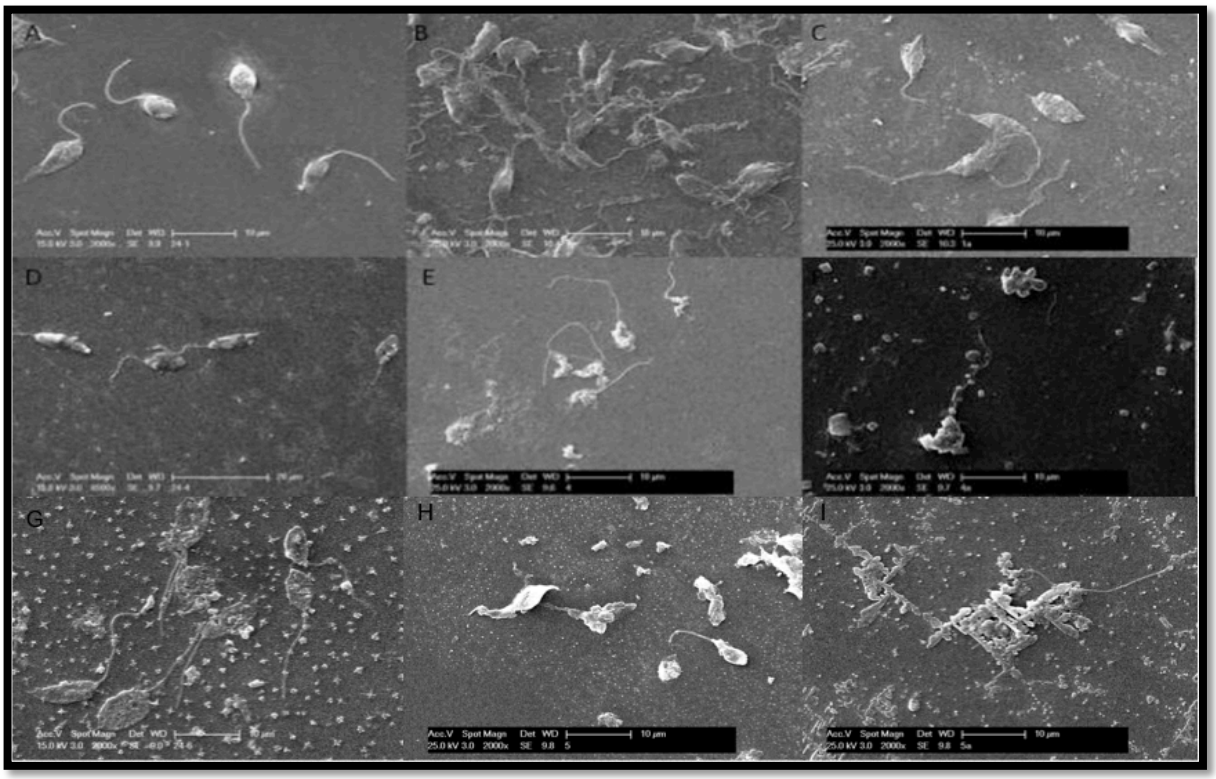


Figure 4.4: Assessment of treated and untreated *L. donovani* promastigote morphology using scanning electron microscope at 24hrs, 48hrs and 72hrs time interval. a, b, c indicate control parasites, d, e, f indicates F5 treated parasites and g, h, i indicates F6 treated parasites.

4.2.3 Externalization of phosphatidylserine and Permeation of PI

Externalization of phosphatidylserine to the outer leaflet of the plasma membrane is one of the hallmarks of apoptosis in the metazoans and unicellular cells. Annexin V exhibits high binding affinity to phosphatidylserine externalized onto the plasma membrane of an organism during apoptosis. Hence, it is commonly used for the detection of apoptotic cells. But, there is also an equal chance of it binding to necrotic cells whose membrane integrity is lost. The addition of PI provides a solution in overcoming this disadvantage by discriminating apoptotic and necrotic cells, as it does not permeate the former.

To investigate whether the cell death triggered by withanolides is via apoptosis or necrosis, promastigotes were treated with the withanolides (F5, F6) for 15 hrs and double stained with annexin V-FITC and PI. As shown in **Figure 4.5**, a significant percentage of cells (F5- 24.77%, F6- 36.77%) were single positively stained for annexin V (lower right quadrant) which represent apoptotic cells with intact membrane and significant percentage of cells (F5- 23.55%, F6- 5.46%) were double positively stained for annexin V and PI (upper right quadrant) which signify late apoptotic or necrotic cells when compared to the

control cells. Withanolides treated promastigotes showed higher percentage of annexin V-stained (positive) cells compared to miltefosine treated (25µM for 72hrs) (11.32%) which was used as a positive control for its antileishmanial activity via apoptosis. The lower left quadrant symbolizes live parasites and upper left quadrant stand for dead cells, which are only PI positive. These results provide evidence that withanolides from F5 and F6 exert their antileishmanial activity by the externalization of phosphatidylserine.

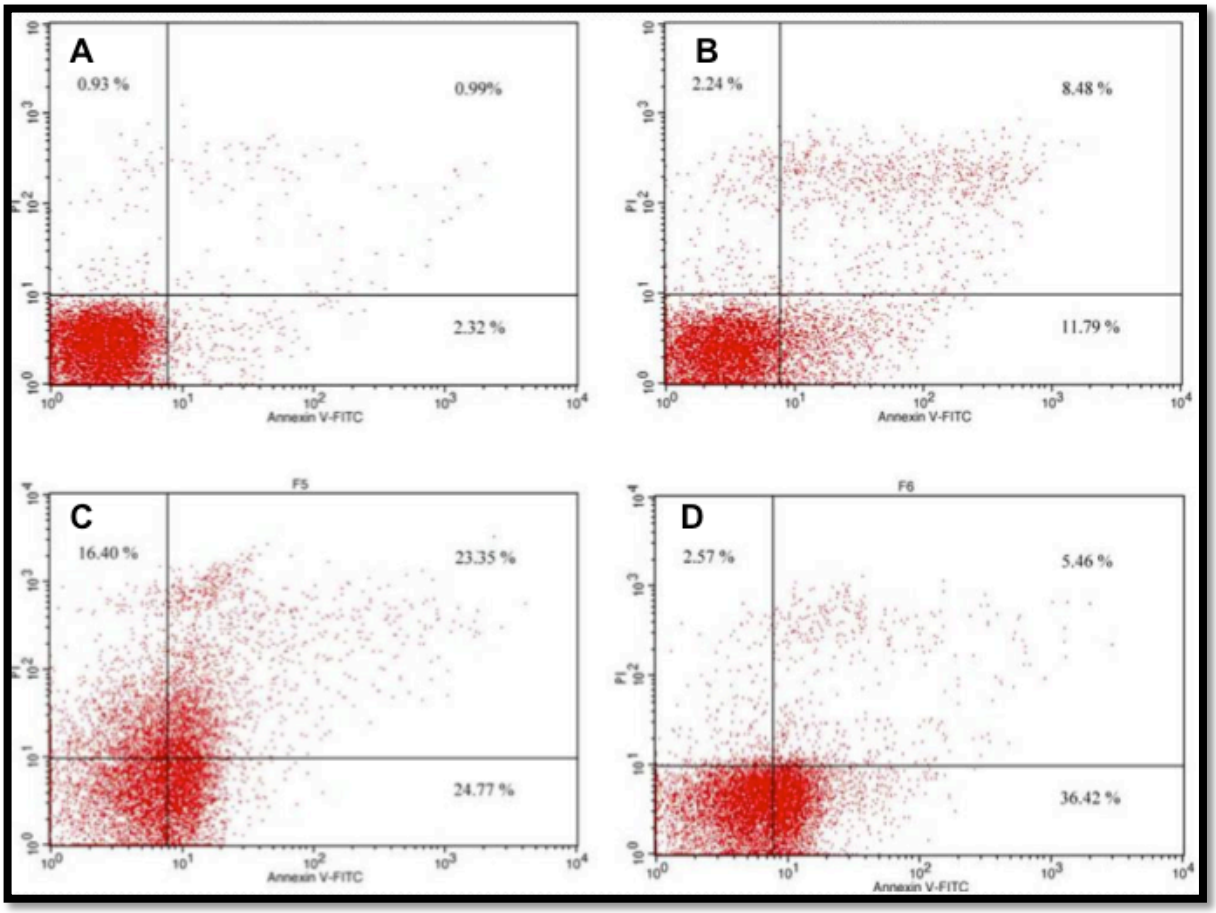


Figure 4.5: Determination of externalization of phosphatidylserine by flow cytometry. Parasites treated with IC_{50} concentrations of F5 (C) and F6 (D) are represented in a dot plot along with control (A) and positive control Miltefosine (B) treated parasites. The lower left and right quadrants indicate the live parasites and annexin V positive parasites respectively.

3.2.4 Cell cycle arrest at sub G0/G1 population in withanolides treated parasites

To determine the percentage of sub G0/G1 phase parasites present, promastigotes were treated with IC_{50} concentrations of F5 and F6 for 6, 24 and 48 hrs, stained with PI and then analyzed by flow cytometry. In comparison to control parasites, the percentage of F5 treated parasites in sub G0/G1 was observed to increase in time-dependent manner from 0.44% at 6 hrs to 6.7% at 48 hrs. Likewise, F6 treated parasites showed an increase in the

percentage of sub G0/G1 parasites in a time-dependent manner from 2.44% at 6 hrs to 18.79% at 48 hrs. However, DMSO treated parasites showed no change compared to control parasites (**Figure 4.6**). This data suggests that withanolides from F5 and F6 arrest cell cycle of parasites at sub G0/G1 phase which ultimately leads to apoptosis.

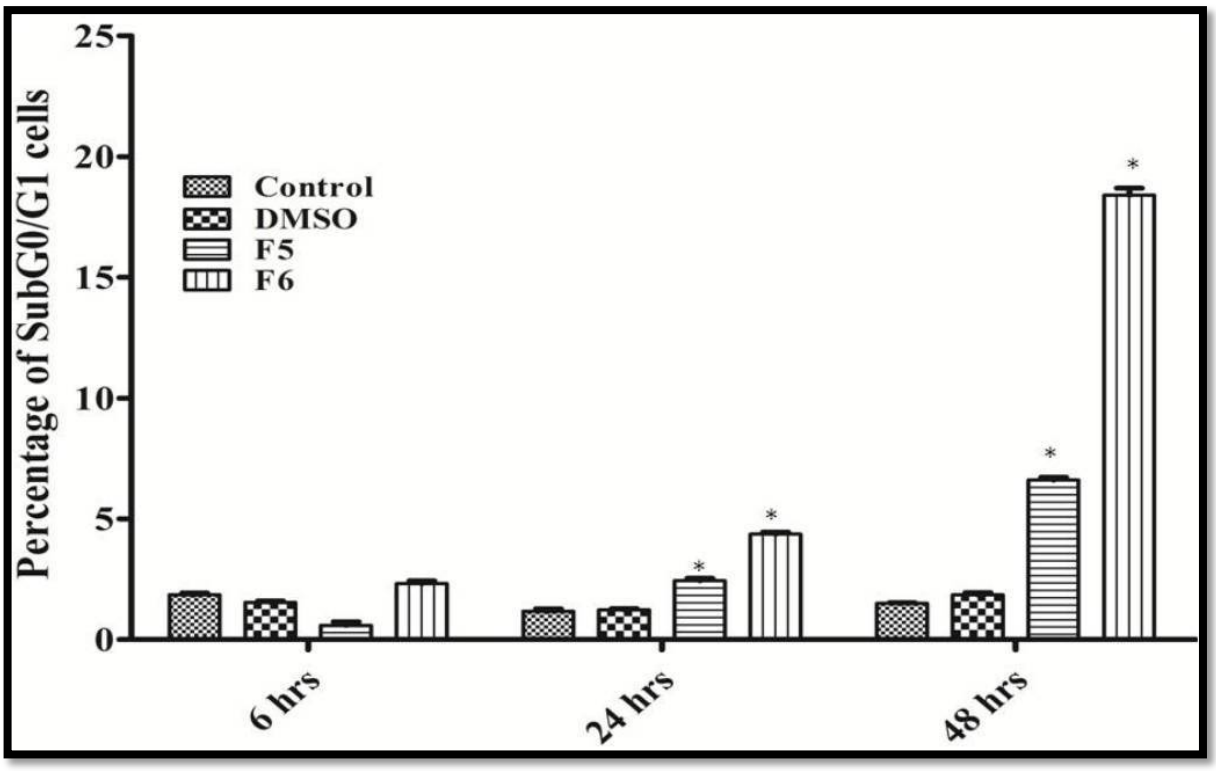


Figure 4.6: Quantification of apoptotic sub G_0/G_1 cells by cell cycle analysis using PI staining. The above bar graph depicts the percentage of sub G_0/G_1 cells of control and treated promastigotes at different time periods. * indicates P<0.01.

4.2.5 DNA fragmentation analysis using TUNEL assay

DNA fragmentation by endogenous nucleases is another hallmark of apoptosis. Fragmented DNA carries 3' hydroxyl groups which forms substrate for binding of fluorescein-dUTP using TUNEL assay and provide a better evaluation of DNA damage in the cell. Treatment with IC_{50} concentrations of both F5 and F6 for 72 hrs induced DNA fragmentation in parasites visualized by TUNEL assay. Miltefosine at a concentration of 25μM for 72 hrs was used positive control, which also showed significant DNA damage in *L. donovani* promastigotes. DNA nicking in the treated parasites is visualized as orange to yellowish in color (**Figure 4.7**). Thus, it shows that withanolides induce apoptotic-like death by introducing DNA breaks in the genome of the parasite.

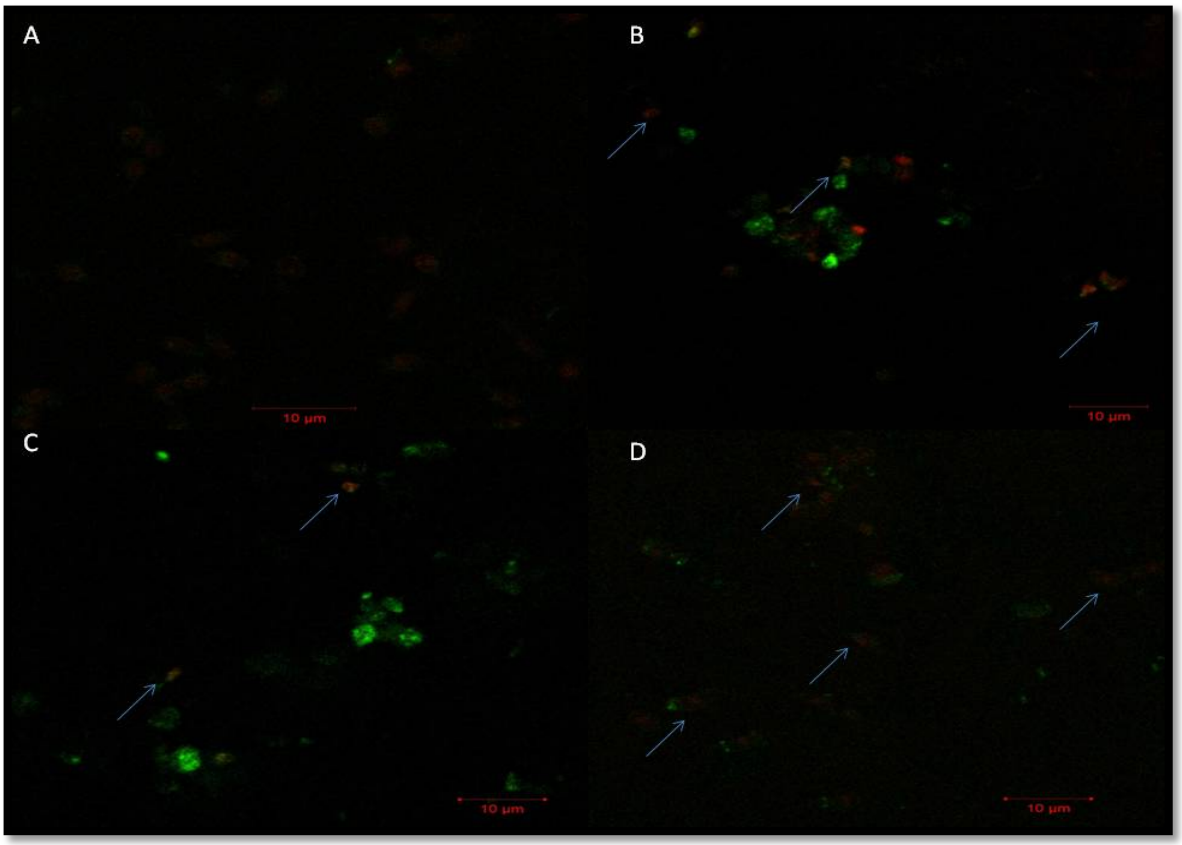


Figure 4.7 (A): Analysis of DNA fragmentation by TUNEL assay. Parasites were treated with IC_{50} concentrations of F5 and F6 for 72hrs and stained using TUNEL method. Parasites were visualized under fluorescence microscopy. Merge image of FITC and PI channel A- Control, B- F5, C- F6 and D- Miltefosine.

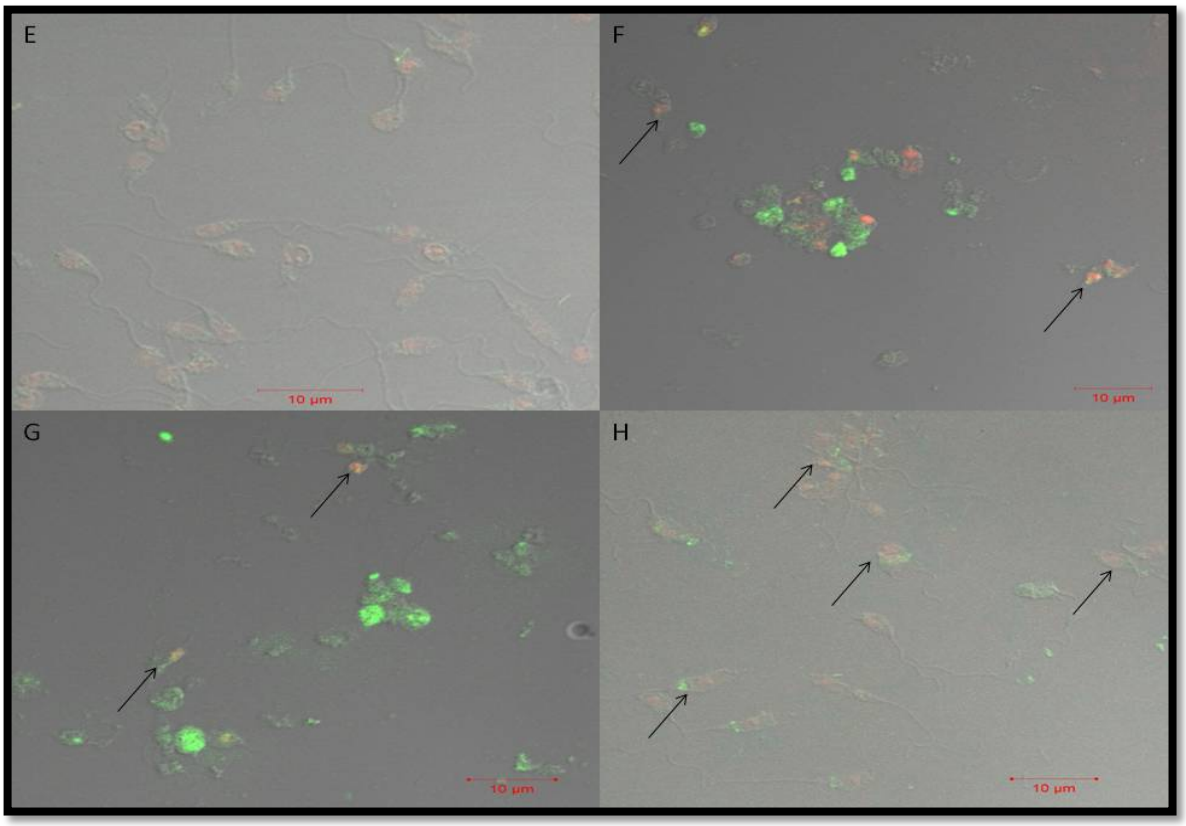


Figure 4.7 (B): Analysis of DNA fragmentation by TUNEL assay. Parasites were treated with IC_{50} concentrations of F5 and F6 for 72hrs and stained using TUNEL method. Parasites were visualized under fluorescence microscopy. Merge image of DIC and FITC/PI dual pass filter E- Control, F- F5, G- F6, H- Miltefosine. The arrows indicate parasites having DNA nicks.

4.2.6 Depolarization of mitochondrial membrane potential

Oxidative damage to the cells results in the depolarization of mitochondrial membrane due to the leaky membranes. In haemo flagellate protozoans, mitochondrial dysfunction is one of the characteristic features of apoptosis and is succeeded by the DNA fragmentation. The J-aggregates inside the mitochondria, which fluoresce red, indicate the normal status of mitochondria whereas the monomers in cytosol indicate the disruption of membrane potential. We have measured the JC-1 fluorescence using flow cytometry and ratio of mean fluorescence intensity of 590/530 nm is expressed as a bar graph. As shown in **Figure 4.8**, 24hrs treatment with both F5 and F6 a induced reduction in Ψm compared to the control cells with the values being 16.1±0.3 and 13.5±0.34 versus 28.8±0.41 respectively. At 48 hrs, Ψm of treated cells decreased considerably compared to that of control cells with the values being 39.1±0.3 and 24.5±0.39 versus 64.5±0.35 respectively. At 72 hrs, the decrease was further drastic in treated compared to control cells being 51.5±0.5 and 53±0.26

versus 130±0.48 respectively. The vehicle control, DMSO treated parasites, showed no significant decrease in membrane potential compared to that of negative control during all time points. Miltefosine, an antileishmanial drug, is used as a positive control at 25µM, and it showed a significant decrease in membrane potential compared to control cells at all time points.

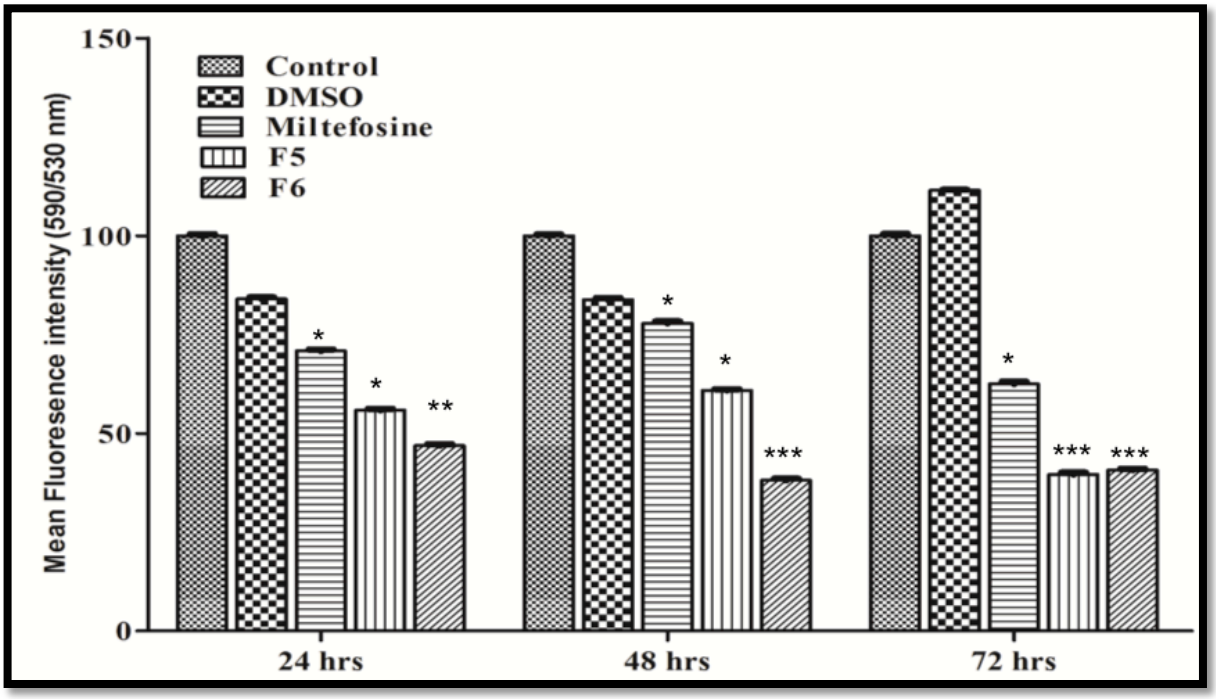


Figure 4.8: Estimation of Ψ_m (membrane potential) in *L. donovani* promastigotes. Control and withanolide treated parasites were incubated for 24hrs, 48hrs and 72hrs respectively and subsequently stained with JC-1 dye (10µg/ml). The change in the relative Ψ_m values are expressed as the ratio of fluorescence measurement at 590nm versus 530nm. Miltefosine is used as positive control. * indicates P<0.05, ** P<0.01, *** P<0.001.

4.2.7 Generation of ROS in withanolides treated promastigotes

To determine whether withanolides in F5 and F6 fractions are able to produce ROS within promastigotes, we used a fluorescent probe, H_2DCFDA, which fluoresce green upon cleavage with H_2O_2, hydroxyl radicals, and O_2H-. This green fluorescence is used as an indicator of ROS production inside the cells. The MFI of the treated cells was compared with that of control cells at 24 hrs, 48 hrs, and 72 hrs respectively. Initially, there was a gradual increase in ROS production at 24 hrs in treated cells compared to that of control cells (F5-108.14±0.41, F6-106.18±0.62 versus control 21.88±0.25). But, drastic increase in ROS was observed at 48 hrs in treated cells when compared to that of control cells (F5-160.88±0.33, F6-321.33±0.57 versus control 20.33±0.51) (**Figure 4.9**). Generation of ROS

in the cells is an indication of a cell undergoing the process of apoptosis, and these results indicate that withanolides are capable of inducing ROS in promastigotes during apoptosis. These above markers indicate that withanolides present in F5 and F6 induce antileishmanial activity through apoptotic-like death in the promastigote stage of the parasite. These results affirm the investigation of these withanolides in the mammalian stage of the parasite i.e. amastigotes.

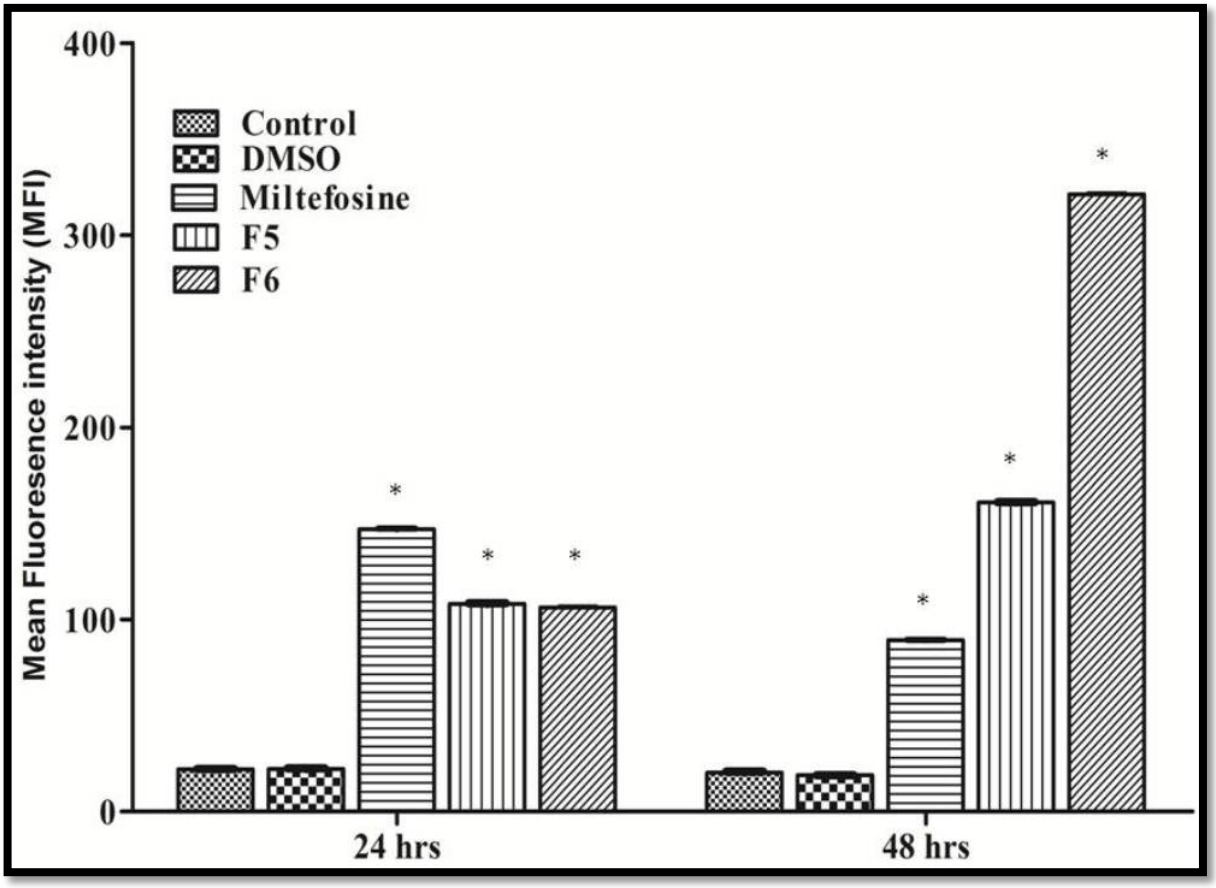


Figure 4.9: Generation of ROS (Reactive Oxygen Species) in *L. donovani* promastigotes. Treated and untreated parasites were incubated with IC_{50} concentration of withanolides, positive control Miltefosine for different time periods and subsequently stained with H_2DCFDA. Dose dependent changes are expressed as mean fluorescence intensity. * indicates P<0.01

4.3 Antileishmanial and immunomodulatory activities of F5, F6, and Withaferin-A *ex vivo*

Amastigotes are the human form of the parasites, which resides in the macrophages and mononuclear phagocytes. These parasite forms thrive in the harsh environment of phagolysosomes inside these cells. The antileishmanial effect of any compound would increase effect the macrophages microbicidal effector molecules like NO and ROS while the immunomodulatory effect would increase the cytokines responsible for the activation of the

macrophages. In this direction, we used the PMM as the model host system for evaluating the antileishmanial and immunomodulatory effect of these fractions and also the pure compound Withaferin-A, which being the abundant withanolide in WS leaves.

4.3.1 Cytotoxic effects of WS fractions and withaferin A on peritoneal macrophages

To test whether the WS fractions and withaferin A show any adverse effects on naive PMM, we used MTT assay to determine the highest concentration till there is no effect on the naive PMM. We used different concentrations of F5 (5, 10, 25, 50 and 75µg/ml), F6 (5, 10, 25, and 50µg/ml) and Withaferin-A (1-20µM) on PMM for 72 hrs. The results indicated that treatment of F5 decreased the cell survivability of PMM by just 40% at the highest concentration of 75µg/ml, in F6 treatment the decrease was about 40% at the maximum concentration of 50µg/ml. In case of withaferin A there was decrease of survivability by 2%, 10%, 12%, 12%, 15%, and 40% at 1, 2, 3, 4, 5 and 7.5µM concentration respectively. After this, there was the more pronounced death of PMM with an increase in Withaferin-A concentration. So we used the above concentrations for determining its antileishmanial activity in PMM (**Figure 4.10**)

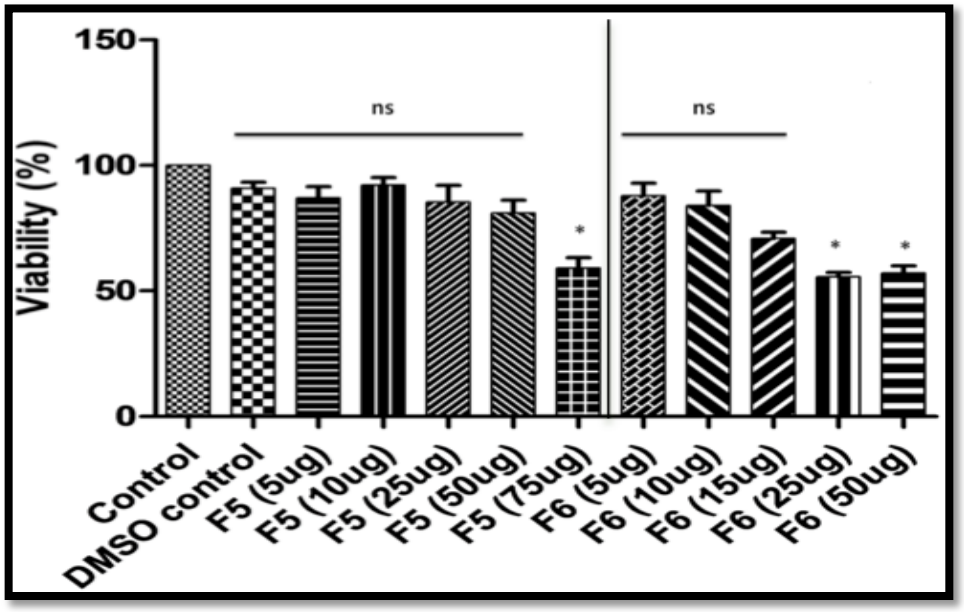


Figure 4.10 (A): Cytotoxic assay on PMM using MTT assay with different concentrations of F5 & F6. The results are represented as percentage viability compared to control (untreated PMM) on the y-axis. Unpaired student t-test was performed between control and treated samples (*P<0.05, **P<0.01 and ***P<0.001). The values are representative of three independent experiments.

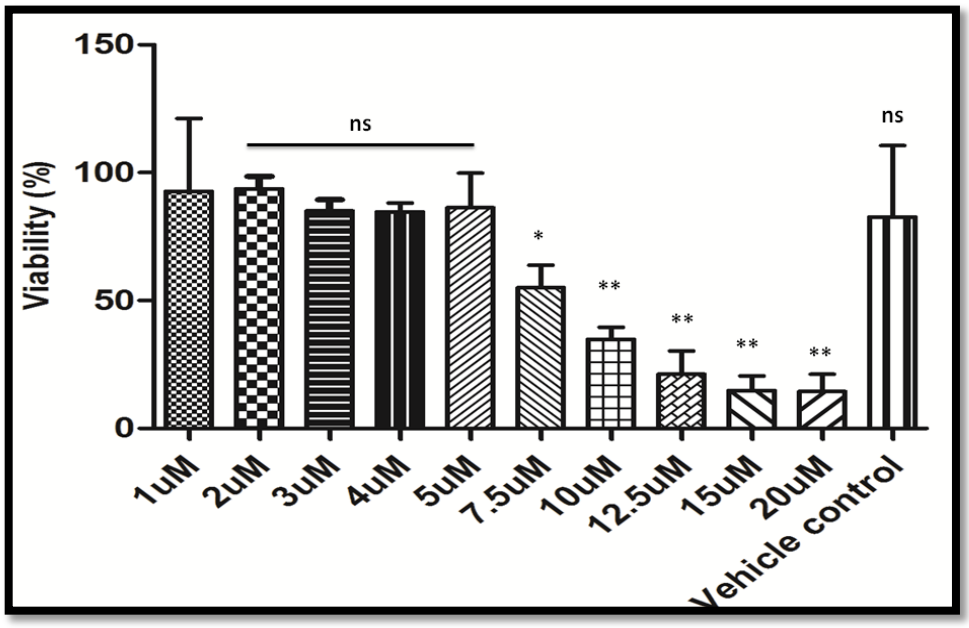


Figure 4.10 (B): Cytotoxic assay on PMM using MTT assay with increasing concentrations of Withaferin-A. The results are represented as percentage viability compared to control (untreated PMM) on the y-axis. Unpaired student t-test was performed between control and treated samples (*P<0.05, **P<0.01 and ***P<0.001). The values are representative of three independent experiments.

4.3.2 Intracellular parasite load in *L. donovani* infected PMM

PMM are used as best models for ex vivo macrophages. The efficacy of F5, F6 and Withaferin-A in reducing the intracellular parasite load was monitored by counting the number of amastigotes in the treated PMM after 72 hrs using Giemsa staining. We used different concentrations of F5 and F6 treatment in infected PMM, we found that F5 at 15µg/ml was able to reduce the parasite load by ~50% ($P<0.001$) and F6 at 10µg/ml was able to reduce ~ 60% ($P<0.001$) of parasite load compared to untreated infected PMM. Further, in case of Withaferin-A, we observed a dose-dependent decrease in the amastigote number with increase in concentrations (0.5, 1.0 and 1.5µM). The decrease was almost ~80% ($P<0.001$) with 1.5µM concentration and we used this concentration for further studies as it showed minimum cytotoxicity against the naive PMM. These results indicate that F5, F6 and Withaferin-A show antileishmanial activity on the intracellular stage of the parasite with decrease in parasite load was more than 50% in all cases (**Figure 4.11**)

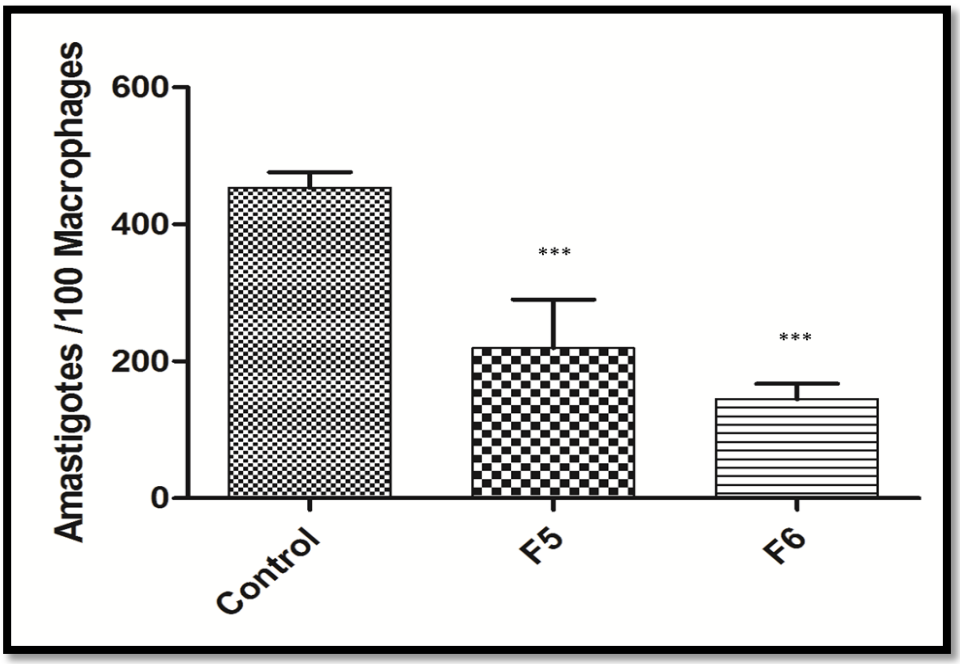


Figure 4.11 (A): *Ex vivo* antileishmanial activity of F5 and F6 on intracellular amastigotes in PMM using giemsa staining. The graph shows the amastigote numbers for 100 macrophages F5 (15µg/ml) & F6 (10µg/ml). The values are from three independent experiments and significance values between controls and treated were calculated using unpaired student t-test (*P<0.05, **P<0.01 and ***P<0.001).

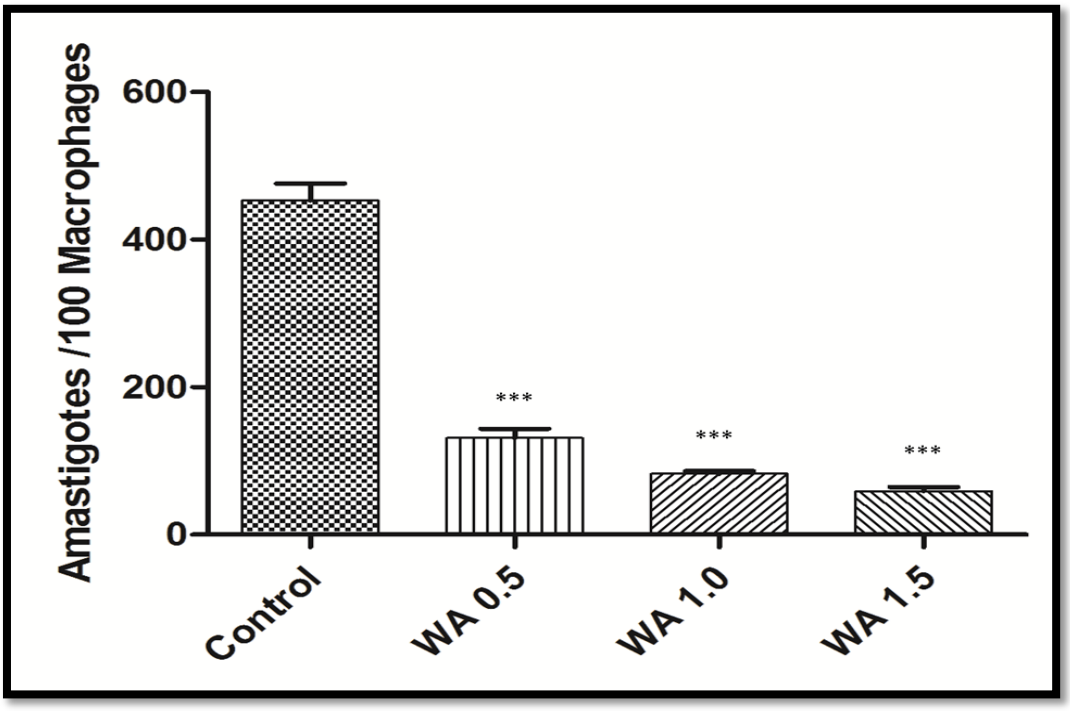


Figure 4.11 (B): *Ex vivo* antileishmanial activity of withaferin A on intracellular amastigotes in PMM using giemsa staining. The graph shows the amastigote numbers for 100 macrophages after Withaferin A (0.5µM, 1.0µM & 1.5µM) treatment. The values are from three independent experiments and significance values between controls and treated were calculated using unpaired student t-test (*P<0.05, **P<0.01 and ***P<0.001).

4.3.3 Nitric oxide (NO) levels in treated PMM supernatants

To identify the mode of clearance of parasites from host macrophages, we measured the nitrite levels present in the culture supernatants of infected and treated macrophages after 48hrs. Since NO and ROS are the major microbicidal molecules responsible for clearance of *Leishmania* parasites in the macrophages, we estimated the amount of NO produced by the treatment with F5, F6 and Withaferin-A. We used the same concentrations used for the estimating the parasite load and found that there was no difference between infected and treated macrophages in F5 and F6. In Withaferin-A treatment, the dose-dependent increase in the concentration was also not able to induce any significant NO levels when compared to its infected counterpart. LPS was used as a positive stimulator for the NO production. So we concluded that the mode of action of the above treatments was not through the induction of NO (**Figure 4.12**).

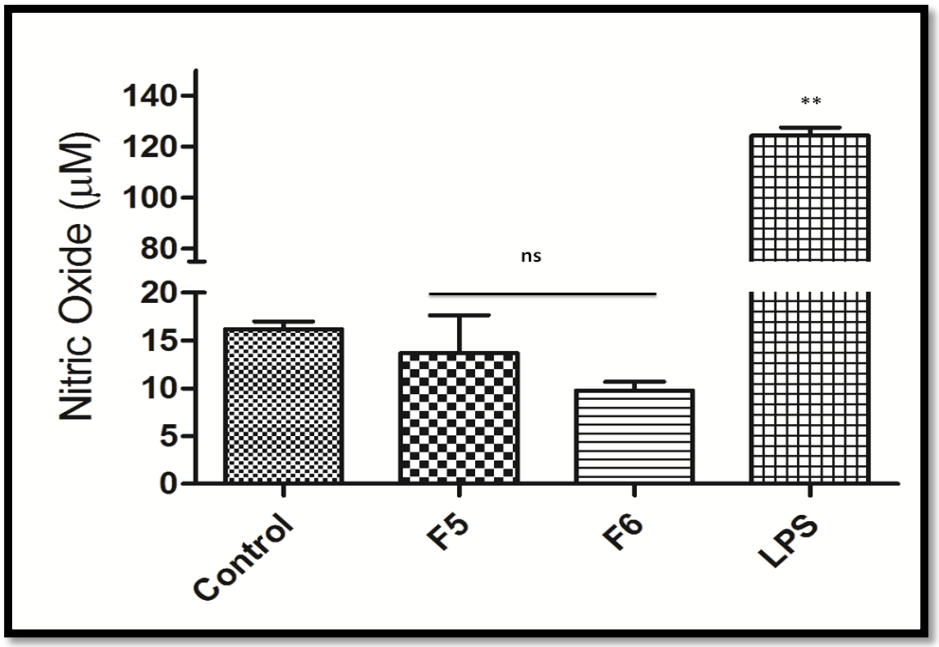


Figure 4.12 (A): NO estimation using griess assay in control and F5 (15µg/ml) & F6 (10µg/ml) treated PMM supernatants after 72 hrs of incubation. LPS was used as a positive stimulant. The values were represented in concentration of nitrite (µM) produced. The values are represented from three different experiments and significance was calculated between control and treated PMM (*P<0.05, **P<0.01 and ***P<0.001).

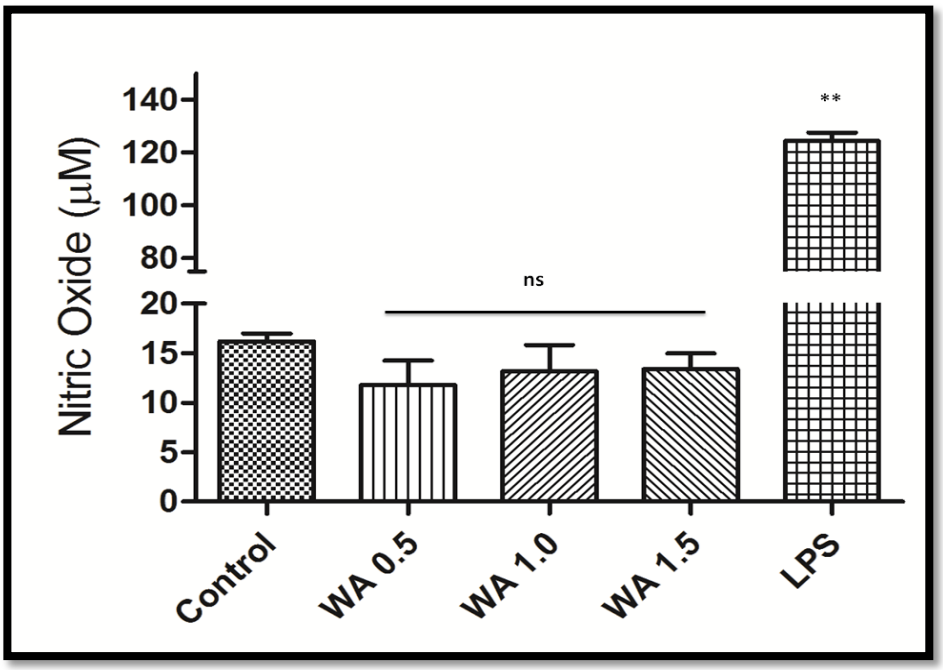


Figure 4.12 (B): NO estimation using griess assay in control and Withaferin A (0.5µM, 1.0µM & 1.5µM) treated PMM supernatants after 72 hrs of incubation. LPS was used as a positive stimulant. The values were represented in concentration of nitrite (µM) produced. The values are represented from three different experiments and significance was calculated between control and treated PMM (*P<0.05, **P<0.01 and ***P<0.001).

4.3.4 Assessment of ROS production

ROS is one of the antimicrobicidal molecule employed by the macrophages for its defense against the pathogens. Our study didn't find any significant NO production in treated macrophages, which prompted us to examine the ROS levels in the macrophages. We treated the macrophages with F5, F6 and Withaferin-A for 6 hrs, and ROS production was done using H_2DCFDA dye. The results demonstrate considerable increase in the mean fluorescence intensity (MFI) in F5 (850), F6 (914), Withaferin-A (724) compared to infected macrophages (538). Miltefosine was used a positive control for treating *Leishmania* infections was also able to induce ROS levels (917) in PMM. LPS being a bacterial cell wall extracts was able to produce ROS in very large amounts. So, these results indicate that F5, F6, and Withaferin-A show the antileishmanial activity by inducing the ROS (**Figure 4.13**).

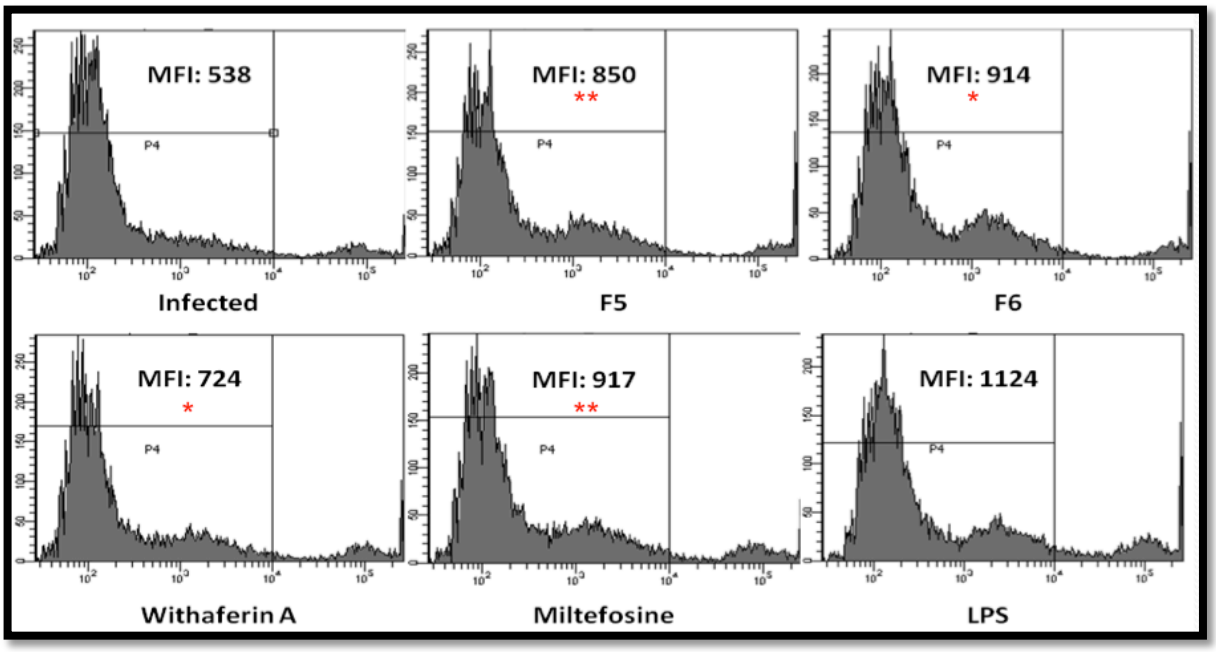


Figure 4.13: Estimation of ROS production in PMM using a fluorescence dye H_2DCFDA. The graph shows the MFI values of control (infected) and F5 (15µg/ml), F6 (10µg/ml), Withaferin A (1.5µM), Miltefosine (3.2µM) and LPS (100ng/ml) treated PMM. The experiment was repeated three times showing the same trend and the graph shows one such experiment.

4.3.5 Th1/Th2 cytokine estimation from PMM

The outcome of the *Leishmania* disease depends on the balance between Th1 and Th2 cytokines of which the Th1 favors the clearance of the parasites from the macrophages. For this, we have seen the expression of IFN-γ (Th1) and IL-10 (Th2) cytokines using RT-qPCR in infected and treated PMM. The increase in the IFN-γ expression was found to be insignificant among all the treated PMM compared to the infected PMM. But the expression of IL-10, which is crucial for the parasite persistence in the macrophages was found be significantly downregulated in the F5, F6 and Withaferin-A treated PMM when compared to infected PMM (**Figure 4.14A**). When the ratio of IFN-γ to IL-10 was taken into consideration we found that there was increase in the ratio in all treated PMM when compared to infected PMM, which clearly suggests that there is parasite clearance from the macrophages and IFN-γ/IL-10 ratio is considered as a good indication of the infection cure (**Figure 4.14B**).

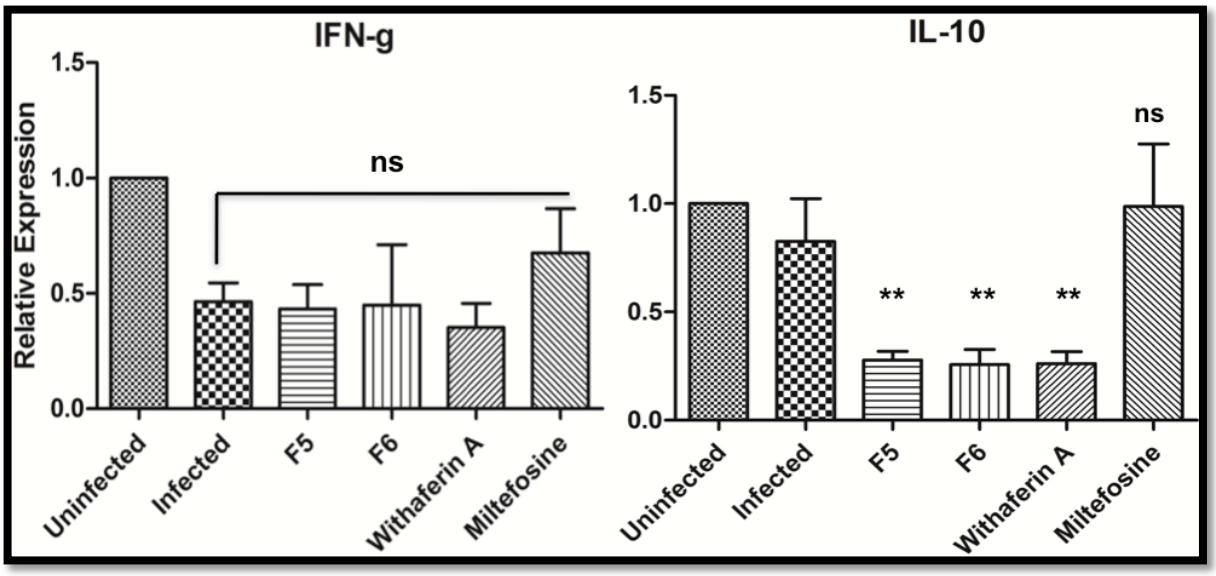


Figure 4.14 (A): Immunomodulatory effect of the withanolides in control (infected) and treated PMM *ex vivo*. RT-qPCR analysis of IFN-γ (Th1) and IL-10 (Th2) cytokine. Unpaired student t-test was performed between the infected and other samples (*P<0.05, **P<0.01 and ***P<0.001).

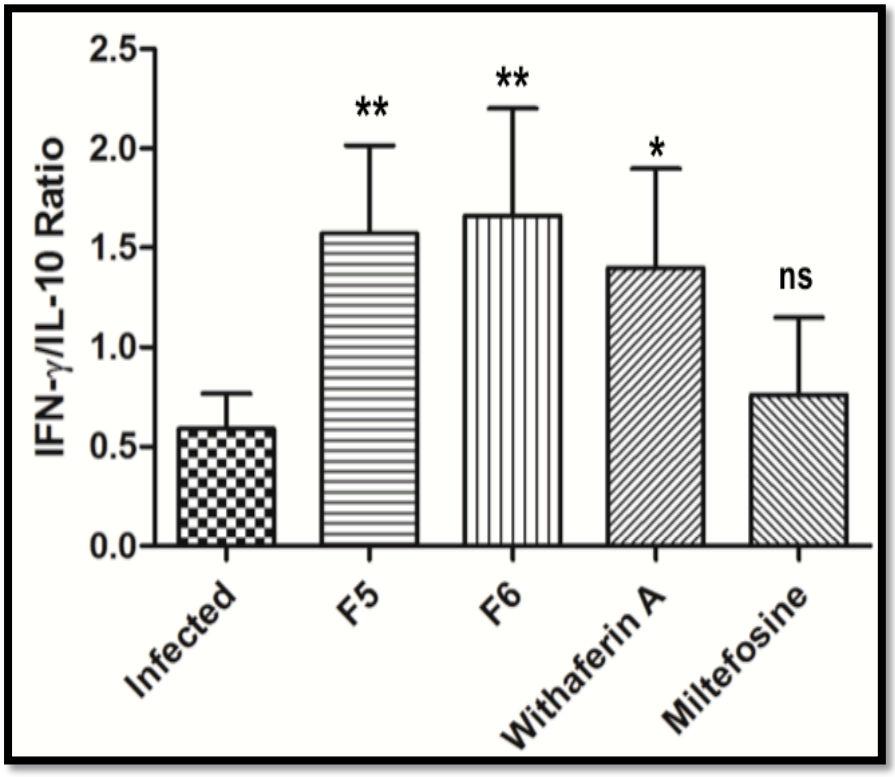


Figure 4.14 (B): Immunomodulatory effect of the withanolides in control (infected) and treated PMM *ex vivo*. The graph shows the IFN-γ/IL-10 ratio representing the parasite clearance from PMM. Unpaired student t-test was performed between the infected and other samples (*P<0.05, **P<0.01 and ***P<0.001).

We measured the cytokine levels (IFN-γ & TNF-γ) in culture supernatants of PMM using CBA kit. In accordance with our RT-qPCR results, we didn't find any significant increase in IFN-γ levels in the treated PMM compared to infected PMM. In case of TNF-α levels, only F6 was able to induce significant levels compared to the infected PMM. Other treatments didn't induce any TNF-α (**Figure 4.14C**).

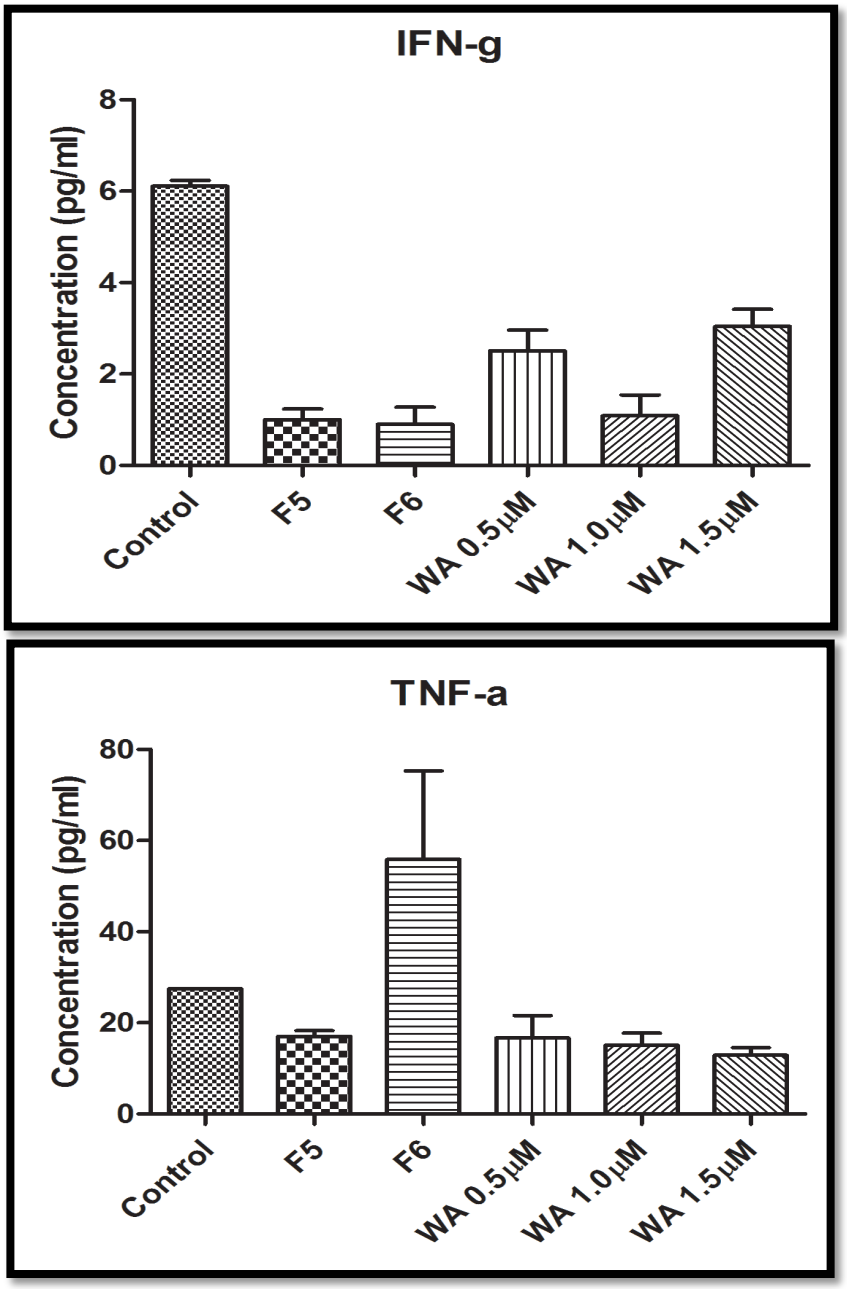


Figure 4.14 (C): Immunomodulatory effect of the withanolides in control (infected) and treated PMM *ex vivo*. The graph represents IFN-γ and TNF-α protein levels in PMM culture supernatants assessed using CBA analysis.

4.4 *In vivo* evaluation of antileishmanial and immunomodulatory potential of withanolides

BALB/C mice are used as the animal model for visceral leishmaniasis studies which being susceptible to parasite infection compared to C57BL/6. The parasite mainly resides in the visceral organs like spleen, liver and bone marrow. The chronic infection in the mice sets in 4 weeks after the infection resides in spleen forever, but in the liver the host clears the infection after 8 weeks by the granulomatous response. During this infection there is suppression of critical cytokines like IFN-γ, IL-12 from macrophages, which helps in the resistance against the disease and increase in production of immunoregulatory cytokine, like IL-10 which helps in propagation and dissemination parasites by deactivating the macrophages. So any compound, which is able to perform the dual action of killing the parasite and restoring the cell-mediated immunity, is the need for the hour.

4.4.1 *In-vivo* assessment of infection

The efficacy of F5, F6, and withaferin A were evaluated in BALB/C model of chronic visceral leishmaniasis by estimating the splenic and hepatic burden after 45days post infection/post treatment period. The results demonstrate that there was no decrease in the splenic parasite burden when treated with F5 (25mg/kg/b.wt) but it showed significant reduction in hepatic parasite burden ($P<0.05$), but when we administered F5 (50mg/kg/b.wt) it showed significant decrease in splenic ($P<0.001$) and hepatic parasite burden ($P<0.001$). F6 (25mg & 50mg/kg/b.wt) showed significant decrease in both splenic ($P<0.001$) and hepatic parasite burden ($P<0.001$, $P<0.01$). Withaferin-A (2mg/kg/b.wt) showed a drastic decrease in hepatic burden ($P<0.001$) compared to splenic burden ($P<0.01$). Miltefosine was used a positive control drug showed a very drastic reduction in parasite burden in spleen and liver (**Figure 4.15A, B**). Further, we calculated the percentage reduction in parasite burden in spleen and liver compared to infected group (0%). The reduction of parasite in spleen and liver was 2% and 30% in F5 (25mg/kg/b.wt), 50% and 75% in F5 (50mg/kg/b.wt), 44% and 55% in F6 (25mg/kg/b.wt), 50% and 65% in F6 (50mg/kg/b.wt), 32% and 62% in Withaferin-A (2mg/kg/b.wt) respectively. The results clearly indicate that the treatment is showing parasite clearance in both spleen and liver (**Figure 4.15C**).

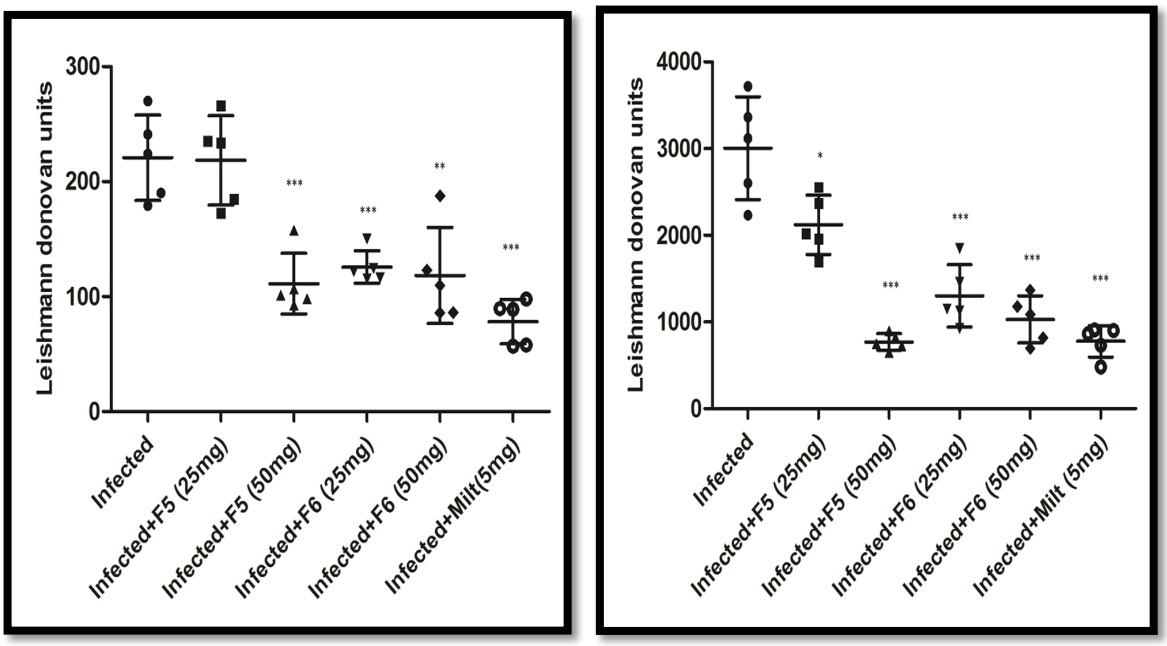


Figure 4.15 (A): Antileishmanial effect of withanolides in BALB/C model of visceral leishmaniasis. LDU in spleen (left) and liver (right) of control (infected) and F5 and F6 treated mice groups after 45 days post infection/post treatment. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

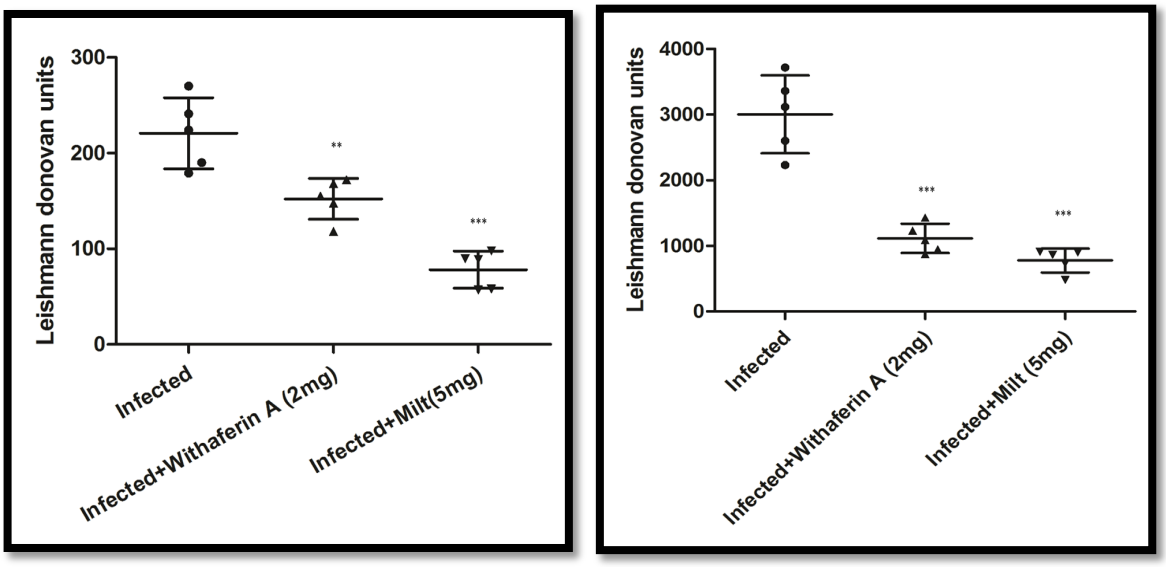


Figure 4.15 (B): Antileishmanial effect of withanolides in BALB/C model of visceral leishmaniasis. LDU in spleen (left) and liver (right) of control (infected) and withaferin A treated mice groups after 45 days post infection/post treatment. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

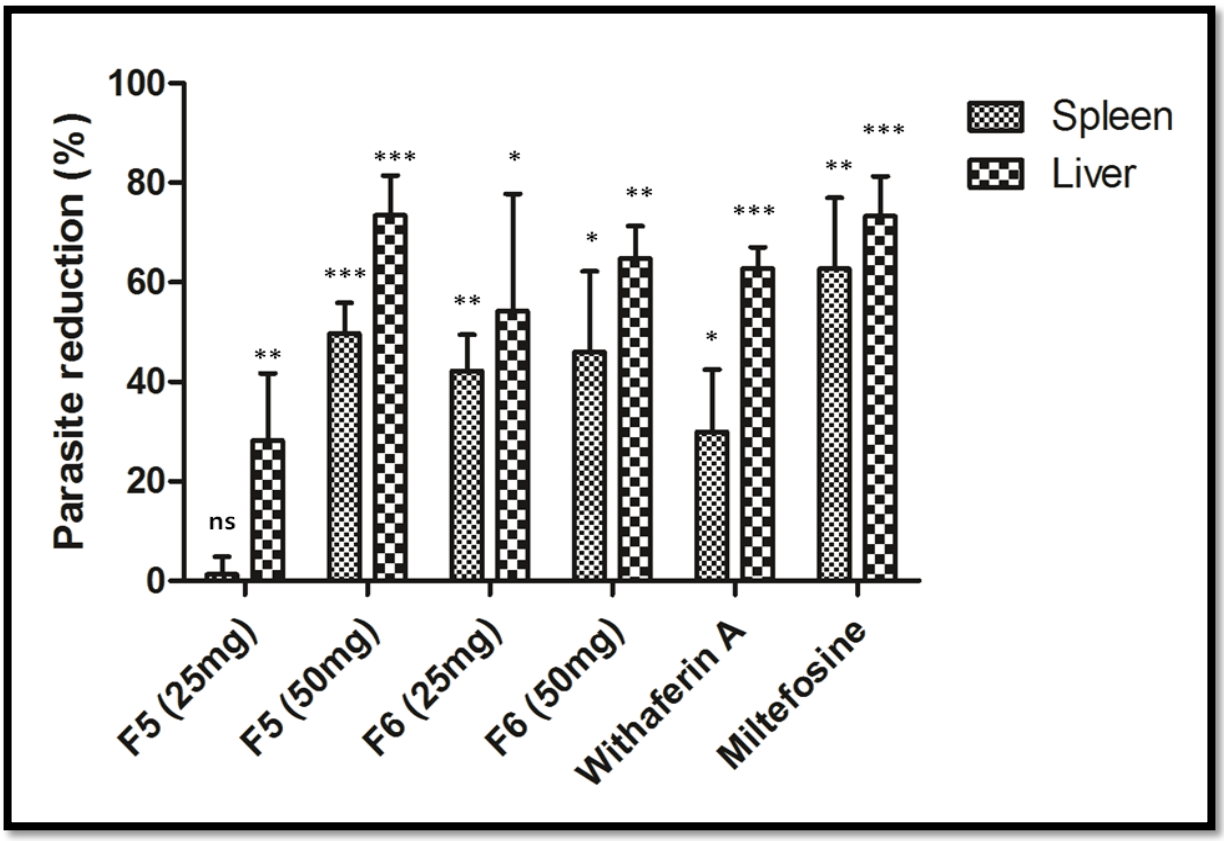


Figure 4.15 (C): Antileishmanial effect of withanolides in BALB/C model of visceral leishmaniasis. The bar graph represents the percentage parasite reduction compared to control (infected) group mice. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

4.4.2 Estimation of IgG subtypes IgG1 and IgG2a

The immune activation during the *Leishmania* determines the outcome of the disease and humoral response in the form of antibody production plays an important role. It's a known fact that IFN-γ and IL-4 direct the immunoglobulin class switching of IgG2a and IgG1 respectively. We estimated the amounts of these immunoglobulin's present in the mouse sera from all groups. We found significant increase in the amount of IgG2a ($P<0.01$, $P<0.001$), a marker of Th1 polarization in all the treated groups except the withaferin A treatment, which didn't show the increase compared to the infected group. The Th2 marker IgG1 was increased in the infected group, but the decrease of this IgG in the treated group was insignificant when compared to the infected (**Figure 4.16**).

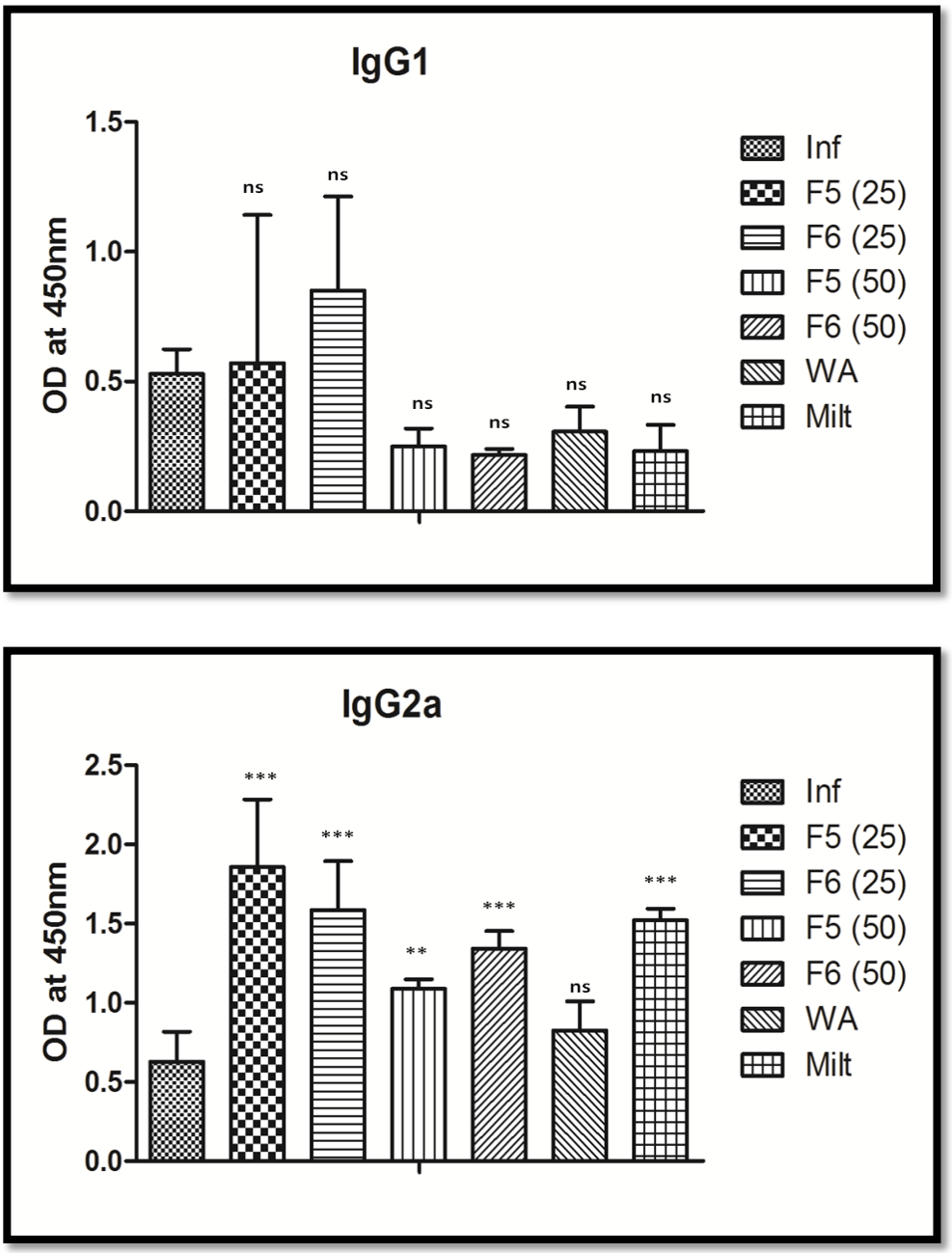


Figure 4.16: The IgG1 and IgG2a antibody titer levels in different mice treatment groups. The results are expressed as mean ± SD of five mice. The significance was calculated between control (infected) and treated mice groups using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

4.4.3 Histopathological sectioning of liver tissue

The liver sectioning of the infected mice showed fatty degeneration of the tissue, which was restored with the treatment of F5, F6 and withaferin A. With *L. donovani* infection there is infiltration of T lymphocytes, which subsequently develops into a granuloma harboring the amastigotes, thereby the infection is cleared in the liver with time. In the treated groups, F5 (25mg/kg/b.wt) forms very loose and scattered granuloma. In F6

(25 & 50mg/kg/b.wt) treatment, the granuloma structure is dense, tight and more in number compared to the infected livers. Withaferin-A treatment also shows the same architecture as compared to other treatment. These granuloma structures didn't harbor any parasite as compared to infected group (**Figure 4.17**). We also counted the number of granuloma structure in all treated groups and compared it with the infected group. There was a significant difference only in F6 (50mg/kg/b.wt) ($P<0.05$) group but other groups had more or less same number compared to infected groups.

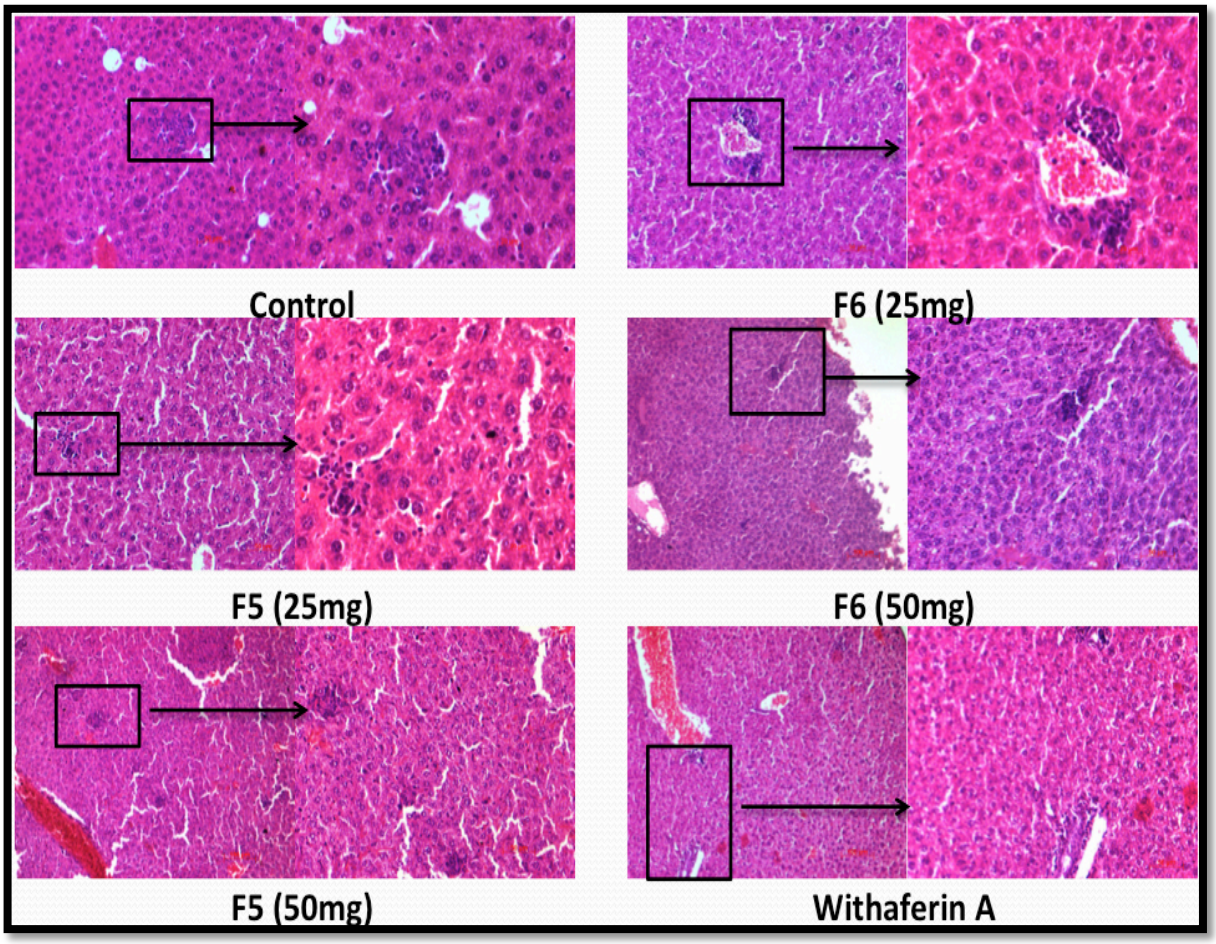


Figure 4.17: Effect of F5, F6 and withaferin A on histological changes in liver. Hematoxylin and eosin stained liver sections of different treatment mice groups.

4.4.4 RT-qPCR analysis of Th1/Th2 cytokines and iNOS mRNA

The disease outcome in the *Leishmania* infection is dependent on the cytokine environment (Seder et al., 1993; Reiner, 1994). We investigated the mRNA expression levels of Th1 (IFN-γ, IL-12), Th2 (IL-10, IL-4 & TGF-β) and iNOS from spleen tissue of infected and treated mice using RT-qPCR. The iNOS transcript, which is important for the control leishmaniasis, was significantly upregulated only in F6 (50mg/kg/b.wt) ($P<0.001$)

and miltefosine (P<0.01) treated mice, in all other treatment the up-regulation was insignificant. The pro-inflammatory cytokine expression, IFN-γ, which is essential for parasite clearance was same as in treated as well as control mice groups but the expression of IL-12p40 expression levels was increased significantly only miltefosine treated groups whereas F5, F6, and Withaferin-A groups didn't show any significant increase when compared to infected groups. IL-10, a Th2 cytokine is important for the parasite persistence whose expression is increased in the infected group but with treatment the expression levels drastically decreased in F5 (50mg/kg/b.wt) (P<0.001), F6 (25 & 50mg/kg/b.wt) (P<0.01, P<0.001) and Withaferin-A (P<0.01). Other Th2 cytokines IL-4 (P<0.01) and TGF-β (P<0.001) were also decreased in treated groups compared to an infected group where its expression is increased (**Figure 4.18A, B**). The IFN- γ to IL-10 expression ratio in the mice groups also signifies the extent of cure in F5, F6, and Withaferin-A treated groups compared to infected group (**Figure 4.18C**).

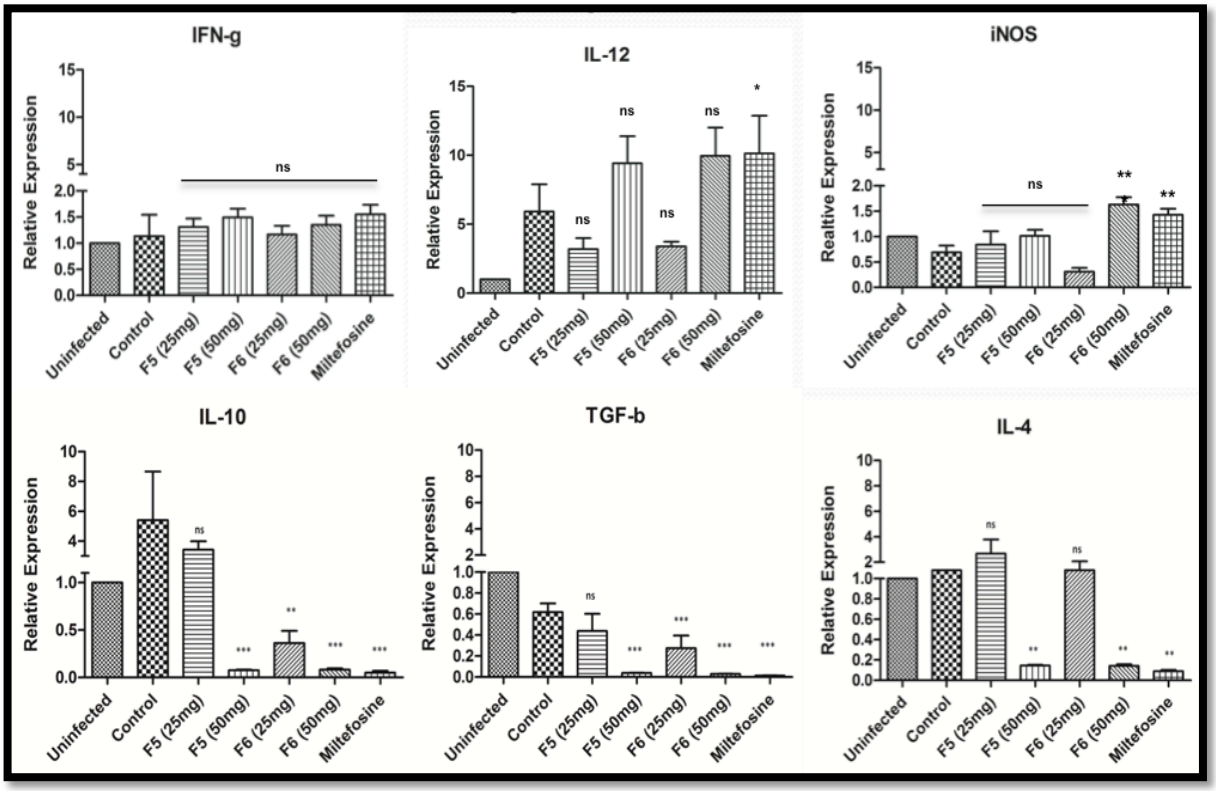


Figure 4.18 (A): Immunomodulatory effects of F5 and F6 in BALB/C mice spleen tissue using RT-qPCR analysis. mRNA expression analysis of Th1, Th2 cytokines and iNOS gene expression in F5 & F6 treated mice after 45 days post infection/post treatment. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

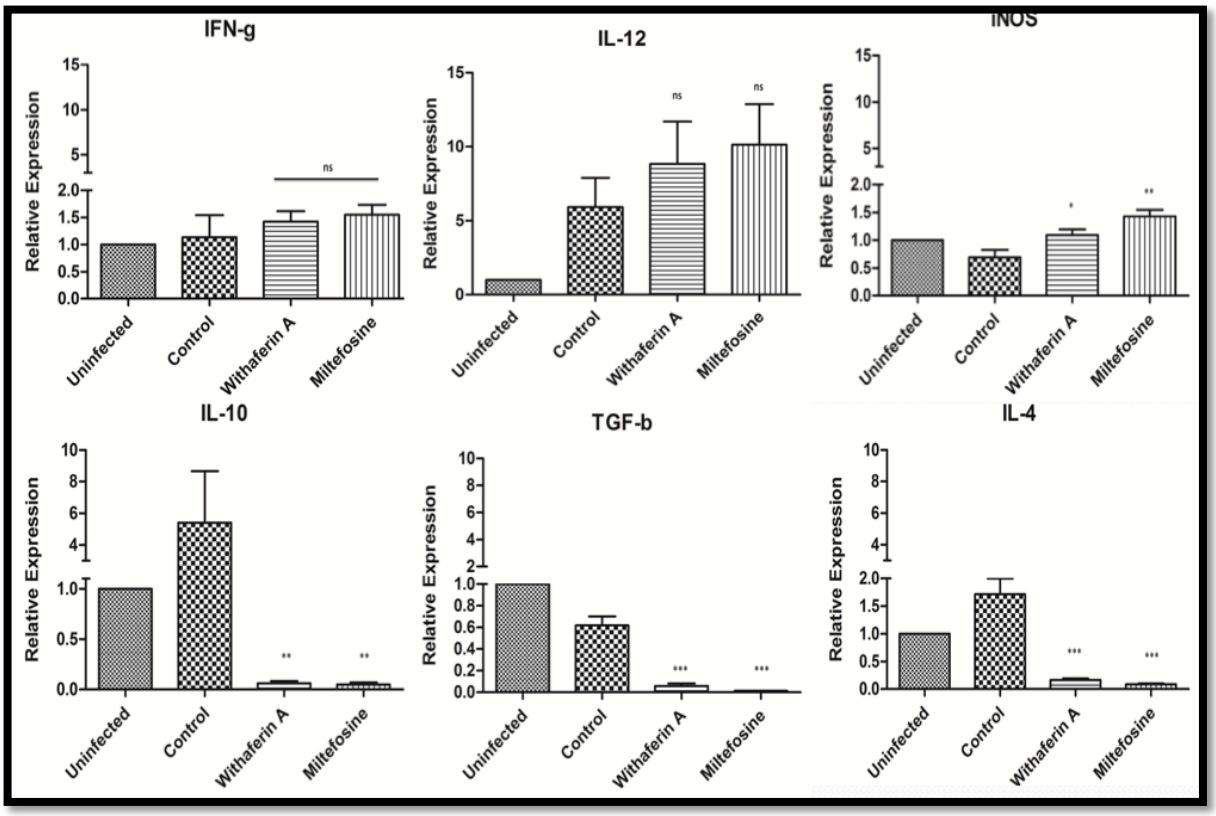


Figure 4.18 (B): Immunomodulatory effects of F5, F6 and withaferin A in BALB/C mice spleen tissue using RT-qPCR analysis. mRNA expression analysis of Th1, Th2 cytokines and iNOS gene expression in withaferin A treated mice. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

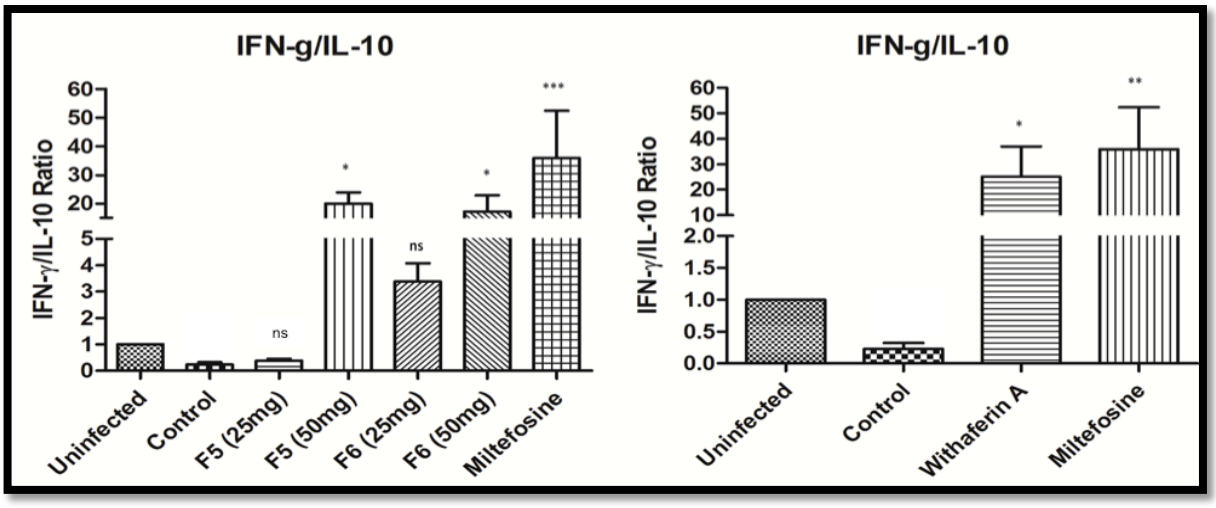


Figure 4.18 (C): Immunomodulatory effects of F5, F6 and withaferin A in BALB/C mice spleen tissue using RT-qPCR analysis. The graph depicts the IFN-γ/IL-10 ratio in F5, F6 and withaferin A treated mice. The experiment was done with five mice per group and significance between control (infected) and treated groups was calculated using one-way ANOVA with dunnett's post test (*P<0.05, **P<0.01 and ***P<0.001).

4.5 *Ld*PTR1 being the possible mode of action of withaferin A

Withaferin-A is an abundant withanolide present in *Withania somnifera* leaves and to some extent in roots. It has been known for its profound anti-cancer properties, but its role in counteracting the *Leishmania donovani* infection has to be explored. Pteridine reductase 1 (PTR1) is involved in pteridine salvage, and an important enzyme for the parasite growth could be targeted for an efficient antileishmanial drug. All trypanosomatid protozoans including *Leishmania* are unable to synthesize reduced pteridines (pterins and folates), which are necessary for nucleic acid and protein biosynthesis. The predominant role of PTR1 is to salvage oxidized pterins rather than to reduce folates, and for the *in vivo* growth of *Leishmania*, this enzyme is necessary for the reduction of biopterin.

4.5.1 Effect of withaferin A on *L. donovani* promastigotes

MTT assay was performed to evaluate the antileishmanial effect of Withaferin-A on the promastigote stage of the parasite. Withaferin-A inhibited the growth of parasites in dose-dependent manner, and its IC_{50} concentration was found to be 1.2μM (**Figure 4.19**). This indicates that Withaferin-A was able to induce death in the *L. donovani* promastigotes.

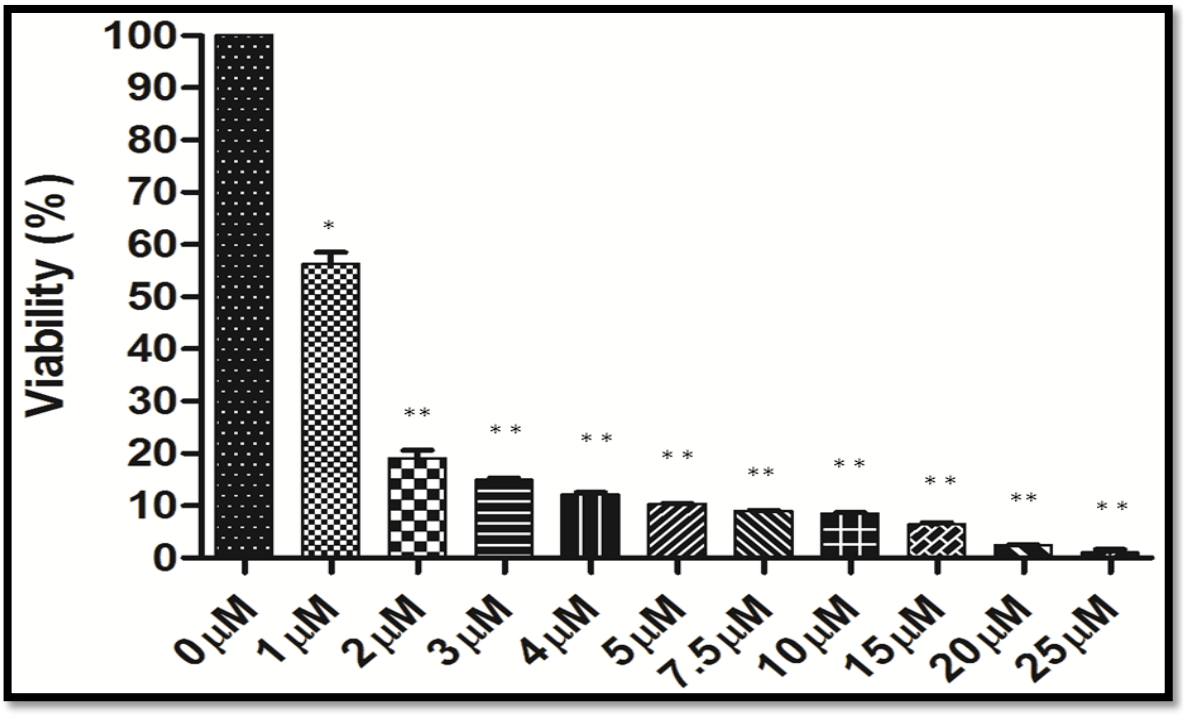
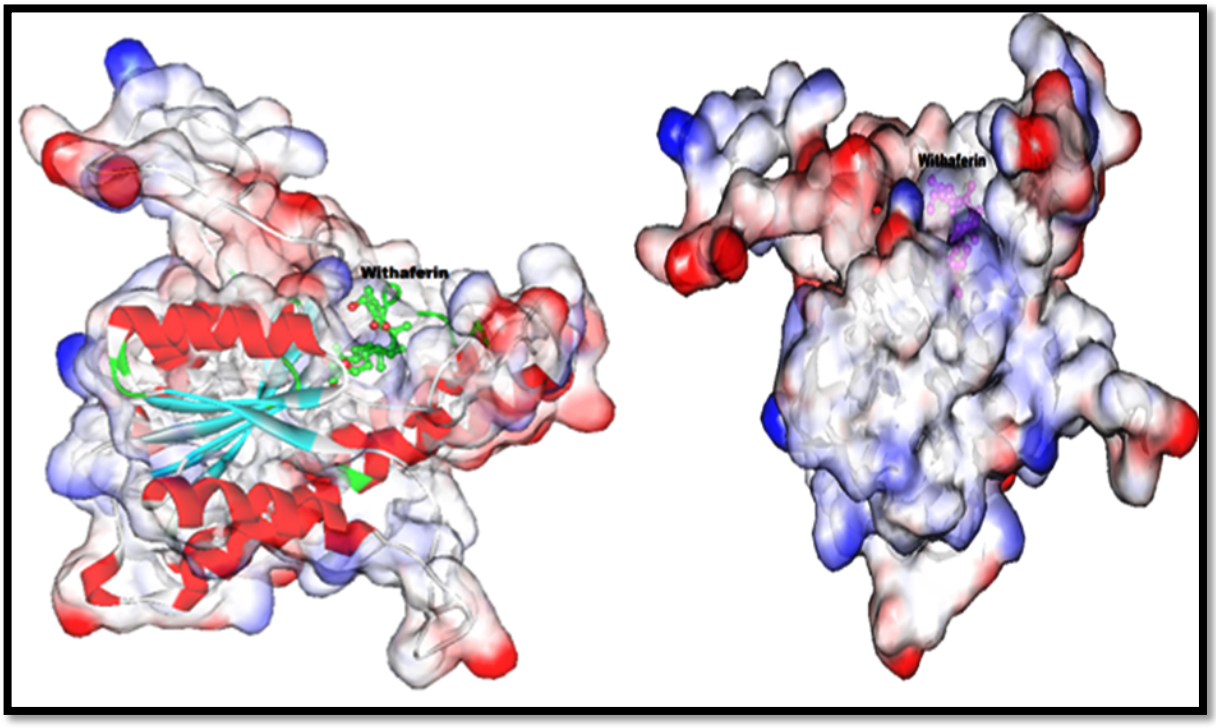


Figure 4.19: The antileishmanial effects of Withaferin-A on promastigotes after 72hrs of treatment using MTT assay. The graph depicts the percentage viability of the parasites in the dose dependent concentrations of Withaferin-A. The values are representative of three values ± SD (* P< 0.05, ** P< 0.01).

4.5.2 *In silico* docking of withaferin A for PTR1 inhibition

Molecular docking methods are commonly used for predicting the binding modes and energies for proteins or ligands. Docking study provided insight into the prediction of the affinity, activity, binding and orientation of Withaferin-A to our target protein molecule *Ld*PTR1. The analysis was based on E-total or free energy of binding and lowest docked energy was calculated. Withaferin-A was found to bind *Ld*PTR1 with the lowest binding energy of -6.73 KJ/Mol. Free binding energy is calculated as the sum of four energy terms namely, intermolecular energy, total internal energy, torsional free energy and unbound system energy. The major interactions shown in the *Ld*PTR1 binding site are the important H-bonds with Gly9, Arg13, His34, and Arg35. The other residues which form a pocket around withaferin A include Gly9, Lys12, Arg13, Gly15, tyr33, his34, Arg35, Leu62, Asn105, Ala106, Ser107, Phe109, Leu139, Asp177, Ser181, Gln182, Pro183, and Leu184. The docking of *Ld*PTR1 and Withaferin-A in different modes are shown in **Figure 3.20.** Our *in-silico* experiments demonstrate that Withaferin-A binds to *Ld*PTR1 and may inhibit its function and thus may act as a potential antileishmanial drug.



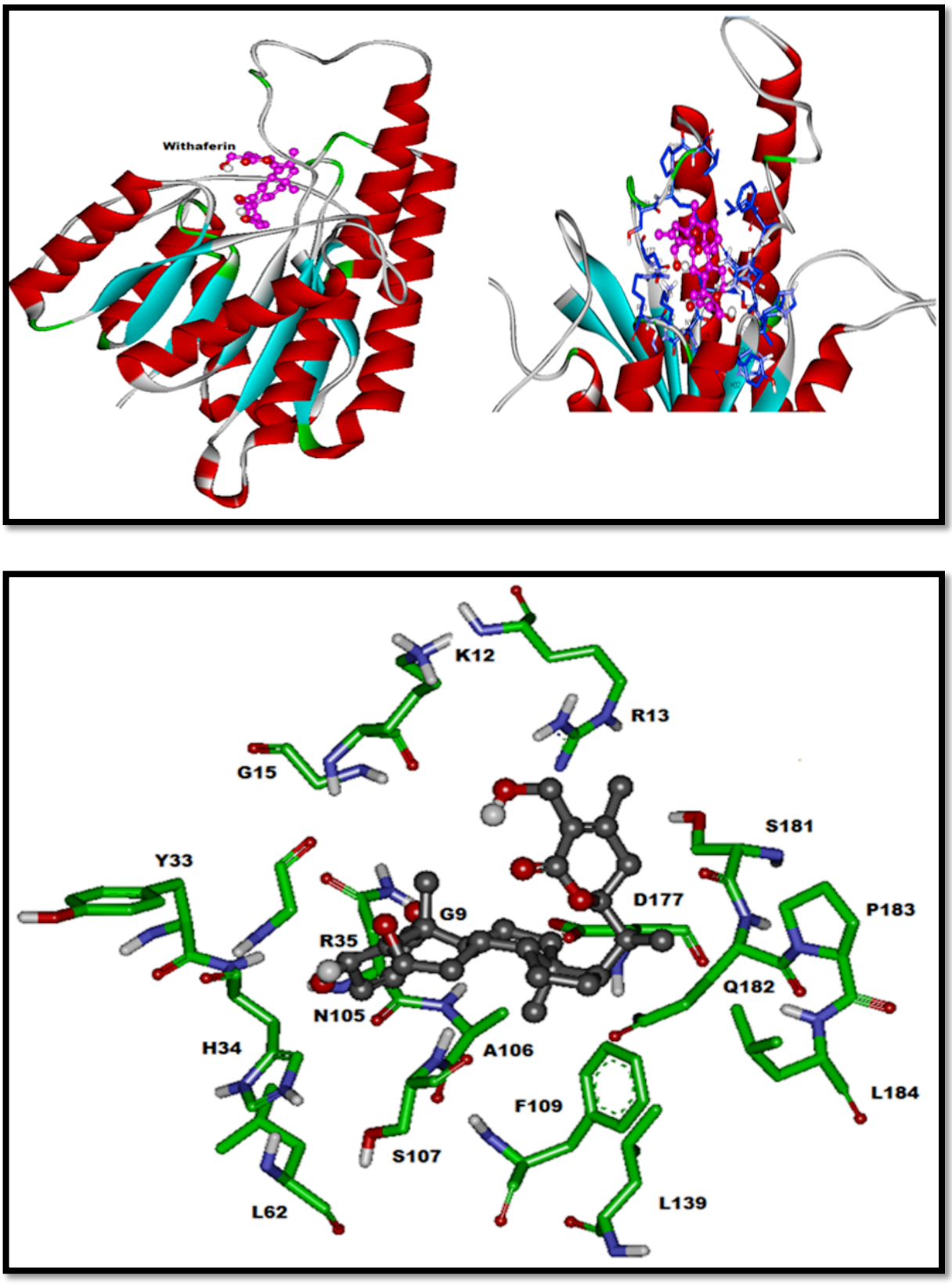


Figure 4.20: Predicted interaction model of Withaferin-A with *Ld*PTR1 a) space fill model of *Ld*PTR1 with Withaferin-A docked to the binding site. b) Red color shows alpha helices, blue indicates beta sheets, dark blue indicates the residues interacting with Withaferin-A and pink indicates the Withaferin-A docked in the binding site. c) Ball and stick model of the interacting residues in the *Ld*PTR1 with the Withaferin-A.

4.5.3 Cloning of *Ld*PTR1

The PTR1 gene, 867bp, was amplified from genomic DNA of DD8 strain of *L. donovani*. Both the amplicon and pET28a cloning/expression vector were double digested with EcoRI and XhoI (**Figure 4.21a**). These were ligated with T4 DNA ligase at 16^{o}C overnight. The ligated product was then transformed into *E. coli* DH5α competent cells and transformed cells were plated onto LB agar with kanamycin as the selection marker. The resultant colony was further screened for the presence of the plasmid with our desired gene. Three positive colonies for the presence of the PTR1 gene insert were confirmed by double digestion for the release of 867 bp insert and were visualized on 1% agarose gel and one representative plasmid is shown in the **Figure 4.21b**. The positive plasmids obtained were further confirmed by sequencing.

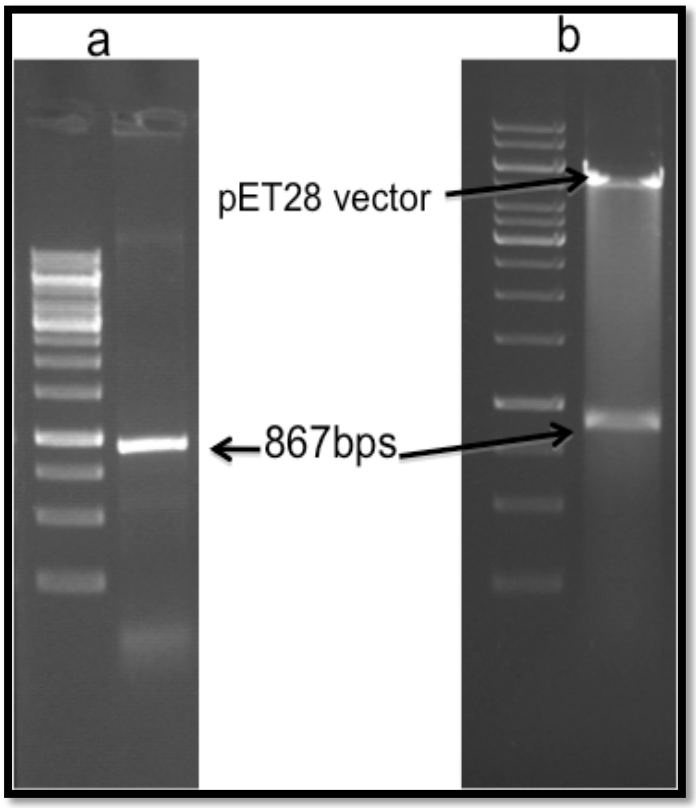


Figure 4.21: Agarose gel electrophoresis of the *Ld*PTR1 gene product. a) The figure shows the 867 bps amplified LdPTR1 product from the genomic DNA along with DNA ladder. b) Restriction digestion of the positive clone plasmid showing the free insert (867bps) and the plasmid (5.5Kb) with DNA ladder.

4.5.4 Expression and purification of *Ld*PTR1

The PTR1 gene encodes for a protein of 288aa with a molecular weight of ≈ 33KDa, and the PTR1 plasmid was transformed into BL21 (DE3) *E. coli* bacterial strain.

Small-scale cultures of the positive clones (selected on the basis of PCR screening) were subjected to IPTG induction to identify clones capable of expressing the predicted ≈33 KDa recombinant protein. Cultures were induced with 1mM IPTG at several time points (2-6hrs). The protein expression was found to be significantly induced at all the time points considered, the highest being at 4hrs. Hence, 4hrs induction was considered for further purification studies (**Figure 4.22a**). Expression of recombinant protein was also validated by western blot analysis using anti-His antibody (Sigma). The predicted ≈33KDa recombinant protein band was visualized on the membrane using BCPIP/NBT reagent (**Figure 4.22b**) that also indicates the recombinant protein was in frame with His-tag.

The relative distribution of protein of interest was examined in both soluble and insoluble fractions of bacterial cell lysates. After the sonication, both the fractions obtained were subjected to SDS- PAGE to detect the recombinant protein and almost 80% of recombinant protein was observed to present in the supernatant fraction. The recombinant protein was purified based on its His_6 tag by affinity chromatography using a Ni^{2+} chelating Sepharose column as described in materials and methods section. The purification conditions were standardized using increasing concentrations of imidazole (50mM-200mM) in the elution buffer after three washes with wash buffer containing 20mM imidazole. The pre-equilibration step of Ni^{2+} column with wash buffer containing 20mM Imidazole removed all the non-specifically bound proteins. In addition, elutions by imidazole gradient facilitated the release of more tightly bound non-specific proteins in the early fractions of elution. The purity of the desired protein was more than 95% in 200mM imidazole elution, and further large-scale purification was done using the same imidazole concentration (**Figure 4.23**). The various eluted fractions at 200mM imidazole concentration were pooled and dialyzed overnight at 4^oC in 1L of dialysis buffer that had been changed for every 6hrs intervals. The purified protein was stored at -80^oC for further studies.

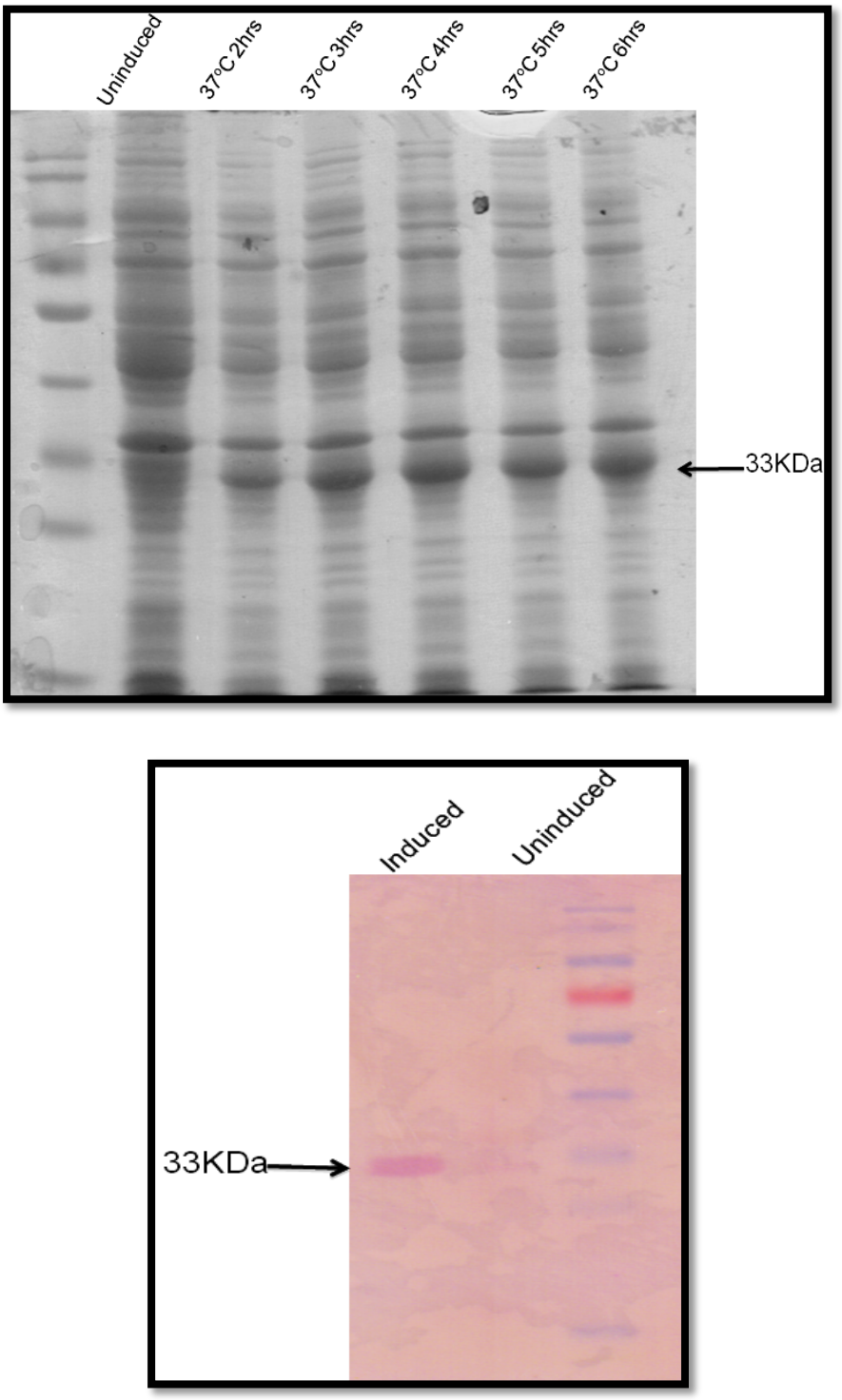


Figure 4.22: Induction and western blot of *Ld*PTR1. a) Proteins were separated on 12% SDS-PAGE before and after induction with 1mM IPTG at different time points. The induced *Ld*PTR1 (33kDa) was represented in the figure. b) Western blot analysis using anti-his antibody using the uninduced and induced crude lysate along with protein marker. The blot was developed using anti mouse ALP conjugate.

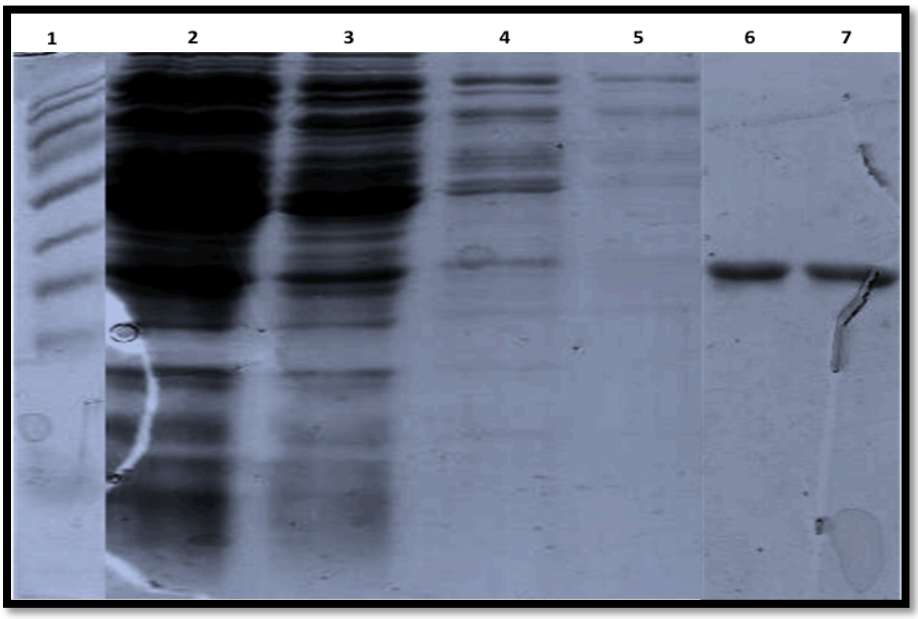


Figure 4.23: The expressed His-tag fusion protein was purified using a Ni^{2+} chelating Sepharose column affinity chromatography. The purified proteins were separated on 12% SDS-PAGE and stained with coomassie blue. Lane 1: Protein marker, Lane 2: flow through, Lane 3-5: washes with the wash buffer, Lane 6-7: Elution of the protein with 200mM imidazole.

4.5.5 Enzyme inhibition studies of recombinant PTR1 with withaferin A

PTR1 enzyme kinetics was performed using Michaelis-Menten reaction. First, we optimized the enzyme concentration for carrying out the reaction using biopterin (100µM) as substrate and NADPH (100µM) as co-factor to determine the Vmax, Km for the biopterin substrate. The velocity of PTR1 increased up to 0.696µM of the substrate after which there was no change in the velocity of the reaction with an increase in the substrate concentration (**Figure 4.24a**). Hence, we used 0.696µM as the optimum enzyme concentration for further reactions. The enzyme assay was performed with various concentrations of substrate, 6-biopterin (0-120µM) and found the Vmax and Km of the reaction to be 2.5 µmol/min/mg and 35 µM respectively (**Figure 4.24b**). The enzyme kinetics was performed using Withaferin-A as an inhibitor and using Lineweaver-Burk plot, the Ki value was determined to be 0.9µM and using Methotrexate (a positive control) as inhibitor, the Ki value was found to be 1.126µM. (**Figure 4.24c**). It followed an uncompetitive mode of inhibition.

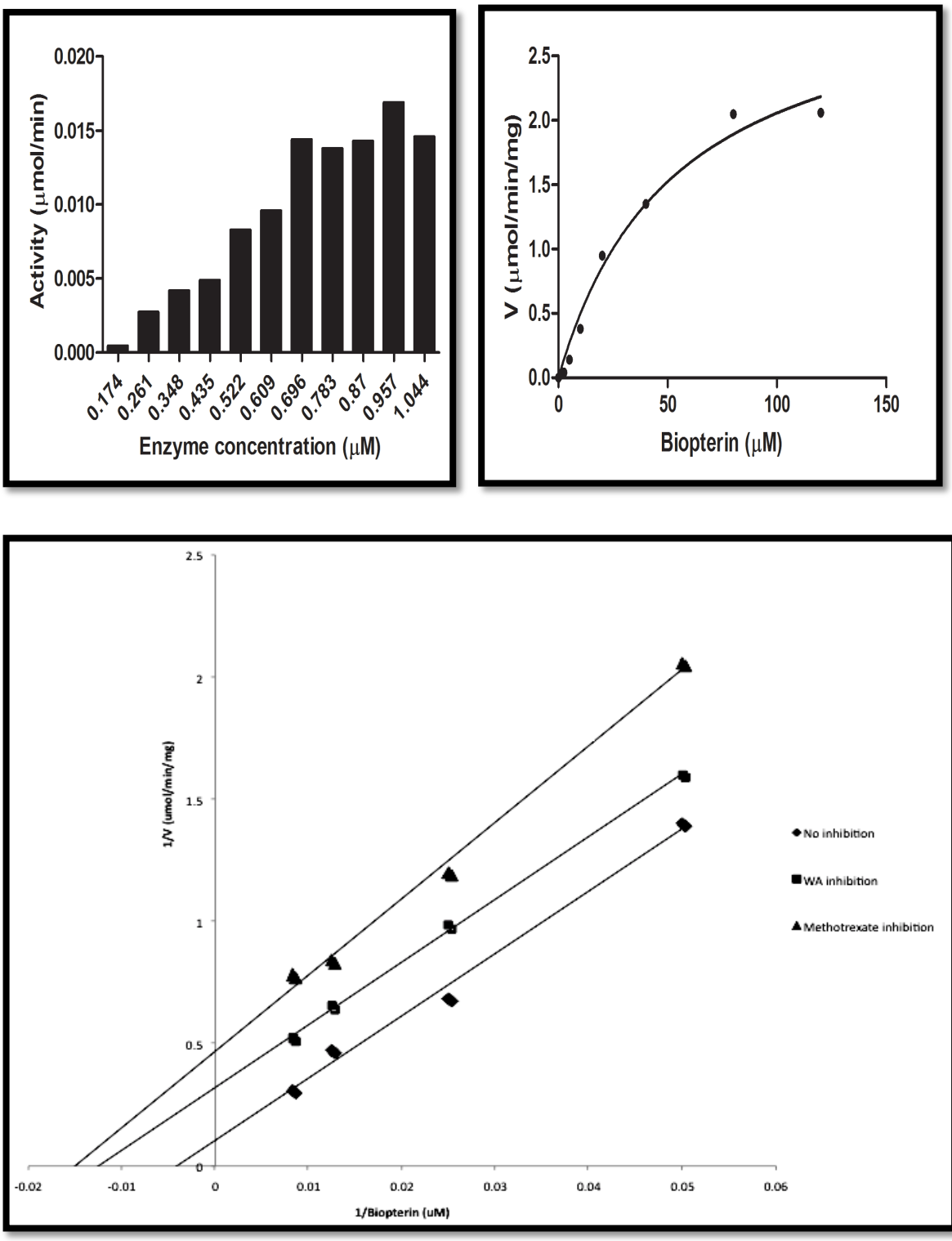


Figure 4.24: Enzyme kinetics of purified *Ld*PTR1 a) The optimum concentration of the enzyme was carried using 100µM NADPH and 100µM biopterin. It was found to be 0.696µM, which was used for further assays. b) The recombinant *Ld*PTR1 enzyme assay was performed using 20mM sodium acetate buffer (pH 4.8) for the different concentrations of substrate biopterin. c) The enzyme inhibition studies were performed for methotrexate and Withaferin-A using lineweaver burk plot. The Ki values for each compound were calculated from the graph. The experiments were repeated three times and figure represents the result from one such experiment.

4.5.6 Enzyme inhibition assay on parasite crude lysates

To prove the PTR1 enzyme inhibition in the parasites upon Withaferin-A treatment, we performed the PTR1 enzyme assay in the total protein lysates isolated from untreated and Withaferin-A treated parasites. Though there was no significant difference at 6hrs treatment, the enzyme inhibition was observed to increase with time. . At 12hrs, the inhibition was more significant than 6hrs treatment (p=0.041) whereas, at 24hrs, the enzyme inhibition was much more significant (p=0.033). The experiment was repeated three times and data represents the mean of three independent experimental values ± SD (**Figure 4.25**). This reveals that Withaferin-A indeed inhibits the *Ld*PTR1 enzyme *in vitro*.

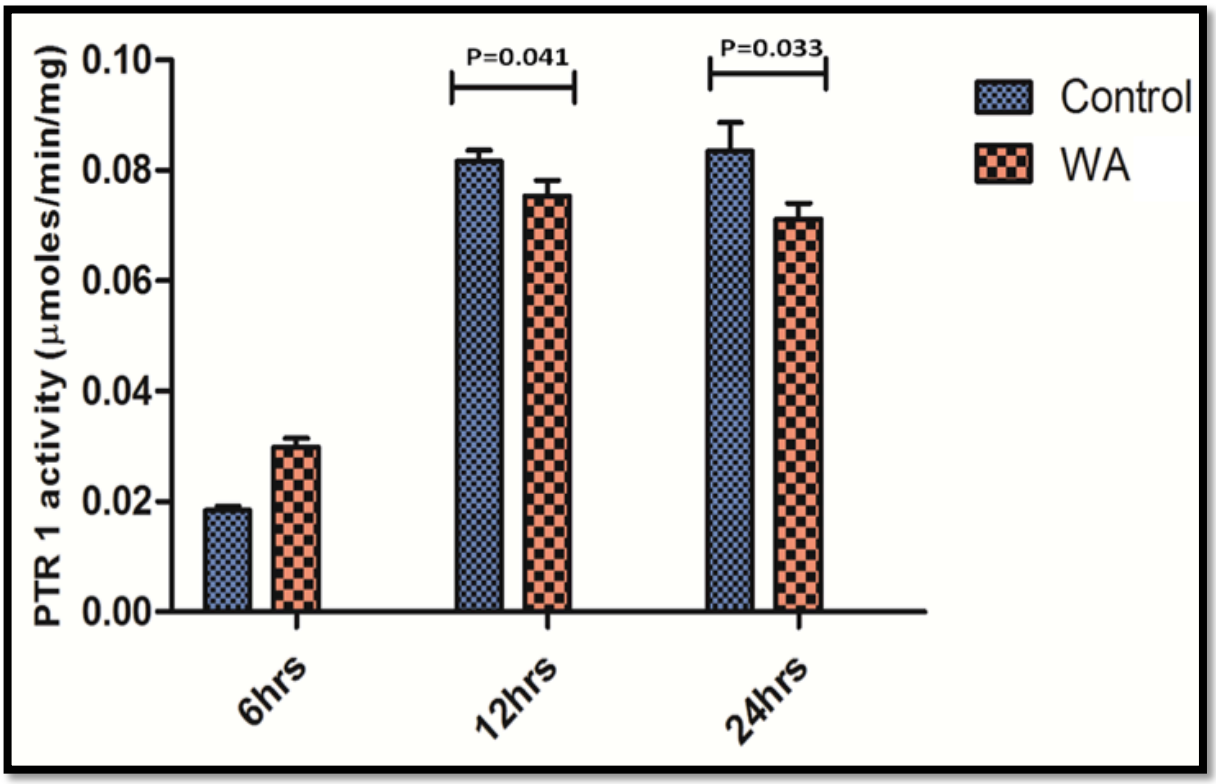
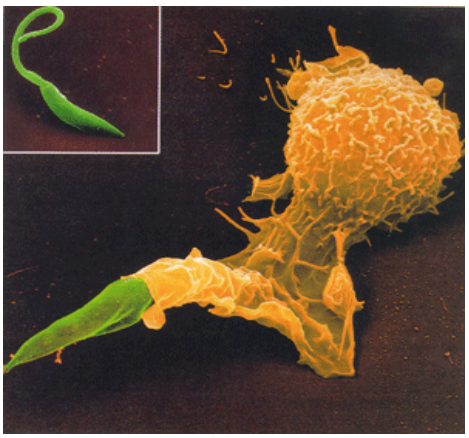
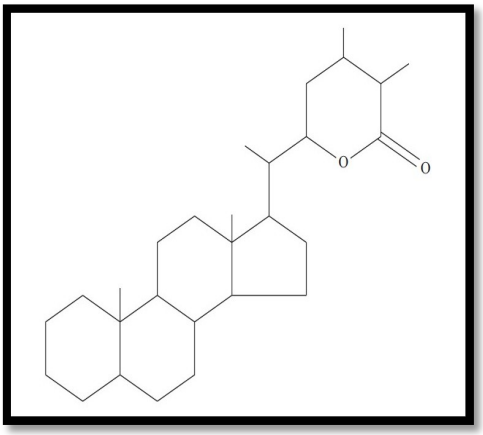


Figure 4.25: PTR1 enzyme activity in the crude parasite lysates of control and Withaferin-A treated at different time intervals. The values are representative of three independent experiments and significant values indicate the comparison of control with treated samples at that particular time point.

Chapter V:

Discussion





Separation and characterization of different phytochemicals from herbs, and screening of plant extracts for novel leads is highly dependent on the extraction method used. Solvent extraction is the most frequently used procedure for preparation of extracts from plant materials. Nature of extracting solvents (polarity), extraction time and temperature are dependent on the extract yield and resulting biological activities of plant material, also on sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples (Shabir et al. 2011; Sultana et al. 2009). In the sequential extraction, solvents, such as methanol, ethanol, ethyl acetate, acetone, chloroform and their combinations are suitable solvents and have been used for the extraction of therapeutically desired constituents from plant materials, often with different proportions of water to get better extraction efficiency. We used the solvents in the increasing order of their polarity (Hexane < Ethyl acetate < Ethanol < water) for the extraction of the withanolides from the *W. somnifera* leaves and we found almost all withanolides being extracted in the ethanolic fraction. Using an ethanolic extract of *Withania somnifera* leaves which was further fractionated using silica gel column yielded seven fractions out which fraction 5 & 6 had the presence of withanolides. LC-MS/MS analysis of fractions F5 and F6 confirmed the presence of withanolides by comparing the mass fragmentation patterns from PubChem library.

We explored the leishmanicidal activity of these withanolides from *Withania somnifera* fractions in *L. donovani* promastigotes *in vitro*. The apoptosis mechanism in *Leishmania* species is not well defined. But, in response to antileishmanial agents like amphotericin B, miltefosine and sodium stibogluconate, apoptotic events like DNA fragmentation, ROS production, disruption of mitochondrial membrane potential, etc., are observed (Lee et al., 2002). *Withania somnifera,* routinely used as ayurvedic medicine in Indian subcontinent, is attributed with various medicinal properties (Rajani et al., 2006; Sharma et al., 2009) and these properties are due to the compounds called withanolides. Here, we have demonstrated the role of withanolides in inducing apoptotic-like death in promastigote stage of parasites The cytotoxic effects of both F5 and F6 on the parasites demonstrated using PI dye exclusion test showed significant activity on the promastigote stage of parasite. For the first time, we showed that withanolides extracted from *Withania somnifera* leaves also have a profound effect on the morphology of the parasites, treated parasites exhibited apoptotic-like features such as shrinkage of the cell membrane, rounding of the parasite which was previously shown by Sharma *et al.* 2009 in WS roots.

During early apoptosis, there is a translocation of phosphatidylserine from inner membrane to the outer membrane and this event has been evident in many metazoans and unicellular organisms like *Leishmania* (Mehta and Shaha, 2009; Sudhandiran and Shaha, 2003; Koonin and Aravind, 2002) and this can be detected by using annexin V which specifically binds to PS. Both the fractions (F5 and F6) containing withanolides induced the externalization of phosphatidylserine on the outer membrane after 15 hrs of incubation. Cell cycle arrest at sub G_0/G_1, which is an indication of the cells entering into the apoptotic mode of death. Withanolides treated parasites showed an increase in sub G_0/G_1 population in time-dependent manner from 6-48 hrs of incubation. F6 treatment showed more increase with the percentage reaching 18.48 after 48 hrs of incubation. In the late stages of apoptosis, cleavage of DNA into its nucleosomal components and introduction of DNA nicks are the characteristic features (Verma and Dey, 2004). Withanolide treated parasites showed TUNEL positive cells after 72 hrs of incubation which proved that withanolides induce apoptotic-like death in *Leishmania donovani* promastigotes *in vitro* and this is a preceding event for various changes in the cell.

Withaferin-A, being abundant in withanolides, has been shown to possess antileishmanial activity as previously reported by Sen. *et al* 2007 who have showed that Withaferin-A is a potent inhibitor of protein kinase C in *L. donovani* promastigotes. They also showed that Withaferin-A directly targets mitochondria to release ROS thereby disrupting the mitochondrial membrane potential leading to apoptosis. In accordance with these findings, our results show that withanolides have a profound effect on the mitochondrial status of the parasites. Induction of ROS inside the cell during the process of apoptosis is an important event that in turn drives the neighboring cells into apoptosis (Chipuk and Green, 2005). Withanolides induce ROS production in treated parasites during the first 24 hrs of incubation compared to control cells with a drastic increase after 48 hrs of incubation. Disruption of mitochondrial membrane potential is an event followed by the production of ROS, which is also found to be promoted by withanolides. These features, in addition to the externalization of phosphatidylserine and permeation of PI, provide an evidence for the apoptosis inducing property of withanolides in *Leishmania*. Thus, our findings prove that withanolides exert the antileishmanial activity through apoptotic-like pathway.

In the recent times the emergence of resistance to the present existing drugs against leishmaniasis and increase in HIV co-infection cases with *Leishmania* are posing a great

challenge for the development of new therapeutics (Redhu et al., 2006). Plant based products make an excellent repertoire for developing bioactive compounds against leishmaniasis (Patricia et al., 2007). In the recent times, plant products have been used as immunomodulators in various infectious diseases (Salem, 2005; Mittal and Singh, 2009; Fulzele, 2003; Lai, 2002; Samjon et al., 2007). During leishmaniasis, the immune system is severely depressed leading to exacerbation of the disease. In this context an antileishmanial drug, which can effectively reverse the immunosuppression, besides killing the parasite is most desirable. It is also known that therapeutic efficacy of antiparasitic drugs works in synchronization with the immune system (Berger and Fairlamb, 1992). *Withania somnifera* in the Indian traditional medicine system is considered as a wonder drug, which has many properties ranging from antibacterial to anticancer (Owais et al., 2005; Ghosh, 2009; Rajani et al., 2006). To further explore the activity of these withanolides on amastigotes stage of the parasite in peritoneal macrophages and BALB/C model, we used F5, F6 and Withaferin-A at different concentrations in peritoneal macrophage model. We observed that more than 50% decrease in the parasite load in all treated macrophages compared to the infected control macrophages. Our result was in accordance with the previous report showing that HPLC purified A6 fraction isolated from ashwagandha showed an IC_{50} value of 9.5µg/ml in the amastigotes stage of the parasite (Sharma et al., 2009). In the *in vivo* set up, we developed chronic VL in BALB/C mice by infecting with *L. donovani* promastigotes for 28 days and treated the mice with F5 (25 & 50mg/kg/b.wt), F6 (25 & 50mg/kg/b.wt) for 10 days orally. There was a drastic reduction in parasite burden in spleen and liver in F5 (50mg/kg/b.wt) and F6 (25 & 50mg/kg/b.wt), but in case of F5 (25mg/kg/b.wt) we didn't find any reduction in parasite burden in spleen. We found the parasite reduction only in liver tissue, which may be due ineffective activation of Th1 cytokines in spleen by F5 at that concentration. With Withaferin-A (2mg/kg/b.wt), treatment the reduction in spleen parasite burden was only 32%, which is comparatively less than F5 and F6. Next we tried to compare the spleen and liver weights of infected and treated groups to investigate whether the treatment has any effect on the biological parameters, but our results showed that there was no statistical difference between the groups.

Macrophages activation results in the increase in the microbicidal activity, which plays a major role in resistance against *Leishmania* infection, of which ROS and NO are important players (Melby et al., 2001; Shah et al., 2002). Our results suggest that F5, F6 and withaferin A exert their antileishmanial activity via production ROS in peritoneal

macrophages compared to infected PMM but not through NO production as we didn't find any significant increase between control and treated. Depression in cell-mediated immunity (CMI) and B-cell activation are consequences of VL (Basak et al., 1992). CMI is important in mediating an effective Th1 type of immune response, which is important in controlling the *Leishmania* infection (Kaye et al., 1991). In Th1 response, IFN-γ is a signature cytokine, which helps in macrophage activation, thereby generating the microbicidal response along with TNF-α for effective killing of the parasite through activation of iNOS transcript for the production of NO (Liew et al., 1990a, 1990b). During *Leishmania* infection, increase in expression of Th2 cytokines IL-4 and IL-10, which exacerbate the severity of VL (Ghalib et al., 1993; Karp et al., 1993; Kaye et al., 1991) and is responsible for the progression of VL (Karp et al., 1993). In peritoneal macrophages with the treatment of F5, F6 and withaferin A, we didn't find any statistical increase in the IFN-γ mRNA expression but the IL-10 mRNA expression was decreased significantly. When the ratio of IFN-γ/IL-10 was observed, there was a drastic increase in all the treated macrophages compared to control, which indicates the parasite clearance from the macrophages (Paul et al., 2012). In culture supernatants of PMM, there was no increase in IFN-γ levels supporting the mRNA expression levels, but there was increase in TNF-α levels in F6 treated, which might be the reason for the increase in ROS levels. In BALB/C model of infection our studies confirmed that F5, F6, and Withaferin-A indeed upregulate Th1 cytokines (IFN-γ, IL-12) and downregulate Th2 cytokines (IL-10, IL-4, TGF-β) supporting the WS role as immunomodulator. The increase in IFN-γ/IL-10 ratio also supports the antileishmanial effects of this above treatment.

The immunological status of mice were evaluated using parasite specific IgG1 and IgG2a levels which give an indication of Th1 or Th2 type of immune response (Siddiqui et al., 2012). We found that Th1 specific IgG2a levels were increased in treated mice groups compared to control groups, the but there was no significance in a decrease of Th2 specific IgG1 levels in treated groups. This might indicate that withanolides from WS might clear the parasites by specifically augmenting Th1 type of immune response. Histopathological changes in liver tissues reflect the successful clearance of parasites. During the hepatic immune response against the parasite, there is considerable infiltration of immune cells at the infection site, which is followed by formation of granuloma structure. These granuloma structures clear the parasite with the time and disappearance of granuloma is observed (Baker and Kaye, 1999). In infected mice liver there was huge infiltration of monocytes

surrounding the parasites is observed and with treatment of F5, F6 and Withaferin-A there was no change in granuloma numbers but there was decrease in parasite numbers in these granuloma, suggesting the efficacy of these treatment.

During the 21st century, the plant-based compounds have gathered much importance in the trials of discovering new antileishmanial drugs with high efficacy and fewer side effects. Our results show that withanolides extracted from *W. somnifera* leaves exhibit potent antileishmanial activity against the promastigote stage of *L. donovani* parasites, but the mode of action of these withanolides has not been elucidated yet. A previous study in the *L. donovani* showed that Withaferin-A inhibits protein kinase, which induces apoptosis through apoptotic topoisomerase I- DNA complex (Sen et al., 2007). A recent study also showed that the antileishmanial activity of the herbal drug from *W. somnifera* is exhibited through blocking of protein kinase C signaling pathway (Grover et al., 2012). Till now, no report has demonstrated its antileishmanial activity on any of the critical pathways that are essential for the growth of the parasite. We focused on the mode of action of Withaferin-A, an abundant withanolide in the leaves of *W. somnifera*, on the Pteridine salvage enzyme of the parasite Pteridine reductase 1.

The novel pathways exclusively present in trypanosomatids would provide for excellent drug targets among which pteridine reductase pathway stands one. *L. donovani* parasites are auxotrophs for pteridines that play a significant role in reducing pteridines and also functions as a metabolic bypass for DHFR-TS by reducing the folates. Thus, an inhibitor targeting Pteridine reductase 1 could also possibly target the DHFR-TS enzyme due to the structural similarity of the substrates. A single compound might be found with good efficacy against both enzymes due to the structural similarities of the substrates (Nare et al., 1997a, b; Knighton et al., 1998; Zuccotto et al., 1998). The reduced bioavailability of pterins makes the parasites more susceptible to the oxidant damage, which correlates with our previous experiments that showed the enhancement of the production of cellular reactive oxygen species.

In this regard, we determined the effect of Withaferin-A on promastigote stage of the parasite, and it was found to exhibit the antileishmanial effect in a dose-dependent manner. The IC_{50} value was determined to be 1.2μM from the graph. In order to decipher its mode of action on the parasites, we performed an in-silico docking analysis of parasite's Pteridine reductase 1 enzyme with Withaferin-A, which revealed its binding residues. Previous

docking study with monastrol by Kaur et al. (2010) revealed that hydrophobic residues namely, Arg17, Asn109, Ser111, Asp181, Tyr191, Tyr194, Lys198, Leu226 and Ala230 mainly enfold the active sites of *Ld*PTR1, but Withaferin A binds with *Ld*PTR1 and forms hydrogen bonding with Gly9, Arg13, His34 and Arg35 whereas the residues involved in forming a pocket around the withaferin A are Gly9, Lys12, Arg13, Gly15, Tyr33, His34, Arg35, Leu62, Asn105, Ala106, Ser107, Phe109, Leu139, Asp177, Ser181, Gln182, Pro183 and Leu184. The binding energy of withaferin A with *Ld*PTR1 was found to be -6.73KJ/mol. The determined high binding affinity of Withaferin-A with *Ld*PTR1 might provide an insinuation for the mechanism of biological activity of Withaferin-A.

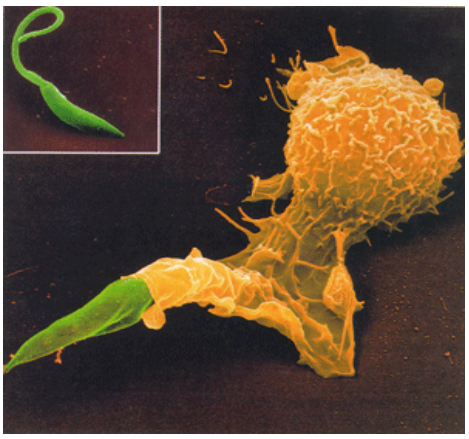
Subsequently, the PTR1 gene was amplified from DD8 strain of *L. donovani* and cloned into pET28a vector. The protein was expressed in BL21 (DE3) strain of *E.coli*. The protein was purified from the soluble fraction through Ni^{2+} chelating Sepharose column (Kumar et al., 2004). In the gradient elution of 50mM-200mM imidazole, the peak fraction was eluted at 200mM imidazole concentration containing with the purity of protein more than 95%.

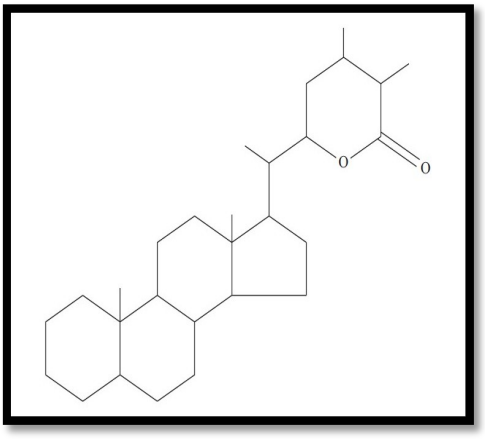
The docking results were validated using the inhibition studies of recombinant enzyme *Ld*PTR1 with Withaferin-A. The optimal concentration of *Ld*PTR1 for its activity was found to be 0.696µM. We have used the sodium acetate buffer pH 4.8 for determining the activity of *Ld*PTR1 as previously reported (Kaur et al., 2011). The subsequent assays were performed at this optimum pH and enzyme concentration. We estimated the PTR1 activity with oxidized biopterin using standard Michaelis-Menten kinetics. Km and Vmax values for biopterin substrate were derived from Lineweaver-Burk plot. Methotrexate is a known antifolate inhibitor of Plasmodium falciparum DHFR (Shallom et al., 1999). We used methotrexate as inhibitor for *Ld*PTR1 and found its Ki value as 1.126µM against the substrate biopterin. Further inhibition studies of *Ld*PTR1 performed with withaferin A yielded a Ki value of 0.9µM. In addition to this, the time-dependent inhibition (at IC_{50} concentration) of *Ld*PTR1 in withaferin-A treated parasite lysates suggests its inhibitory role in promastigote stage *in-vitro*. This might be due to the independent role of pterins in *Leishmania* growth apart from folate biosynthesis (Nare et al., 1997a,b). Additional preclinical experiments of withaferin A in mice model for visceral infection would shed light on the mode of action *in vivo*.

Inventory of antileishmanial compounds in clinical use is very minuscule, there is an urgent need for new compounds, which are cheap, affordable, safe and effective, and in these natural products from plants are the forerunners. Plant-based compounds are an important alternative and may be complimentary to the existing drugs. In conclusion F5, F6 and withaferin A had two synergistic actions on *L. donovani* infection. Firstly, it was able to induce the cytotoxic effect in both *ex vivo* and *in vivo* model and secondly, it was able to induce the immune system towards the protective Th1 arm by suppressing the susceptible Th2 arm of the immune system. The study of other active principles, their mechanisms and effect of these withanolides on various immune cells are in progress in our laboratory. Further studies will confirm whether these withanolides can be used as a mixture of compounds or as single entities for effective antileishmanial and immunomodulatory activities.

Chapter VI:

Summary





Keeping in view about the toxicity and emergence of resistance with the available drugs to treat visceral leishmaniasis in the endemic regions, there seems to be an urgent need for the alternative treatment strategies to efficiently counteract this disease. In this direction, compounds from the plant origin might be good option in the search for an efficient antileishmanial. Visceral leishmaniasis is associated with immune suppression predominately Th2 type immune response that supports the propagation of parasites inside the host macrophages and resistance is associated with induction of Th1 type response. Previous reports have demonstrated the immune modulating role of withanolides from *Withania somnifera* in various disease conditions. Even though *W. somnifera* has many health promoting properties, so far it has not been evaluated for its antileishmanial activity in visceral leishmaniasis. The objective of the present study was therefore to evaluate the antileishmanial and immunomodulatory properties of withanolides isolated from *W. somnifera* leaves using both peritoneal mouse macrophages as *ex vivo* host system and BALB/c mice as *in vivo* model.

The results of the sequential extraction of *Withania somnifera* leaf powder with n-hexane, ethyl acetate, ethanol and water showed that most of the withanolides are extracted in the ethanol solvent. Thin layer chromatography and identification of purple colour withanolides by staining it with p-anisaldehyde sulphuric acid, confirmed the presence of withanolides. Further, the active ethanolic extract was fractionated using silica gel chromatography with the solvent gradient of ethyl acetate and ethanol. The fractionation yielded total of seven fractions, which were further screened for the antileishmanial activity on the promastigotes using MTT assay. Fraction 5 and 6 were most active against the parasites and also they showed the presence of abundant withanolides compared to other fractions. We also made an effort to identify the components in the active fractions by LC-MS/MS analysis by comparing the compounds present in the mass bank using their molecular masses. Further, we investigated the antileishmanial effects of F5 and F6 on the promastigote stage of the parasite. Using PI staining, we found the IC_{50} value of F5 and F6 to be 52µg/ml and 13µg/ml respectively. Further the mode of action of these fractions on the promastigotes was investigated using DNA cell cycle analysis, DNA fragmentation by TUNEL assay, externalization of phosphatidylserine, measurement of ROS and mitochondrial membrane potential and we concluded that these fractions induce apoptotic like death in the parasite. As *Leishmania* being a eukaryote, it lacks the proteins like

caspases, which are involved in the apoptosis. So other features that are also exhibited during apoptosis are shown in *Leishmania*. Hence, we termed it as apoptotic like death.

We next investigated the antileishmanial and immunomodulatory effects of the active fractions F5 and F6 in intracellular amastigotes using mouse peritoneal macrophages as host system. Cytotoxic assay of the active fractions on the macrophages revealed that it doesn't have any effect up to certain concentration. The infection rate of parasites in the macrophages has significantly decreased with the treatment of F5 and F6. To identify the mode of clearance of parasites from host macrophages, we measured the nitrite levels present in the culture supernatants of infected and treated macrophages after 48hrs and found to be unaltered. ROS and NO are key mediators of microbicidal molecule employed by the macrophages for its defense against the pathogens. In our inverstigation, we didn't find any significant NO production in treated macrophages, which intended us to examine the ROS levels in the macrophages. So we tried to estimate the amount of ROS produced inside the cells using H_2DCFDA dye and found that active fractions F5 and F6 significantly upregulated the production of ROS. The mode of killing in the macrophages was through the production of ROS instead of NO. The immunomodulatory activity of these active fractions were analyzed by qRT-PCR of IFN-γ and IL-10 mRNA expression and we found no change in IFN-γ expression but there was a significant decrease in IL-10 expression. Overall the IFN-γ/IL-10 ratio is increased with treatment with fractions compared control implies Th1 protective response. Withaferin-A being the abundant withanolide in WS leaves, we also investigated its antileishmanial and immunomodulatory effect on the intracellular amastigotes. In mouse peritoneal macrophages, Withaferin-A decreased the parasite burden in a dose-dependent manner. There was significant production of ROS with an unaltered production of NO in Withaferin-A treated macrophages. The immunomodulatory activity of withaferin A showed the decrease in IL-10 mRNA expression and the increase in IFN-γ/IL-10 ratio suggesting the protective Th1 response.

The chronic visceral leishmaniasis can be mimicked in BALB/c mice which is susceptible to *Leishmania* infection. The experimental visceral leishmaniasis can be generated by infecting mice with *L. donovani* promastigotes for 28 days. The antileishmanial and immunomodulatory activities of these withanolides were tested in BALB/C infection model. Mice were infected with *L. donovani* metacyclic promastigotes for 28days and then we treated with F5 (25mg & 50mg/kg/B.wt), F6 (25mg & 50mg/kg/B.wt), Withaferin-A (2mg/kg/B.wt) and Miltefosine (5mg/kg/B.wt) for 10

consecutive days. Active fractions were given orally and Withaferin-A was given intraperitoneally. We estimated the parasite burden in spleen and liver by counting the amastigotes, we found that there was a significant decrease in parasite load in all the treated groups except F5 (25mg/kg/B.wt). In the liver, the parasite burden was reduced in all the treated groups. Next we estimated the amount of IgG1 (Th2 specific) and IgG2a (Th1 specific) antibodies titers in mice serum through ELISA and found that withanolides treatment increased the IgG2a titers, but showed no significant change in IgG1 titers. Granuloma formation in the liver is the hallmark for the clearance of the parasite and we estimated the granuloma formation in liver histology sections in treated samples. The liver section of the infected mice showed fatty degeneration of the tissue, which was restored with the treatment of F5, F6 and Withaferin-A. There is infiltration of T lymphocytes, which subsequently develops into a granuloma harboring the amastigotes through which the infection is cleared in the liver with time. In the treated groups, formation of loose and scattered granuloma indicates the clearance of parasites and in the infected group the granuloma structure is dense and tight. Withaferin-A treatment also shows the same architecture as compared to other treatment. These granuloma structures didn't harbor any parasite as compared to the infected group.

The disease outcome in the *Leishmania* infection is dependent on the cytokine environment (Seder et al., 1993; Reiner, 1994). We investigated the mRNA expression levels of Th1 (IFN-γ, IL-12), Th2 (IL-10, IL-4 & TGF-β) and iNOS from spleen tissue of infected and treated mice using RT-qPCR. The iNOS transcript, which is important for the control of leishmaniasis, was significantly upregulated only in F6 (50mg/kg/b.wt) and Miltefosine treated mice and in all other treatments the expression was unaltered. The pro-inflammatory cytokine expression, IFN-γ, which is important for parasite clearance was similar in treated as well as control mice. While, the expression of IL-12p40 expression levels was increased significantly only in Miltefosine treated mice whereas F5, F6 and Withaferin-A mice didn't show any significant increase as compared to infected mice. The anti-inflammatory cytokine expression (IL-10), which is important for the parasite persistence was decreased in F5 (50mg/kg/b.wt), F6 (25 & 50mg/kg/b.wt) and Withaferin-A treated mice compared to control mice. Other Th2 cytokines IL-4 and TGF-β were also decreased in treated groups compared to the infected mice. The IFN- γ to IL-10 expression ratio also signifies the extent of healing of VL, which is increased in F5, F6 and Withaferin-

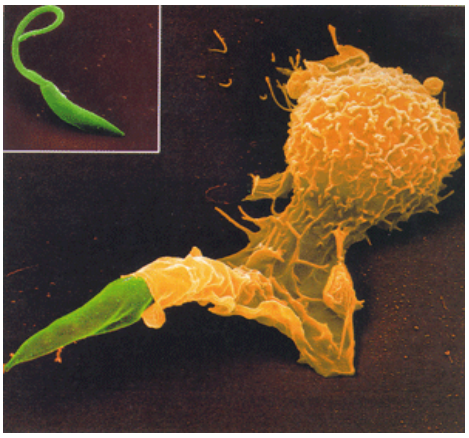
A treated groups compared to the infected mice. The withanolides treatment decreased the Th2 cytokine expression suggesting the immunomodulatory role in *L. donovani* infection.

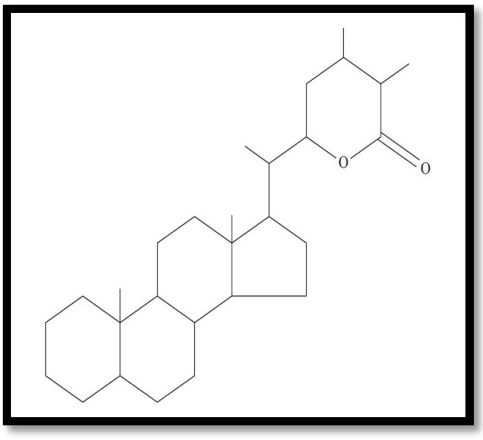
We earlier showed that withanolides induce apoptotic like death in the *L. donovani* parasites, following this we tried to find the mode of action of Withaferin-A which being an abundant withanolide in *W. somnifera* leaves. In this direction, we explored the Pteridine reductase-1 as the target enzyme because it being an essential enzyme in the parasite for its survival. Using molecular docking approach, we found that Withaferin-A has the potential binding capability with Pteridine reductase-1 enzyme *in-silico*. To prove this *in vitro*, we cloned, expressed and purified this enzyme to near homogeneity from *L. donovani* genome. We performed Michaelis-Menten equation to determine its V_{max} as 2.4µmol/min/mg of protein and K_m as 35µM and we also determined the optimum enzyme concentration as 0.696µM. Further using Withaferin-A as inhibitor we performed the inhibition assays using Lineweaver -Burk plot and deduced the Ki value as 0.9µM. These studies clearly demonstrate that Pteridine reductase-1 as the possible target of Withaferin-A. Then to prove this inhibition in the parasites, we isolated the whole protein from untreated and withaferin A treated parasites and performed the PTR1 enzyme assay. We found with increase in treatment time interval there was significant decrease in the PTR1 enzyme activity proving that indeed withaferin A is inhibiting the PTR1 enzyme in the parasites.

Our study for the first time showed both the antileishmanial and immunomodulatory activities of withanolides in *ex vivo* and *in vivo* model of experimental visceral leishmaniasis. We also proved that Pteridine reductase 1 enzyme from *L. donovani* parasites might be one of the targets of withaferin A, but further studies are needed to decipher the other targets of withaferin A in the parasites. Withanolides from this plant has to be investigated further

1. To evaluate the bioavailability of withanolides in the mice model.
2. To understand the role of these withanolides on the immune cell functions in mice model of infection.
3. The signaling pathways induced by the withanolides in host during the treatment.
4. The feasibility of using withaferin A in combination with presently available chemotherapeutic drugs.

Bibliography





Akula KK, Dhir A, Kulkarni SK (2009). Effect of various antiepileptic drugs in a pentylenetetrazol-induced seizure model in mice. Methods and Findings in Experimental and Clinical Pharmacology 31: 423-32.

Alexander J, Carter KC, Al-Fasi N, Satoskar A, Brombacher F (2000). Endogenous IL-4 is necessary for effective drug therapy against visceral leishmaniasis. Eur J Immunol 10: 2935-43.

Alexander J, Satoskar AR, Russell DG (1999) *Leishmania* species: models of intracellular parasitism. J Cell Sci 18:2993-3002.

Ali SA and Meitei KV (2011) On the action and mechanism of withaferin-A from *Withania somnifera*, a novel and potent melanin dispersing agent in frog melanophores. Journal of Receptors and signal transduction Research 31: 359-366.

Almeida R, Norrish A, Levick M, Vetrie D, Freeman T, Vilo J, Ivens A, Lange D, Stober C, McCann S, Blackwell JM (2002) From genomes to vaccines: *Leishmania* as a model. Philos Trans R Soc Lond B Bioi Sci 357: 5-11.

Alvar J, Canavate C, Molina R, Moreno J, Nieto J (2004) Canine leishmaniasis. Adv Parasitol 57: 1–88.

Alvar J, Yactayo S, Bern C (2006) Leishmaniasis and poverty. Trends Parasitol 22: 552.

Andrade C (2009). Ashwagandha for anxiety disorders. World Journal of Biological Psychiatry 10: 686-687.

Ansari NA, Kumar R, Gautam S, Nylen S, Singh OP, Sundar S, Sacks D (2011). IL-27 and IL-21 are associated with T cell IL-10 responses in human visceral leishmaniasis. J Immunol 186: 3977–3985.

Anwer T, Sharma M, Pillai KK, Iqbal M (2008) Effect of *Withania somnifera* on insulin sensitivity in non-insulin-dependent diabetes mellitus rats. Basic and Clinical Pharmacology and Toxicology 102: 498-503.

Ahmad MK, Mahdi AA, Shukla KK, Islam N, Rajender S, Madhukar D, Shankhwas SN, Ahamad S (2010) *Withania somnifera* improves semen quality by regulating reproductive hormone levels and oxidative stress in seminal plasma of infertile males. Fertility and Sterility 94: 989-996.

Arango V, Robledo S, Seon-Meniel B, Figadere B, Cardona W, Saez J, et al (1990) Coumarins from Galipea panamensis and their activity against *Leishmania panamensis.* J Nat Prod 28:1012–1014.

Arnot DE and Barker DC (1981) Biochemical identification of cutaneous leishmaniasis by

analysis of kinetoplast DNA. II. Sequence homologies in *Leishmania* kDNA. Molecular Biochemistry & Parasitology 3: 47-56.

Ato M, Stager S, Engwerda CR, Kaye PM (2002) Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis. Nat Immunol 3: 1185–1191.

Attar AJ, Chance ML, ElSafi S, Carney J, Azazy A, El Hadi M, Duorado C, Hommel M (2001) Latex test for the detection of urinary antigens in visceral leishmaniasis. Acta Tropica 78:11–16.

Banchereau J, Bazan F, Blanchard D, Brière F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S (1994) The CD40 antigen and its ligand. Annu Rev Immunol 12: 881-922.

Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R, Johnson WD Jr (1986). New perspectives on a sub-clinical form of visceral leishmaniasis. J Infect Dis 154: 1003–1011.

Baker RCP and Kaye PM (1999) Leishmaniasis. In: Zumla, D.G.J.A. (Ed.), The Granulomatous Disorders. Chembridge Univ press, UK: 212–234.

Banarjee BD (2010) Propoxur-induced acetylcholine esterase inhibition and impairment of cognitive function: attenuation by *Withania somnifera*. Indian Journal of Biochemical Biophysics 47: 117-120.

Baneth G, Koutinas AF, SolanoGallego L, Bourdeau P, Ferrer L (2008) Canine leishmaniosis–new concepts and insights on an expanding zoonosis: partone. Trends Parasitol 24: 324–330.

Bani S, Gautam M, Sheikh FA, Khana B, Satti NK, Suri KA, Qazi GN, Patwardhan B (2006) Selective Th1 up-regulating activity of *Withania somnifera* aqueous extract in an experimental system using flow cytometry. Journal of Ethnopharmacology 107: 107–115.

Basak SK, Saha B, Bhattacharya A, Roy S (1992) Immunobiological studies on experimental visceral leishmaniasis. II. Adherent cell-mediated down-regulation of delayed-type hypersensitivity response and up-regulation of B cell activation. Eur J Immunol 22: 2041–2045.

Bekersky I, Fielding RM, Dressler DE, et al. (2002) Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. Antimicrobial Agents and Chemotherapy 46: 828-833.

Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, Sacks DL (2001) The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med 194: 1497–1506.

Bello AR, Nare B, Freedman D, Hardy L, Beverley SM (1994) PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci USA 91: 11442-11446.

Belosevic M, Finbloom DS, Van Der Meide PH, Slayter MV, Nacy CA (1989) Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. J Immunol 143: 266-274.

Berger BJ and Fairlamb AH (1992) Interactions between immunity and chemotherapy in the treatment of trypanosomiases and leishmaniases. Parasitology 105: S71–78.

Berhe N, Wolday D, Hailu A, et al. (1999) HIV viral load and response to antileishmanial chemotherapy in co-infected patients. AIDS 13: 1921-1925.

Berman JD (1997) Human leishmaniasis, clinical, diagnostic and chemotherapeutic developments in the last 10 years. Clinical Infectious Diseases 24: 684–703.

Berman JD, Badaro R, Thakur CP, et al. (1998) Efficacy and safety of liposomal amphotericin B (AmBisome) for visceral leishmaniasis in endemic developing countries. Bulletin of World Health Organisation 76: 25–32.

Beutler JA (2009) Natural products as a foundation for drug discovery. Curr Protoc Pharmacol 46: 9.11.1

Bhatnagar M, Sharma D, Salvi M (2009). Neuroprotective effects of *Withania somnifera* dunal.: A possible mechanism. Neurochemical Research 34: 1975-1983.

Bhattacharya SK, Rinzin N, Chusak P, Dash AP, Chowdhury R, Tobgay T, et al. (2010) Occurrence and significance of kala-azar in Bhutan. Indian J Med Res 132: 337-338.

Bhattarai JP, Ah Park S, Han SK (2010) The methanolic extract of *Withania somnifera* ACTS on GABAA receptors in gonadotropin releasing hormone (GnRH) neurons in mice. Phytotherapy Research 24: 1147-1150.

Billo M, Fournet A, Cabalion P, Waikedre J, Bories C, Loiseau P, et al. (2005) Screening of New Caledonian and Vanuatu medicinal plants for antiprotozoal activity. J Ethnopharmacol 15: 569–575.

Bittencourt AL and Barral-Netto M (1995) Leishmaniasis. Tropical Pathology, In Doerr W,

Seifert G, eds. Berlin, Springer-Verlag.

Blackwell JM (1983) *Leishmania donovani* infection in heterozygous and recombinant H-2 haplotype mice. Immunogenetics 18: 101–109.

Blackwell JM, Roach TI, Kiderlen A, Kaye PM (1989) Role of Lsh in regulating macrophage priming/activation. Res Immunol 140: 798–805.

Blair PJ, Riley JL, Harlan DM, Abe R, Tadaki DK, Hoffmann SC, White L, Francomano T, Perfetto SJ, Kirk AD, June CH (2000) CD40 ligand (CD154) triggers a short-term CD4(+) T cell activation response that results in secretion of immunomodulatory cytokines and apoptosis. J Exp Med 191: 651-660.

Bogitsh BJ and Cheng TC (1990) In vitro antileishmanial effect of antibacterial diterpenes from two Ethiopian premna species: P. schimperi and P. oligotrica. BMC Pharmacology 3: 6.

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Braga FG, Bouzada ML, Fabri RL, de O, Matos M, Moreira FO, Scio E, et al. (2007) Antileishmanial and antifungal activity of plants used in traditional medicine in Brazil J Ethnopharmacol 4: 396–402.

Brainard GC, Podolin PL, Leivy SW, et al. (1986) Near-ultraviolet radiation suppresses pineal melatonin content. Endocrinology 119: 2201-2205.

Bray RS (1974) *Leishmania*. Annual Review of Microbiology 28: 189-217.

Brenzan MA, Nakamura CV, Dias Filho BP, Ueda-Nakamura T, Young MC, Cortez DA. (2007) Antileishmanial activity of crude extract and coumarin from Calophyllum brasiliense leaves against *Leishmania amazonensis*. Parasitol Res 101: 715–722.

Callahan HL, Portal AC, Devereaux R, Grogl M. (1997) An axenic amastigote system for drug screening. Antimicrob Agents Chemother 41: 818–822.

Carrión J, Nieto A, Iborra S, Iniesta V, Soto M, Folgueira C, Abanades DR, Requena JM, Alonso C (2006) Immunohistological features of visceral leishmaniasis in BALB/c mice. Parasite Immunol 28: 173–183.

Carvalho EM, Barral A, PedralSampaio D, Barral-Netto M, Badaro R, Rocha H, Johnson WD Jr (1992) Immunologic markers of clinical evolution in children recently infected with *Leishmania donovani chagasi*. J Infect Dis 165: 535–540.

Castillo D, Arevalo J, Herrera F, Ruiz C, Rojas R, Rengifo E, et al. (2007) Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional

remedy made with Himatanthus sucuuba (Apocynaceae). J Ethnopharmacol 13: 410–414.

Caux C, Massacrier C, Vanbervliet B, Dubois B, Van Kooten C, Durand I, Banchereau J. (1994) Activation of human dendritic cells through CD40 cross-linking. J Exp Med 180: 1263-1272.

Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. (1996) Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. J Exp Med 184: 747-752.

Chan MM, Bulinski JC, Chang KP, Fong D. (2003) A microplate assay for *Leishmania amazonensis* promastigotes expressing multimeric green fluorescent protein. Parasitol Res 89: 266–271.

Chappuis F, Sundar S, Hailu A, et al. (2007) Visceral leishmaniasis, what are the needs for diagnosis, treatment and control? Nature Reviews of Microbiology 5: 873-882.

Chatterji S, Srivastava S, Khalid A, Singh N, Sangwan RS, Sidhu OP, Roy R, Khetrapal CL, Tuli R (2010) Comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts. Phytochemistry 71: 1085-1094.

Chen XW, Serag ES, Sneed KB, Zhou SF (2011) Herbal bioactivation, molecular targets and the toxicity relevance. Chemico Biological Interactions 192: 161-176.

Chipuk JE, Green DR. (2005) Do inducers of apoptosis trigger caspase-independent cell death? Nature Reviews Molecular Cell Biology 6: 268–275.

Choi MJ, Park EJ, Min KJ, Park JW, Kwon TK (2011) Endoplasmic reticulum stress mediates withaferin A-induced apoptosis in human renal carcinoma cells. Toxicology *In vitro* 25: 692-698.

Choudhary MI, Hussain S, Yousuf S, Dar A, Mudassar AR (2010) Chlorinated and diepoxy withanolides from *Withania somnifera* and their cytotoxic effects against human lung cancer cell line. Phytochemistry 71: 2205-2209.

Chunge CN, Owate J, Pamba HO, et al. (1990) Treatment of visceral leishmaniasis in Kenya by aminosidine alone or combined with sodium stibogluconate. Transactions of Royal Society for Tropical Medicine and Hygiene 84: 221–225.

Cox FEG (2002) History of human leishmaniasis. Clin Microbiol Rev 15: 595–612.

Crocker PR, Blackwell JM, Bradley DJ (1984) Expression of the natural resistance gene Lsh in resident liver macrophages. Infect Immun 43: 1033–1040.

Croft S, Neal R, Pendergast W, et al. (1987) The activity of alkyl phosphocholines and related derivates against *Leishmania donovani*. Biochemical Pharmacology 36: 2633–2636.

Croft SL, Seifert K, Yardley V (2006a) Current scenario of drug development for leishmaniasis. Indian Journal of Medical Research 123: 399-410.

Croft SL, Sundar S, Fairlamb AH (2006b) Drug resistance in leishmaniasis. Clin Microbiol Rev 19: 111–126.

Cua DJ and Stohlman SA (1997) *In vivo* effects of T helper cell type 2 cytokines on macrophage antigen-presenting cell induction of T helper subsets. J Immunol 159: 5834-5840.

D Andrea A, Ma X, Aste-Amezaga M, Paganin C, Trinchieri G (1995) Stimulatory and inhibitory effects of interleukin (lL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production. J Exp Med 181: 537-546.

Danelli MG, Soares DC, Abreu HS, Pec¸anha LM, Saraiva EM (2009) Leishmanicidal effect of LLD-3 (1), a nor-triterpene isolated from Lophanthera lactescens. Phytochemistry 70: 608–614.

Danese S, Sans M, and Fiocchi C (2004) The CD40/CD40L co-stimulatory pathway in inflammatory bowel disease. Gut 53: 1035–1043.

Das K, Samanta TT, Samanta P, Nandi DK (2010) Effect of extract of *Withania somnifera* on dehydration-induced oxidative stress-related uremia in male rats. Saudi Journal of Kidney Disease and Transplantation 21: 75-80.

Davidson RN, Boerb MD, Ritmeijerb K (2009) Paromomycin. Trans Roy Soc Trop Med Hyg 103: 653-660.

De Waal Malefyt R, Figdor CG, Huijbens R, Mohan-Peterson S, Bennett B, Culpepper 1, Dang W, Zurawski G, de Vries JE (1993) Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-gamma or IL-I0. J Immunol 151: 6370-6381.

Das L, Datta N, Bandyopadhyay S et al. (2001) Successful therapy of lethal murine visceral leishmaniasis with cystatin involves up regulation of nitric oxide and a favorable T cell response. J Immunol 166: 4020–4028.

De Ibarra AA, Howard JG, Snary D (1982) Monoclonal antibodies to *Leishmania tropica* major, specificities and antigen location. Parasitology 85: 523-531.

Desjeux P (1996) Leishmaniasis: public health aspects and control. Clinics in Dermatology 14: 417–423.

Desjeux P (2001) The increase in risk factors for the leishmaniasis Worldwide. Trans Roy Soc Trop Med Hyg 95: 239–243.

Desjeux P (2004) Leishmaniasis, current situation and new perspectives. Comparative Immunology, Microbiology and Infectious Diseases 27: 305-318.

Desrivot J, Waikedre J, Cabalion P, Herrenknecht C, Bories C, Hocquemiller R, et al. (2007) Antiparasitic activity of some New Caledonian medicinal plants. J Ethnopharmacol 30: 7–12.

Di Giorgio C, Faraut-Gambarelli F, Imbert A, et al. (1999) Flow cytometric assessment of amphotericin B susceptibility in *Leishmania infantum* isolates from patients with visceral leishmaniasis. Journal of Antimicrobial Chemotherapy 44: 71–76.

DNDi Annual Report 2007-2008.

Duenas-Romero AM, Loiseau PM, Saint-Pierre-Chazalet M (2007) Interaction of sitamaquine with membrane lipids of *Leishmania donovani* promastigotes. Biochimica et Biophysica Acta 17: 246-252.

Eloff JN (1998) Which extractant should be used for the screening and isolation of antimicrobial components from plants? J Ethnopharmacol 60: 1–8.

Engwerda CR, Ato M, Kaye PM (2004) Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis. Trends Parasitol 20: 524–530.

Engwerda CR, Murphy ML, Cotterell SE, Smelt SC, Kaye PM (1998) Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of *Leishmania donovani*. Eur J Immunol. 28: 669-680.

Ephros M, Bitnun A, Shaked P, et al. (1999) Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. Antimicrobial Agents and Chemotherapy 43: 278-282.

Escobar P, Yardley V, Croft SL (2001) Activities of hexadecylphosphocholine (miltefosine), AmBisome, and sodium stibogluconate (Pentostam) against *Leishmania donovani* in immunodeficient SCID mice. Antimicrobial Agents and Chemotherapy 45: 1872–1875.

Espuelas S, Legrand P, Loiseau PM, et al. (2000) In vitro reversion of amphotericin B resistance in *Leishmania donovani* by Poloxamer 188. Antimicrobal Agents and Chemotherapy 44: 2190-2192.

Estevez Y, Castillo D, Pisango MT, Arevalo J, Rojas R, Alban J, et al. (2007) Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group. J Ethnopharmacol 1: 254–259.

Foey AD, Feldmann M, Brennan FM (2000) Route of monocyte differentiation determines their cytokine production profile: CD40 ligation induces interleukin 10 expression. Cytokine 12: 1496-1505.

Fong D, Chan MM, Rodriguez R, et al. (1994) Paromomycin resistance in *Leishmania tropica*: lack of correlation with mutation in the small subunit ribosomal RNA gene. American Journal of Tropical Medicine and Hygine 51: 758-766.

Fulzele SV (2003) Study of Immunomodulatory activity of *Haridradi Ghrita* in rats. Indian J Pharmacol 35: 51–54.

Gachet MS, Lecaro JS, Kaiser M, Brun R, Navarrete H, Munoz RA, et al. (2010) Assessment of anti-protozoal activity of plants traditionally used in Ecuador in the treatment of leishmaniasis. J Ethnopharmacol 2: 184–197.

Ganeasan K, Sehgal PK, Mandal AB, Syeed S (2011) Protective Effect of *Withania somnifera* and *Cardiospermum halicacabum* Extracts Against Collagenolytic Degradation of Collagen. Applied Biochemistry and Biotechnology 165: 1075-1091.

Ganguly S, Bandyopadhyay S, Sarkar A, Chatterjee M (2006) Development of a semiautomated colorimetric assay for screening anti-leishmanial agents. J Microbiol Methods 66: 79–86.

Gautam S, Kumar R, Maurya R, Nylen S, Ansari N, Rai M, Sundar S, Sacks D (2011) IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis. J Infect Dis 204: 1134–1137.

Gautam S, Kumar R, Singh N, Singh AK, Rai M, Sacks D, Sundar S, Nylén S (2014) CD8 T cell exhaustion in human visceral leishmaniasis. J Infect Dis 209: 290-299.

Geeta KM and Murugan V (2008) Antistress activity of an herbal extract. Acta horticulturae 786: 99-103.

Ghalib HW, Piuvezam MR Skeiky YA, Siddig M, Hashim FA, El-Hassan AM, Russo DM, Reed SG (1993) Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. J Clin Invest 92: 324-329.

Ghalib HW, Whittle JA, Kubin M, Hashim FA, El-Hassan AM, Grabstein KH, Trinchieri G, Reed SG (1995) IL-12 enhances Th1-type responses in human *Leishmania donovani* infections. J Immunol 154: 4623–4629.

Ghosh AK and Ghosh DK (1987) Infection pattern of leishmaniasis in hamsters produced by recent isolates from kala-azar patients in India. Indian J Med Res 86: 14–19.

Ghosh M (2009) Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*. Fitoterapia 80: 91–95.

Gidwani K, Jones S, Kumar R, Boelaert M, Sundar S (2011a). Interferon-gamma release assay (modified QuantiFERON) as a potential marker of infection for *Leishmania donovani*, a proof of concept study. PLoS Negl Trop Dis 5, e1042.doi: 10.1371/0001042.

Grover A , Katiyar SP, Jeyakanthan J, Dubey VK, Sundar D (2012) Blocking Protein kinase C signaling pathway: mechanistic insights into the anti-leishmanial activity of prospective herbal drugs from *Withania somnifera*. BMC Genomics 13 Suppl 7:S20. doi: 10.1186/1471-2164-13-S7-S20.

Green LC, Wagmer DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem 126: 131-138.

Guerin PJ, Olliaro P, Sundar S, et al. (2002) Visceral leishmaniasis, current status of tconrol, diagnosis, and treatment, and a proposed research and development agenda. Lancet Infectious Diseases 2: 494-501.

Gupta GL and Rana AC (2007) Protective effects of *Withania somnifera* dunal root extract against protracted social isolation induced behavior in rats. Indian Journal of Physiology and Pharmacology 51: 345-353.

Gupta GL and Rana AC (2008) Effect of *Withania somnifera Dunal* in ethanolinduced anxiolysis and withdrawal anxiety in rats. Indian Journal of Experimental Biology 46: 470-475.

Hahm ER, Lee J, Huang Y, Singh SV (2011) Withaferin A suppresses estrogen receptor-α expression in human breast cancer cells. Molecular Carcinogenesis 50: 614-624.

Handman E (2001) Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 14: 229-243.

Handman E and Curtis JM (1982) *Leishmania tropica*, surface antigens of intracellular and flagellate forms. Experimental Parasitology 54: 243-249.

Heinzel FP, Schoenhaut DS, Rerko RM, Rosser LE, Gately MK (1993) Recombinant interleukin 12 cures mice infected with *Leishmania major*. J Exp Med 177: 1505-1509.

Heinzel FP, Rerko RM, Ahmed F, Pearlman E (1995) Endogenous IL-12 is required for control of Th2 cytokine responses capable of exacerbating leishmaniasis in normally resistant mice. J Immunol 155: 730-739.

Herrmann H and Gercken G (1982) Metabolism of 1-O-[1´-14C]octadecyl-sn-glycerol in *Leishmania donovani* promastigotes. Ether lipid synthesis and degradation of the ether bond. Molecular and Biochemical Parasitology 5: 65–76.

Herwaldt BL (1999) Leishmaniasis. Lancet 354: 1191-1199.

Hersh E (1982) Perspective for immunological and biological therapeutic intervention in human cancer In E. Michich (ed) John Wiley, New York: 506 -540.

Heyman HM, Hussein AA, Lall N (2009) Antibacterial activity of south African medicinal plants against methicillin resistant *Staphylococcus aureus* Pharmaceutical Biology 47: 67-71.

Ho CS, Lam CW, Chan MH, Cheung RCK, et al. (2003) Electrospray ionisation mass spectrometry: Principles and Clinical applications. Clin Biochem Rev 24: 3-12.

Hodgkinson VH, Soong L, Duboise SM, McMahon-Pratt D (1996) *Leishmania amazonensis*: cultivation and characterization of axenic amastigote-like organisms. Exp Parasitol 83: 94–105.

Holaday BJ, Pompeu MM, Jeronimo S, Texeira MJ, Sousa Ade A, Vasconcelos AW, Pearson RD, Abrams IS, Locksley RM (1993) Potential role of interleukin-10 in the immunosuppression associated with kala azar. J Clin Invest 92: 2626-2632.

Holaday BJ, Sadick MD, Wang ZE, Reiner SL, Heinzel FP, Parslow TG, Locksley RM (1991) Reconstitution of *Leishmania* immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines. J Immunol 147: 1653-1658.

Howard M and O'Garra A (1992) Biological properties of interleukin 10. Immunol Today 13: 198-200.

Idowu OR, Peggins JO, Brewer TG (1995) Side-chain hydroxylation in the metabolism of 8-aminoquinoline antiparasitic agents. Drug Metabolism and Disposition: The Biological Fate of Chemicals 23: 18-27.

Iranshahi M, Arfa P, Ramezani M, Jaafari MR, Sadeghian H, Bassarello C, et al. (2007) Sesquiterpene coumarins from Ferula szowitsiana and *in vitro* antileishmanial activity of 7-prenyloxycoumarins against promastigotes. Phytochemistry 68: 554–561.

Iwu MM, Jackson JE, Schuster BG (1994) Medicinal plants in the fight against leishmaniasis. Parasitol Today 10: 65–68.

Jatwa R and Kar A (2009) Amelioration of metformin-induced hypothyroidism by *Withania somnifera* and *Bauhinia purpurea* extracts in Type 2 diabetic mice. Phytotherapy Research 23: 1140-1145.

Jayaprakasam B, Padmanabhan K, Nair MG (2010) Withanamides in Withania somnifera fruit protect PC-12 cells from beta-amyloid responsible for Alzheimer's disease. Phytotherapy Research 24: 859-863.

Jeyanthi T and Subramanian P (2009) Nephroprotective effect of *Withania somnifera*: a dose-dependent study. Renal Failure 31: 814-821.

Jeyanthi T and Subramanian P (2010) Protective effect of *Withania somnifera* root powder on lipid peroxidation and antioxidant status in gentamicin-induced nephrotoxic rats. Journal of Basic Physiology and Pharmacology 21: 61-78.

Jha TK, Sundar S, Thakur CP, et al. (2005) A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. American Journal of Tropical Medicine and Hygiene 73: 1005-1011.

Jullian V, Bonduelle C, Valentin A, Acebey L, Duigou AG, Prevost MF, et al. (2005) New clerodane diterpenoids from Laetia procera (Poepp.) Eichler (Flacourtiaceae), with antiplasmodial and antileishmanial activities. Bioorg Med Chem Lett 15: 5065–5070.

Kalthur G and Pathirissery UD (2010) Enhancement of the response of B16F1 melanoma to fractionated radiotherapy and prolongation of survival by withaferin A and/or hyperthermia. Integrative Cancer Therapy 9: 370-377

Kamogawa Y, Minasi LA, Carding SR, Bottomly K, Flavell RA (1993) The relationship of IL-4 and IFN gamma-producing T cells studied by lineage ablation of IL-4-producing cells. Cell 75: 985-995.

Karioti A, Skaltsa H, Kaiser M, Tasdemir D (2009) Trypanocidal, leishmanicidal and cytotoxic effects of anthecotulide-type linear sesquiterpene lactones from Anthemis auriculata. Phytomedicine 16: 783-787.

Karp CL, el-Safi SH, Wynn TA, Satti MM, Kordofani AM, Hashim FA, Hag-Ali M, Neva FA, Nutman TB, Sacks DL (1993) *In vivo* cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-l0 and interferon-gamma. J Clin Invest 91: 1644-1648.

Kasture S, Barhate S, Mohan M, Ballero M, Sanna C, Maxia A (2009) Caffeine withdrawal retains anticataleptic activity but *Withania somnifera* withdrawal potentiates haloperidol-induced catalepsy in mice. Natural Product Research 23: 724-728.

Kataria H, Shah N, Kaul SC, Wadhwa R, Kaur G (2009) Water extract of Ashwagandha leaves limits proliferation and migration, and induces differentiation in Glioma cells. Evidence Based Complementary and Alternative Medicine (ahead of print) doi: 10.1093/ecam/nep188

Kaul MK, Kumar A, Sharma A (2005) Reproductive biology of *Withania somnifera* (L.) Dunal. Current Science 88: 1375–1377.

Kaur J, Kumar P, Tyagi S, Pathak R, Batra S, Singh P, Singh N (2011) *In silico* screening, structure-activity relationship, and biologic evaluation of selective pteridine reductase inhibitors targeting visceral leishmaniasis. Antimicrob Agents Chemother 55: 659-666.

Kaur J, Sundar S, Singh N (2010) Molecular docking, structure–activity relationship and biological evaluation of the anticancer drug monastrol as a pteridine reductase inhibitor in a clinical isolate of *Leishmania donovani*. J Antimicrob Chemother 65: 1742–1748.

Kaye PM (1995) Costimulation and the regulation of antimicrobial immunity. Immunol Today 16: 423-427.

Kaye PM, Curry AJ, Blackwell JM (1991) Differential production of Th1 and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis. J Immunol 146: 2763-2770.

Kemp M, Theander TG, Kharazmi A (1996) The contrasting roles of CD4+ T cells in intracellular infections in humans: leishmaniasis as an example. Immunol Today 17: 13-16.

Khan S, Malik F, Suri KA, Singh J (2009) Molecular insight into the immune up-regulatory properties of the leaf extract of Ashwagandha and identification of Th1 immunostimulatory chemical entity. Vaccine 27: 6080-6087.

Kigondu EV, Rukunga GM, Keriko JM, Tonui WK, Gathirwa JW, Kirira PG, et al. (2009) Anti-parasitic activity and cytotoxicity of selected medicinal plants from Kenya. J Ethnopharmacol 25: 504–509.

Kirira PG, Rukunga GM, Wanyonyi AM, Muregi FM, Gathirwa JW, Muthaura CN, Omar SA, Tolo F, Mungai GM, Ndiege IO (2006) Anti-plasmodial activity and toxicity of extracts of plants used in traditional malaria therapy in Meru and Kilifi Districts of Kenya. Journal of Ethnopharmacology 106: 403-407.

Knighton DR, Kan CC, Howland E, Janson CA, Hostomska Z, Welsh KM, Matthews DA

(1998) Structure of and kinetic channelling in bifunctional dihydrofolate reductase-thymidylate synthase. Nat Struct Biol 3: 186-194.

Koduru S, Kumar R, Srinivasan S, Evers MB, Damodaran C (2010) Notch-1 inhibition by Withaferin-A: a therapeutic target against colon carcinogenesis. Molecular Cancer Therapy 9: 202-210.

Koonin EV and Aravind L (2002) Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death and Differentiation 9: 394–404.

Krishnamurthy YL, Shashikala J, Shankar Naik B (2008) Antifungal potential of some natural products against *Aspergillus flavus* in soybean seeds during storage. Journal of Stored Products Research 44: 305-309.

Krummel MF and Allison JP (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med. 182: 459-465.

Kuhlencord A, Maniera T, Eibl H, et al. (1992) Hexadecylphosphocholine, oral treatment of visceral leishmaniasis in mice. Antimicrobial Agents and Chemotherathy 36: 1630–1634.

Kulkarni SK, Akula KK, Dhir A (2008) Effect of *Withania somnifera Dunal* root extract against pentylenetetrazol seizure threshold in mice: possible involvement of GABAergic system. Indian Journal of Experimental Biology 46: 465-469.

Kumar A and Kalonia H (2008) Effect of *Withania somnifera* on Sleep-Wake Cycle in Sleep-Disturbed Rats: Possible GABAergic Mechanism. Indian Journal of Pharmaceutical Sciences 70: 806-810.

Kumar P, Kothari H, Singh N (2004) Overexpression in *Escherichia coli* and purification of pteridine reductase (PTR1) from a clinical isolate of *Leishmania donovani*. Protein Expr Purif 38: 228-236.

Kumar P and Kumar A (2009) Possible neuroprotective effect of *Withania somnifera* root extract against 3-nitropropionic acid-induced behavioral, biochemical, and mitochondrial dysfunction in an animal model of Huntington's disease. Journal of Medicinal Food 12: 591-600.

Kumar P, Seal CJ, Howes MJ, Kite GC, Okello EJ (2010). *In vitro* protective effects of *Withania somnifera (L.) dunal* root extract against hydrogen peroxide and β-amyloid(1-42)-induced cytotoxicity in differentiated PC12 cells. Phytotherapy Research 24: 1567-1574.

Kumar S, Harris RJ, Seal CJ, Okello EJ (2011) An Aqueous Extract of *Withania somnifera*

Root Inhibits Amyloid β Fibril Formation *in-vitro*. Phytotherapy Research available online doi: 10.1002/ptr.3512.

Kuttan G and Kuttan R (1992) Immunomodulatory activity of a peptide isolated from *Viscum album* extract (NSC 635089). Immunological Investigations 21: 285-296.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lai JH (2002) Immunomodulatory effects and mechanisms of plant alkaloid tetrandrine in autoimmune diseases. Acta Pharmacol Sin 23: 1093–1101.

Lakshmi V, Pandey K, Kapil A, Singh N, Samant M, Dube A (2007) *In vitro* and *in vivo* leishmanicidal activity of *Dysoxylum binectariferum* and its fractions against *Leishmania donovani*. Phytomedicine 14: 36–42.

Langreth SG, Berman JD, Riordan GP, et al. (1983) Finestructural alterations in *Leishmania tropica* within human macrophages exposed to antileishmanial drugs *in vitro*. Journal of Protozoology 30: 555-561.

Laskay T, Diefenbach A, Rollinghoff M, Solbach W (1995) Early parasite containment is decisive for resistance to *Leishmania major* infection. Eur J Immunol 25: 2220-2227.

Lee N, Bertholet S, Debrabant A,Muller J, Duncan R, Nakahasi HL (2002) Programmed cell death in the unicellular protozoan parasite *Leishmania*. Cell Death Differ 9: 53–64.

Lee J, Hahm ER, Singh SV (2010) Withaferin-A inhibits activation of signal transducer and activator of transcription 3 in human breast cancer cells. Carcinogenesis 31: 1991-1998.

Liew FY and O'Donnell CA (1993) Immunology of leishmaniasis. Adv Parasitol 43: 161-259.

Liew FY (1991) Role of cytokines In killing of intracellular pathogens. Immunol Lett 30: 193-197.

Liew FY, Li Y, Millott S (1990a) Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J Immunol 145: 4306–4310.

Liew FY, Li Y, Millott S (1990b) Tumour necrosis factor (TNF-alpha) in leishmaniasis. II.TNF-alpha-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology 71: 556–559.

Lin J, Opoku AR, Geheeb-Keller M, Hutchings AD, et al. (1999) Preliminary screening of

some traditional Zulu medicinal plants for anti-inflammatory and antimicrobial activities. J Ethnopharmacol 68: 267-274.

Lopez-Martin C, Perez-Victoria JM, Carvalho L, et al. (2008) Sitamaquine sensitivity in *Leishmania* species is not mediated by drug accumulation in acidocalcisomes. Antimicrobial Agents and Chemotherapy 52: 4030-4036.

Lu L, Liu Y, Zhu W, Shi J, Liu Y, Ling W, Kosten TR (2009) Traditional medicines in the treatment of drug addiction. American Journal of Drug and Alcohol Abuse 35: 1-11.

Lux H, Heise N, Klenner T, et al. (2000) Ether-lipid (alkyl-phospholipid analog) metabolism and the mechanism of action of etherlipid analogs in *Leishmania*. Molecular and Biochemical Parasitology 111: 1-14.

Maarouf M, Adeline MT, Solignac M, et al. (1998) Development and characterization of paromomycin-resistant *Leishmania donovani* promastigotes. Parasite 5: 167-173.

Maasho K, Sanchez F, Schurr E, Hailu A, Akuffo H (1998) Indications of the protective role of natural killer cells in human cutaneous leishmaniasis in an area of endemicity. Infect Immun 66: 2698-2704.

MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. Annu Rev Immunol 15: 323-350.

Mahdi AA, Shukla KK, Ahmad MK, Rajender S, Shankhwar SN, Singh V, Dalela D (2011) *Withania somnifera* Improves Semen Quality in Stress-Related Male Fertility. Evidence Based Complementary and Alternative Medicine available online doi: 10.1093/ecam/nep138

Maitra R, Porter MA, Huang S, Glimour BP (2009) Inhibition of NFkappaB by the natural product Withaferin A in cellular models of cystic fibrosis inflammation. Journal of Inflammation 13: 6-15.

Malviya N, Jain S, Gupta VB, Vyas S (2011) Recent studies on aphrodisiac herbs for the management of male sexual dysfunction--a review. Actam Ploniae Pharmaceutica 68: 3-8.

Malaviya P, Picado A, Singh SP, et al. (2011) Visceral Leishmaniasis in Muzaffarpur District, Bihar, India from 1990 to 2008. PLoS One 6, pp e14751.

Manandhar KD, Yadav TP, Prajapati VK, et al. (2008) Antileishmanial activity of nano-amphotericin B deoxycholate. Journal of Antimicrobial Chemotherapy 62: 376-380.

Mandal C, Dutta A, Mallick A, Chandra S, Misra L, Sangwan RS, Mandal C (2008a) Withaferin A induces apoptosis by activating p38 mitogenactivated protein kinase

signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade. Apoptosis 13: 1450-1464.

Mandal D, Panda N, Kumar S, Banerjee S, Mandal NB, Sahu NP (2006) A triterpenoid saponin possessing antileishmanial activity from the leaves of Careya arborea. Phytochemistry 67:183–190.

Mandal S, Maharjan M, Ganguly S, Chatterjee M, Singh S, Buckner FS, et al. (2009) High-throughput screening of amastigotes of *Leishmania donovani* clinical isolates against drugs using a colorimetric beta-lactamase assay. Indian J Exp Biol 47: 475–479.

Mandal S, Mandal C, Sangwan RS, Chandra S, Mandal C (2010) Withanolide D induces apoptosis in leukemia by targeting the activation of neutral sphingomyelinase-ceramide cascade mediated by synergistic activation of c-Jun N-terminal kinase and p38 mitogen activated protein kinase. Molecular Cancer 13: 239.

MarinKelle CJ (1980) The control of Leishmaniasis. Bull world health organ 58: 807-818.

Marovich MA, McDowell MA, Thomas EK, Nutman TB (2000) IL-12p70 production by *Leishmania major*-harboring human dendritic cells is a CD40/CD40 ligand-dependent process. J Immunol 164: 5858-5865.

Marsden PD and Lumsden WH (1971) Trypanosomiasis and leishmaniasis, Practitioner 207: 181-185.

Martin S, Agarwal R, Murugaiyan G, Saha B (2010) CD40 expression levels modulate regulatory T cells in *Leishmania donovani* infection. J Immunol 185: 551-559.

Mathur RK, Awasthi A, Wadhone P, Ramanamurthy B, Saha B (2004) Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses. Nat Med 10: 540-544

Mauel J and Ransijn A (1997) *Leishmania spp*.: mechanisms of toxicity of nitrogen oxidation products. Exp Parasitol 87: 98-111.

Maurya R, Singh RK, Kumar B, Salotra P, Rai M, Sundar S (2005) Evaluation of PCR for diagnosis of Indian kala-azar and assessment of cure. J Clin Microbiol 43: 3038-3041.

Mayola E, Gallerne C, Esposti DD, Martel C, Pervaiz S, Larue L, Debuire B, Lemoine A, Brenner C, Lemaire C (2011) Withaferin-A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. Apoptosis 16: 1014-1027.

Melby PC, Chandrasekar B, Zhao W, Coe JE (2001) The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in

the face of a prominent Th1 like cytokine response. J Immunol 166: 1912–1920.

Mehta A and Shaha C (2009) Apoptotic death in *Leishmania donovani* promastigotes in response to respiratory chain inhibition: complex II inhibition results in increased pentamidine cytotoxicity. J Biol Chem 279: 11798–11813.

Meyerhoff A (1999) U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. Clinical Infectious Diseases 28: 42-48.

Mikolai J, Erlandsen A, Murison A, Brown KA, Gregory WL, Raman-Caplan P, Zwickey HL (2009) *In vivo* effects of Ashwagandha (*Withania somnifera*) extract on the activation of lymphocytes. Journal of Alternative Complementary Medicine 15: 423-430.

Mikus J and Steverding D (2000) A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. Parasitol Int 48: 265–269.

Miles MA, Lainson R, Shaw JJ, et al. (1981) Leishmaniasis in Brazil, XV. Biochemical distinction of Leishmania mexicana amazonensis, L. braziliensis braziliensis and L. braziliensis guyanensis-aetiological agents of cutaneous leishmaniasis in the Amazon Basin of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 75: 524-529.

Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW (1994) Thl and Th2 cell-associated cytokines in experimental visceral leishmaniasis. Infect Immun 62: 1058-1063.

Mirjalili MH, Moyano E, Bonfill M, Cusido RS, Palazón J (2009) Steroidal lactones from *Withania somnifera*, an ancient plant for novel medicine. Molecules 14: 2373-2393.

Mittal A and Singh RP (2009) Anticancer and immunomodulatory properties of Tinospora. In Ranawat KG (ed.): Herbal Drugs: Ethnomedicine to Modern Medicine. Berlin, Heidelberg, Springer, 195–206.

Modabber F, Buffet PA, Torreele E, et al. (2007) Consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis. Kinetoplastid Biology and Diseases 6: 3.

Modak M, Dixit P, Londhe J, Ghaskadbi S, Paul A, Devasagayam T (2007) Indian herbs and herbal drugs used for the treatment of diabetes. Journal of Clinical Biochemistry and Nutrition 40: 163-173.

Mohanty IR, Arya DS, Gupta SK (2008) *Withania somnifera* provides cardio protection and attenuates ischemia-reperfusion induced apoptosis. Clinical Nutrition 27: 635-642.

Monte-Alegre A, Ouaissi A, Sereno D (2006) *Leishmania* amastigotes as targets for drug screening. Kinetoplastid Biol Dis 23: 5–6.

Monzote L, Garcı´a M, Montalvo AM, Scull R, Miranda M (2010) Chemistry, cytotoxicity and antileishmanial activity of the essential oil from Piper auritum. Mem Inst Oswaldo Cruz 105:168–173.

Mosmann TR and Moore KW (1991) The role of IL-I0 cross regulation of Th1 and Th2 responses. Immunol Today 12: A49-53.

Mulabagal V, Subbaraju GV, Rao CV, Sivaramakrishna C, Dewitt DL, Holmes D, Sung B, Aggarwal BB, Tsay HS, Nair MG (2009) Withanolide sulfoxide from Aswagandha roots inhibits nuclear transcription factor-kappa-B, cyclooxygenase and tumor cell proliferation. Phytotherapy Research 23: 987-992.

Munagala R, Kausar H, Munjal C, Gupta RC (2011) Withaferin A induces p53-dependent apoptosis by repression of HPV oncogenes and upregulation of tumor suppressor proteins in human cervical cancer cells. Carcinogenesis 16: doi: 10.1093/carcin/bgr192.

Muniaraj M. (2014) The lost hope of elimination of Kala-azar (visceral leishmaniasis) by 2010 and cyclic occurrence of its outbreak in India, blame falls on vector control practices or co-infection with human immunodeficiency virus or therapeutic modalities?. Trop Parasitol 4:10-19.

Muralikrishnan G, Amanullah S, Basha MI, Dinda AK, Shakeel F (2010a) Modulating effect of *Withania somnifera* on TCA cycle enzymes and electron transport chain in azoxymethane-induced colon cancer in mice. Immunopharmacology and Immunotoxicology 32: 523-527.

Muralikrishnan G, Dinda AK, Shakeel F (2010b) Immunomodulatory effects of *Withania somnifera* on azoxymethane induced experimental colon cancer in mice. Immunology Investigation 39: 688-698

Muregi FW, Ishih A, Miyase T, Suzuki T, Kino H, Amano T, Gerald M, Mkoji GM, Terada M (2007) Antimalarial activity of methanolic extracts from plants used in Kenyan ethnomedicine and their interactions with chloroquine (CQ) against a CQ- tolerant rodent paracite in mice. Journal of Ethnopharmacology 111: 190-195.

Murray H (2000) Suppression of post-treatment recurrence of experimental visceral leishmaniasis in T-cell-deficient mice by oral miltefosine. Antimicrobial Agents and Chemotherapy 44: 3235–3236.

Murray HW, Jungbluth A, Ritter E, Montelibano C, Marino MW (2000) Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment. Infect Immun 68: 6289–6293.

Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, Coffman RL (2002) Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect Immun 70: 6284–6293.

Murray HW, Flanders KC, Donaldson DD, Sypek JP, Gotwals PJ, Liu J, Ma X (2005) Antagonizing deactivating cytokines to enhance host defense and chemotherapy in experimental visceral leishmaniasis. Infect Immun 73: 3903–3911.

Murray HW, Moreira AL, Lu CM, Devecchio JL, Matsuhashi M, Ma X, Heinzel FP (2003b) Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis. J Infect Dis 188: 458–464.

Murray HW, Stern JJ, Welte K, Rubin BY, Carriero SM, Nathan CF (1987) Experimental visceral leishmaniasis: production of interleukin-2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin-2 and interferon-gamma. J Immunol 138: 2290–2297.

Murray PD (1990) Guide to protein purification. In: (Methods in Enzymology) Academic Press, 182.

Musharraf SG, Ali A, Yousuf S, Rahman AU, Choudhary MI (2011) Analysis and development of structure-fragmentation relationships in withanolides using an electrospray ionization quadropole time-of-flight tandem mass spectrometry hybrid instrument. Rapid Communications Mass Spectrum 25: 104-114.

Muzitano MF, Tinoco LW, Guette C, Kaiser CR, Rossi-Bergmann B, Costa SS (2006) The antileishmanial activity assessment of unusual flavonoids from Kalanchoe pinnata. Phytochemistry 67: 2071–2077.

Nakajima H, Wakabayashi Y, Wakamatsu K, Imokawa G (2011) An Extract of *Withania somnifera* Attenuates Endothelin-1-stimulated Pigmentation in Human Epidermal Equivalents through the Interruption of PKC Activity within Melanocytes. Phytotherapy Research available online doi: 10.1002/ptr.3552.

Nakamura T, Lee RK, Nam SY, Podack ER, Bottomly K, Flavell RA (1997) Roles of IL-4 and IFN-gamma in stabilizing the T helper cell type 1 and 2 phenotype. J Immunol 158: 2648-2653.

Namdeo AG, Sharma A, Yadav KN, Gawande R, Mahadik KR, Lopez-Gressa MP, Kim

HK, Choi YH, Verpoorte R (2011) Metabolic characterization of *Withania somnifera* from different regions of India using NMR spectroscopy. Planta Medica July 4 (ahead of print) DOI: 10.1055/s-0031-1279997.

Nare B, Luba J, Hardy LW, Beverley SM (1997a) New approaches to Leishmania chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. Parasitology 114: S101-110.

Nare B, Hardy L, Beverley SM (1997b) The roles of pteridine reductase 1 and dihydrofolate reductase-thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. J Biol Chem 272: 13883-13891.

Nicoletti S, Seifert K, Gilbert IH (2009) N-(2- hydroxypropyl) methacrylamide-amphotericin B (HPMA-AmB) copolymer conjugates as antileishmanial agents. International Journal of Antimicrobial Agents, 33: 441-448.

Nicoletti S, Seifert K, Gilbert IH (2010) Water-soluble polymer-drug conjugates for combination chemotherapy against visceral leishmaniasis. Bioorganic and Medicinal Chemistry 18: 2559-2565.

Noble A, Macary PA, Kemeny DM (1995) IFN-gamma and IL-4 regulate the growth and differentiation of CD8+ T cells into subpopulations with distinct cytokine profiles. J Immunol 155: 2928-2937.

Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D (2007) Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+ CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. J Exp Med 204: 805–817.

Nylen S and Sacks D (2007) Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 28: 378–384.

Oh JH and Kwon TK (2009a) Withaferin A inhibits tumor necrosis factor-alpha induced expression of cell adhesion molecules by inactivation of Akt and NF-kappaB in human pulmonary epithelial cells. International Immunopharmacology 5: 614-619.

Oh JH, Lee TJ, Kim SH, Choi YH, Lee SH, Lee JM, Kim YH, Park JW, Kwon TK (2008) Induction of apoptosis by withaferin-A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis 13: 1494-1504.

Oskam L, PratIong F, Zijlstra EE, Kroon CC, Dedet M, Kager JP , Schonian PA, Ghalib HW, EI-Hassan AM, Meredith SE (1998) Biochemical and molecular characterization of *Leishmania* parasites isolated from an endemic focus in eastern Sudan. Trans R Soc Trop Med Hyg 92: 120-122.

Osorio E, Arango GJ, Jime´nez N, Alzate F, Ruiz G, Gutierrez D, et al. (2007) Antiprotozoal and cytotoxic activities in vitro of Colombian Annonaceae. J Ethnopharmacol 22: 630–635.

Owais M, Sharad KS, Shehbaz A, Saleemuddin M (2005) Antibacterial efficacy of *Withania somnifera* (ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis. Phytomedicine 12: 229-235.

Oza VP, Trivedi SD, Parmar PP, Subramanian RB (2009) *Withania somnifera* (Ashwagandha): a novel source of L-asparaginase. Journal of Integrative Plant Biology 51: 201-206.

Oza VP, Trivedi SD, Parmar PP, Subramanian RB (2010) Anticancer properties of highly purified L-asparaginase from *Withania somnifera L.* against acute lymphoblastic leukemia. Applied Biochemistry and Biotechnology 160: 1833-1840.

Panjamurthy K, Manoharan S, Menon VP, Nirmal MR, Senthil N (2008) Protective role of withaferin-A on 7, 12-dimethylbenz (a) anthracene induced genotoxicity in bone marrow of Syrian golden hamsters. Journal of Biochemical and Molecular Toxicology 22: 251-258.

Pape LP (2008) Development of new antileishmanial drugs-current knowledge and future prospects. Journal of Enzyme Inhibition and Medicinal Chemistry 23: 708-718.

Paris C, Loiseau P, Bories C, et al. (2004) Miltefosine induces apoptosis like death in *Leishmania donovani* promastigotes. Antimicrobial Agents and Chemotherapy 48: 852–859.

Park HJ, Rayalam S, Della-Fera MA, Ambati S, Yang JY, Baile CA (2008) Withaferin A induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Biofactors 33: 137-148.

Patil D, Gautam M, Jadhav U, Mishra S, Karupothula S, Gairolla S, Jadhav S, Patwardhan B (2010) Physicochemical stability and biological activity of *Withania somnifera* extract under real-time and accelerated storage conditions. Planta Medica 76: 481-488.

Patricia S, Samanta PA, Marcia SCM, Frederico OP, Andre GT (2007) Isolation of anti-leishmanial sterol from the fruits of Cassia fistula using bioguided fractionation. Phytother Res 21: 644–647.

Patz JA, Graczyk TK, Geller N, et al. (2000) Effects of environmental change on emerging parasitic diseases. International Journal for Parasitology 30: 1395–1405.

Paul J, Karmakar S, De T (2012) TLR-mediated distinct IFN-γ/IL-10 pattern induces

protective immunity against murine visceral leishmaniasis Eur J Immunol 42: 2087–2099.

Pawar P, Gilda S, Sharma S, Jagtap S, Paradkar A, Mahadik K, Ranjekar P, Harsulkar A (2011) Rectal gel application of *Withania somnifera* root extract expounds anti-inflammatory and mucorestorative activity in TNBS induced inflammatory bowel disease. BMC Complementary and Alternative Medicine 11: 34.

Pearson RD and Sousa AQ (1996) Clinical spectrum of Leishmaniais. Clinical Infectious Diseases 22: 1–13.

Perez LE, Chandrasekar B, Saldarriaga OA, Zhao W, Arteaga LT, Travi BL, Melby PC (2006) Reduced nitric oxide synthase 2 (NOS2) promoter activity in the Syrian hamster renders the animal functionally deficient in NOS2 activity and unable to control an intracellular pathogen. J Immunol 176: 5519–5528.

Platzer C, Richter G, Uberla K, Hock H, Diamantstein T, Blankenstein T (1992a) Interleukin-4-mediated tumor suppression in nude mice involves interferon-gamma. Eur J Immunol 22: 1729-1733.

Platzer C, Richter G, Uberla K, Muller W, Blocker H, Diamantstein T, Blankenstein T (1992b) Analysis of cytokine mRNA levels in interleukin-4- transgenic mice by quantitative polymerase chain reaction. Eur J Immunol 22: 1179-1184.

Pramanick S, Roy A, Ghosh S, Majumdar HK, Mukhopadhyay S (2008) Withanolide Z, a new chlorinated withanolide from *Withania somnifera*. Planta Medica 74: 1745-1748.

Pratt DM and David JR (1981) Monoclonal antibodies that distinguish between New World species of *Leishmania*. Nature 291: 581-583.

Pretorius E, Oberholzer HM, Viera WA, Smit E (2009) Ultra structure of platelets and fibrin networks of asthmatic mice exposed to selenium and *Withania somnifera*. Anatomical Science Interantional 84: 210-217.

Priyandoko D, Ishii T, Kaul SC, Wadhwa R (2011) Ashwagandha leaf derived withanone protects normal human cells against the toxicity of methoxyacetic acid, a major industrial metabolite. PLoS ONE 6: 1-10.

Rajani M, Gupta SK, Singh N, Mathur S, Kochupillai V, Velpandian T (2006) Evaluation of the effect of *Withania somnifera* root extracts on cell cycle and angiogenesis. J Ethnopharmacol 105: 336–341.

Rajasankar S, Manivasagam T, Surendran S (2009) Ashwagandha leaf extract: a potential agent in treating oxidative damage and physiological abnormalities seen in a mouse

model of Parkinson's disease. Neuroscience Letters 454: 11-15.

Rates SM (2001) Plants as source of drugs. Toxicon 39: 603–613.

Redhu NS, Dey A, Balooni V, Singh S (2006) Leishmania–HIV co-infection: an emerging problem in India. AIDS 20: 1213–1215.

Requena JM, Soto M, Doria MD, Alonso C (2000) Immune and clinical parameters associated with *Leishmania infantum* infection in the golden hamster model. Vet Immunol Immunopathol 76: 269–281.

Rioux JA, Lanotte G, Serres E, et al. (1990) Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification, Annales de Parasitologie Humaine et Comparee 65: 111–125.

Robert CW, McLeod R, Rice DW, et al. (2003) Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypanosomatid parasitic protozoa. Molecular and Biochemical Parasitology 126: 129–142.

Rocha LG, Almeida JR, Macedo RO, Barbosa-Filho JM (2005) A review of natural products with antileishmanial activity. Phytomedicine 12: 514–535.

Rogers KA and Titus RG (2004) Characterization of the early cellular immune response to *L. major* using peripheral blood mononuclear cells from *Leishmania*-naive humans. American journal of Tropical Medicine and Hygiene 71: 568-576.

Rook GA, Onyebujoh P, Stanford IL (1993) Th1/Th2 switching and loss of CD4+ T cells in chronic infections: an immunoendocrinological hypothesis not exclusive to HIV. Immunol Today 14: 568-569.

Ryan L, Vexenat A, Marsden PD, et al. (1990) The importance of rapid diagnosis of new cases of cutaneous leishmaniasis in pin-pointing the sandfly vector, Transactions of the Royal Society of Tropical Medicine and Hygiene 84: 786.

Sacks D and Kamhawi S (2001) Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annual Reviews of Microbiology 55: 453-483.

Sacks D and Noben-Trauth N (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol 2: 845-858.

Sadick MD, Heinzel FP, Holaday BJ, Pu RT, Dawkins RS, Locksley RM (1990) Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T-cell dependent, interferon-gammaindependent mechanism. J Exp Med 171: 115-127.

Salem ML (2005) Immunomodulatory and therapeutic properties of the *Nigella sativa L.* seed. Int Immunopharmacol 5: 1749–1770.

Samant M, Gupta R, Kumari S, et al. (2009) Immunization with the DNA-encoding N-terminal domain of proteophosphoglycan of *Leishmania donovani* generates Th1-type immunoprotective response against experimental visceral leishmaniasis. J Immunol 183: 470–479.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual (2nd Edition.). Cold Spring Harbour Press.

Samjon J, Sheeladevi R, and Ravindran R (2007) Oxidative stress in brain and antioxidant activity of *Ocimum sanctum* in noise exposure. Neurotoxicology 28: 679–685.

Sandhu JS, Shah B, Shenoy S, Chauhan S, Lavekar GS, Padhi MM (2010) Effects of *Withania somnifera* (Ashwagandha) and *Terminalia arjuna* (Arjuna) on physical performance and cardiorespiratory endurance in healthy young adults. International Journal of Ayurveda Research 1:144–149.

Sangwan RS, Das Chaurasiya N, Lal P, Misra L, Tuli R, Sangwan NS (2008) Withanolide A is inherently de novo biosynthesized in roots of the medicinal plat Ashwagandha (*Withania somnifera*). Physiology of Plants 133: 278-287.

Santin MR, Santos AO, Nakamura CV, Dias-Filho BP, Ferreira IC, Ueda-Nakamura T (2009) *In vitro* activity of the essential oil of *Cymbopogon citratus* and its major component (citral) on *Leishmania amazonensis*. Parasitol Res 105: 1489–1496.

Santos AO, Ueda-Nakamura T, Dias-Filho BP, Veiga-Junior VF, Pinto AC, Nakamura CV (2008) Effect of Brazilian copaiba oils on *Leishmania amazonensis*. J Ethnopharmacol 120: 204–208.

Santos AO, Santin AC, Yamaguchi MU, Cortez LE, Ueda-Nakamura T, Dias-Filho BP, et al. (2010) Antileishmanial activity of an essential oil from the leaves and flowers of *Achillea millefolium*. Ann Trop Med Parasitol 104: 475–483.

Sartori A, Roque-Barreira MC, Coe J, Campos-Neto A (1992) Immune complex glomerulonephritis in experimental kala-azar. II: Detection and characterization of parasite antigens and antibodies eluted from kidneys of *Leishmania donovani*-infected hamsters. Clin Exp Immunol 87: 386–392.

Saravia NG, Hazzbon MH, Osorio Y, Valderrama L, Walker J, Santrich C, Cortazar T, Lebowitz JH, Travi BL (2005) Protective immunogenicity of the paraflagellar rod protein 2 of *Leishmania maxicana*. Vaccine 23: 984-995.

Sen N, Banerjee B, Das BB, Ganguly A, Sen T, Pramanik S, et al. (2007) Apoptosis is induced in leishmanial cells by a novel protein kinase inhibitor withaferin A and is

facilitated by apoptotic topoisomerase I–DNA complex. Cell Death and Differentiation 14: 358–367.

Senthilnathan P, Padmavathi R, Magesh V, Sakthisekaran D (2006) Stabilization of membrane bound enzyme profiles and lipid peroxidation by *Withania somnifera* along with paclitaxel on benzo(a)pyrene induced experimental lung cancer. Molecular and Cellular Biochemistry 292: 13–17.

Sereno D, Cordeiro da Silva A, Mathieu-Daude F, Ouaissi A (2007) Advances and perspectives in Leishmania cell based drug-screening procedures. Parasitol Int 56: 3–7.

Shabir G, Anwar F, Sultana B, Khalid ZM, et al. (2011) Antioxidant and antimicrobial attributes and phenolics of different solvent extracts from leaves, flowers and bark of Gold Mohar [Delonix regia (Bojer ex Hook.) Raf]. Molecules 16: 7302-7319.

Shah V, Bayeta E, Lau BHS (2002) Pycnogenol augments macrophage phagocytosis and cytokine secretion. Pak J Nutr 1: 196–201.

Shah N, Kataria H, Kaul SC, Ishii T, Wadhwa R, Kaur G (2009) Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: combinational approach for enhanced differentiation. Cancer Science 100: 1740-1747.

Shallom S, Zhang K, Jiang L, Rathod PK (1999) Essential protein-protein interactions between *Plasmodium falciparum* thymidylate synthase and dihydrofolate reductase domains. J Biol Chem 274: 37781-37786.

Sharma U, Velpandian T, Sharma P, Singh S (2009) Evaluation of antileishmanial activity of selected Indian plants known to have antimicrobial properties. Parasitology Research 105: 1287-1293.

Shukla KK, Mahdi AA, Mishra V, Rajender S, Sankhwas SN (2011) *Withania somnifera* improves semen quality by combating oxidative stress and cell death and improving essential metal concentrations. Reproductive Biomedicine Online 22: 421-427.

Siddiqui NA, Singh S, Siddiquei MM, Khan TH (2012) Immunomodulatory effect of *Withania somnifera, Asparagus racemosus* and *Picrorhiza kurroa* roots. Int J Pharmacol 8: 108–114.

Silva BP, Cortez DA, Violin TY, Dias Filho BP, Nakamura CV, Ueda-Nakamura T, et al. (2010) Antileishmanial activity of a guaianolide from *Tanacetum parthenium* (L.) Schultz Bip. Parasitol Int 59: 643–646.

Silva Filho AA, Resende DO, Fukui MJ, Santos FF, Pauletti PM, Cunha WR, et al. (2009) *In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from Baccharis dracunculifolia D. C. (Asteraceae). Fitoterapia 80: 478–482.

Singh G, Sharma PK, Dudhe R, Singh S (2010) Biological activities of *Withania somnifera*. Annals of Biological Research 1: 56-63.

Singh RH, Narasimhamurthy K, Singh G (2008) Neuronutrient impact of Ayurvedic rasayana therapy in brain aging. Biogerontology 9: 369-374.

Singh S and Kumar S (1998) In: *Withania somnifera*: the Indian ginseng. Ashwagandha. Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Sinha S, Nosalova G, Bandyopadhyay SS, Fleškova D, Ray B (2011) *In vivo* anti-tussive activity and structural features of a polysaccharide fraction from water extracted *Withania somnifera*. Journal of Ethnopharmacology 134: 510-513.

Spath GF and Beverley SM (2001) A lipophosphoglycan independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. Exp Parasitol 99: 97-103.

Srivastava P, Tiwari N, Yadav AK, Kumar V, Shanker K, Verma RK, Gupta MM, Gupta AK, Khanuja SP (2008) Simultaneous quantification of withanolides in *Withania somnifera* by a validated high-performance thin-layer chromatographic method. Journal of Association of Analytical Chemistry International 91: 1154-1161.

Stan SD, Zeng Y, Singh SV (2008) Ayurvedic medicine withaferin-A causes G2 and M Phase cell cycle arrest in human breast cancer cells. Nutrition and Cancer 60: 51-60.

Stanley AC and Engwerda CR (2007) Balancing immunity and pathology in visceral leishmaniasis. Immunol Cell Biol 85: 138–147.

Stuehr DJ and Nathan CF (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J Exp Med 169: 1543-1555.

Subbaraju GV, Vanisree M, Rao CV, Sivaramakrishna C, Sridhar P, Jayaprakasam P, Nair MG (2006) Ashwagandhanolide, a bioactive dimeric thiowithanolide isolated from the roots of *Withania somnifera*. Journal of Natural Products 69: 1790-1792.

Sudhandiran G and Shaha C (2003) Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. J Biol Chem 278: 25120–25132.

Sultana B, Anwar F, Ashraf M (2009) Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 14: 2167-2180.

Sumantran VN, Chandwaskar R, Joshi AK, Boddul S, Patrwardhan B, Chopra A, Wagh UV (2008) The relationship between chondroprotective and antiinflammatory effects of *Withania somnifera* root and glucosamine sulphate on human osteoarthritic cartilage in vitro. Phytotherapy Research 22: 1342-1348.

Sundar S and Chatterjee M (2006) Visceral leishmaniasis-current therapeutic modalities. Indian Journal of Medical Research 123: 345-352.

Sundar S, Jha TK, Thakur CP, et al. (2007b) Injectable paromomycin for visceral leishmaniasis in India. New England Journal of Medicine 356: 2571-2581.

Sundar S, More DK, Singh MK, et al. (2000) Failure of pentavalent antimony in visceral leishmaniasis in India, report from the center of the Indian epidemic. Clinical Infectious Diseases 31: 1104–1107.

Sundar S and Rai M (2002a) Advances in treatment of leishmaniasis. Curr Opin Infect Dis 15: 593-598.

Sundar S and Rai M (2002b) Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol 9: 951-958.

Sundar S, Reed SG, Singh VP, Kumar PC, Murray HW (1998) Rapid accurate field diagnosis of Indian visceral leishmaniasis. Lancet 351: 563–565.

Sundar S, Reed SG, Sharma S, Mehrotra A, Murray HW (1997) Circulating T-helper1 (Th1) cell and Th2 cell associated cytokines in Indian patients with visceral leishmaniasis. Am J Trop Med Hyg 56: 522–525.

Sundar S, Rosenkaimer F, Makharia M, et al. (1998) Trial of miltefosine for visceral leishmaniasis. Lancet 352: 1821–1823.

Sypek JP, Chung CL, Mayor SE, Subramanyam JM, Goldman SJ, Sieburth DS, Wolf SF, Schaub RG (1993) Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. J Exp Med 177: 1797-1802.

Takenaka H, Maruo S, Yamamoto N, Wysocka M, Ono S, Kobayashi M, Yagita H, Okumura K, Hamaoka T, Trinchieri G, Fujiwara H (2002) Regulation of T cell-dependent and -independent IL-12 production by the three Th2-type cytokines IL-I0, IL-6, and IL-4. J Leukoc Bioi. 61: 80-87.

Tasdemir D, Brun R, Franzblau SG, Sezgin Y, Calis I (2008) Evaluation of antiprotozoal and antimycobacterial activities of the resin glycosides and the other metabolites of

Scrophularia cryptophila. Phytomedicine 15: 209–215.

Taylor T, Hawkins DR, Morris GR, et al. (1991) Pharmacokinetics of the anti-leishmanian agent WR 6026 in dogs. European Journal of Drug Metabolism and Pharmacokinetics 3: 136-139.

Teklemariam S, Hiwot AG, Frommel D, et al. (1994) Aminosidine and its combination with sodium stibogluconate in the treatment of diffuse cutaneous leishmaniasis caused by *Leishmania aethiopica*. Transactions of the Royal Society of Tropical Medicine and Hygiene 88: 334–339.

Tekwani BL and Walker LA (2006) 8-Aminoquinolines: future role as antiprotozoal drugs. Current Opinion on Infectious Disease. 19: 623-631.

Tempone AG, Borborema SE, de Andrade Jr HF, de Amorim Gualda NC, Yogi A, Carvalho CS, et al. (2005) Antiprotozoal activity of Brazilian plant extracts from isoquinoline alkaloid-producing families. Phytomedicine 12: 382–390.

Thaiparambil JT, Bender L, Ganesh T, Kline E, Patel P, Liu Y, Tighiouart M, Vertinol PM, Harvey D, Garcia A, Marcus AI (2011) Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. International Journal of Cancer 129: 2744-2755.

Thakur CP, Olliaro P, Gothoskar S, et al. (1992) Treatment of visceral leishmaniasis (kala-azar) with aminosidine (paromomycin)-antimonial combinations, a pilot study in Bihar, India. Transactions of Royal Society of Tropical Medicine and Hygiene 86: 615-616.

Thatte UM and Dahanukar SA (1988) Comparative study of immunomodulating activity of Indian medicinal plants, lithium carbonate and glucan. Methods Find Exp Clin Pharmacol 10: 639-644.

Thatte UM and Dahanukar SA (1986) Ayurveda and contemporary scientific thoughts. Trends Pharmocol Sci 17: 248-257.

Theoharides AD, Chung H, Velazquez H (1985) Metabolism of a potential 8-aminoquinoline antileishmanial drug in rat liver microsomes. Biochemical Pharmacology 34: 181-188.

Tilak JC, Adhikari S, Devasagayam TP (2004) Antioxidant properties of Plumbago zeylanica, an Indian medicinal plant and its active ingredient, Plumbagin. Redox Rep 9: 219-227.

Titus RG, Milon G, Marchal G, Vassali P, Cerottini JC, Louis JA (1987) Involvement of

specific lyt-2+ T cells in the immunological control of experimentally induced murine cutaneous leishmaniasis. Eur J Immunol 17: 1429-1433.

Tiuman TS, Ueda-Nakamura T, Dias Filho BP, Cortez DA, Nakamura CV (2005a) Studies on the effectiveness of *Tanacetum parthenium* against *Leishmania amazonensis*. Acta Protozool 44: 245–251.

Tiuman TS, Ueda-Nakamura T, Cortez DA, Dias Filho BP, Morgado-Dı´az JA, Souza W, et al. (2005b) Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*. Antimicrob Agents Chemother 49: 176–182.

Travi BL, Osorio Y, Melby PC, et al. (2002) Gender is a major determinant of the clinical evolution and immune response in hamsters infected with *Leishmania spp*. Infection and Immununology 70: 2288-2296.

Tuli R, Sangwan RS, Kumar S, Bhattacharya S, Mishra LN, Mandal C, Raghubir R, Nath C, Trivedi PK, Tewari SK, Mishra P, Chaturvedi P, Sangwan NS, Nair KN, Rawat AKS, Srivastava V, Srivastava RK, Ojha SK, Mehrotra S, Khajuria A, Suri KA (2009) Ashwagandha (*Withania somnifera*) An Indian Medicinal Plant.

Udaykumar R, Kasthurirengan S, Mariashibu TS, Rajesh M, Anbazhagan VR, Kim SC, Ganapathi A, Choi CW (2009) Hypoglycaemic and hypolipidaemic effects of *Withania somnifera* root and leaf extracts on alloxan-induced diabetic rats. International Journal of Molecular Sciences 10: 2367-2382.

Udaykumar R, Kasthurirengan S, Vasudevan A, Mariashibu TS, Rayan JJ, Choi CW, Ganapathi A, Kim SC (2010a) Antioxidant effect of dietary supplement *Withania somnifera L.* reduces blood glucose levels in alloxan-induced diabetic rats. Plant Foods and Human Nutrition 65: 91-98.

Ueda-Nakamura T, Mendonc¸a-Filho RR, Morgado-Dı´az JA, Maza PK, Dias-Filho BP, Cortez DA, et al. (2006) Antileishmanial activity of eugenol-rich essential oil from Ocimum gratissimum. Parasitol Int 55: 99–105.

Valadeau C, Pabon A, Deharo E, Alba´ n-Castillo J, Estevez Y, Lores FA, et al. (2009) Medicinal plants from the Yanesha (Peru): evaluation of the leishmanicidal and antimalarial activity of selected extracts. J Ethnopharmacol 123: 413–422.

Van Kooten C and Banchereau J (2000) CD40-CD40 ligand. J Leukoc Biol 67: 2-17

Vendrametto MC, Santos AO, Nakamura CV, Dias-Filho BP, Cortez DA, Ueda-Nakamura T (2010) Evaluation of antileishmanial activity of eupomatenoid-5, a compound isolated from leaves of *Piper regnellii var. pallescens*. Parasitol Int 59: 154–158.

Ven murthy MR, Ranjekar PK, Ramassamy C, Deshpande M (2010) Scientific basis for the use of Indian ayurvedic medicinal plants in the treatment of neurodegenerative didorders: ashwagandha. Central Nervous System Agents in Medicinal Chemistry 10: 238-246.

Vercesi AE and Docampo R (1992) Ca2+ transport by digitonin permeabilized *Leishmania donovani*. Effects of Ca2+, pentamidine and WR-6026 on mitochondrial membrane potential in situ. Biochemical Journal 284: 463-467.

Vercesi AE, Rodrigues CO, Catisti R, et al. (2000) Presence of a Na(+)/H(+) exchanger in acidocalcisomes of *Leishmania donovani* and their alkalization by anti-leishmanial drugs. FEBS Letter 473: 203-206.

Verma NK and Dey CS (2004) Possible mechanism of miltefosine mediated death of *Leishmania donovani*. Antimicrob Agents Chemother 48: 3010–3015.

Vidal S, Tremblay M, Govoni G, Gauthier S, Sebastiani G, Malo D, Skamene E, Olivier M, Jothy S, Gros P (1995) The Ity/Lsh/Bcglocus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1gene. J Exp Med 182: 655–666.

Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, Radhika S, Deepak M (2007) Screening of selected Indian medicinal plants for acetylcholinesterase inhibitory activity. Journal of Ethnopharmacology 109: 359-363.

Vouldoukis I, Riveros-Moreno V, Dugas B, Ouaaz F, Becherel P, Debre P, Moncada S, Mossalayi MD (1995) The killing of *Leishmania major* by human macrophages is mediated by nitric oxide induced after ligation of the Fc epsilon RIIICD23 surface antigen. Proc Natl Acad Sci 92: 7804-7808.

Walker PS, Scharton-Kersten T, Rowton ED, Hengge U, Bouloc A, Udey MC and Vogel JC (1998) Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type 1 immune response and protection in a murine model of leishmaniasis. Hum Gene Ther 9: 1899-1907.

Walnus TL, Len Schow DJ, Bakker EY, Linsley PS, Freeman GJ, Green JM, Thompson CB, Bluestone JA (1994) CTLA-4 can function as a negative regulator of T cell activation. Immunity 405-413.

Wang J, Peng Q, Li G (2009) New compounds of natural resources in 2008. Afr J Biotechnol 8: 4299–4307.

Wasan KM, Wasan EK, Gershkovich P, et al. (2009) Highly effective oral amphotericin B

formulation against murine visceral leishmaniasis. Journal of Infectious Disease 200: 357-360.

Wasunna MK, Rashid JR, Mbui J, et al. (2005) A phase II dose-increasing study of sitamaquine for the treatment of visceral leishmaniasis in Kenya. American Journal of Tropical Medicine and Hygiene 73: 871-876.

Weiner HL (2001) Induction and mechanism of action of transforming growth factor -beta secreting Th3 regulatory cells. Immunol Rev 182: 207-214.

Weniger B, Vonthron-Senecheau C, Kaiser M, Brun R, Anton R (2006) Comparative antiplasmodial, leishmanicidal and antitrypanosomal activities of several biflavonoids. Phytomedicine 13: 176–180.

WHO (1990) Control of the Leishmaniasis. Technical report series, Geneva, pp 793.

WHO (1996) Manual on visceral leishmaniasis control. Geneva, WHO/LEISH/9640. 1996.

WHO (2002) The world health report 2002 - Reducing Risks, Promoting Healthy Life, World Health Organization, Geneva.

WHO (2005) Make every mother and child count, World Health Organization, Geneva.

Widodo N, Takagi Y, Shrestha BG, Ishii T, Kaul SC, Wadhwa R (2008) Selective killing of cancer cells by leaf extract of Ashwagandha: components, activity and pathway analyses. Cancer Letters 262: 37-47.

Widodo N, Shah N, Priyandoko D, Ishii T, Kaul SC, Wadhwa R (2009) Deceleration of senescence in normal human fibroblasts by withanone extracted from ashwagandha leaves. Journal of Gerontology series A Biological Sciences and Medical Sciences 64: 1031-1038.

Wieder T, Reutter W, Orfanos C, et al. (1999) Mechanisms of action of phospholipid analogs as anticancer compounds. Progress in Lipid Research 38: 249–259.

Wilson ME, Jeronimo SM, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microb Pathog 38: 147–160.

Winters M (2006) Ancient Medicine, Modern Use: *Withania somnifera* and its Potential Role in Integrative Oncology Alternative Medicinal Reviews 11: 269-277.

Wirth DF and Pratt DM (1982) Rapid identification of *Leishmania* species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proceedings of National Acadamy of Sciences 79: 6999-7003.

Wyllie S, Cunningham ML, Fairlamb AH (2004) Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. Journal of Biological

Chemistry 279: 39925–39932.

Yadav CS, Kumar V, Suke SG, Ahmed RS, Mediratta PK, Banarjee BD (2010) Propoxur-induced acetylcholine esterase inhibition and impairment of cognitive function: attenuation by *Withania somnifera*. Indian Journal of Biochemical Biophysics 47: 117-120.

Yamada K, Hung P, Park TK, Park PJ, Lim BO (2011) A comparison of the immunostimulatory effects of the medicinal herbs Echinacea, Ashwagandha and Brahmi. Journal of Ethnopharmacology 137: 231-235.

Yamey G and Torreele E (2002) The world's most neglected diseases. British Medical Journal 325: 176–177.

Yang K, Choi MJ, Kim JH, Choi KS, Kwon TK (2011) Withaferin A enhances radiation-induced apoptosis in Caki cells through induction of reactive oxygen species, Bcl-2 downregulation and Akt inhibition. Chemico Biological Interation 190: 9-15.

Yeates C (2002) Sitamaquine (GlaxoSmithKline/Walter Reed Army Institute). Current Opinion in Investigational Drugs 3: 1446–1452.

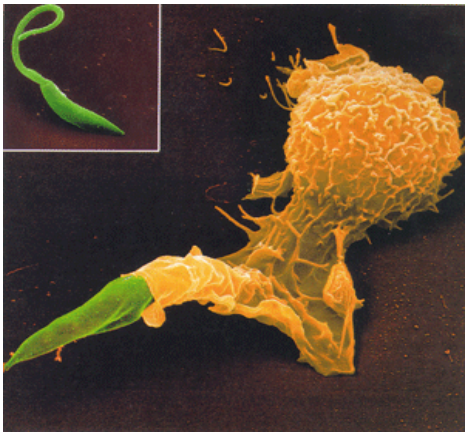
Yu Y, Hamza A, Zhang T, Gu M, Zou P, Newman B, Li Y, Gunatilaka AA, Zhan CG, Sun D (2010) Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochemical Pharmacology 79: 542-551.

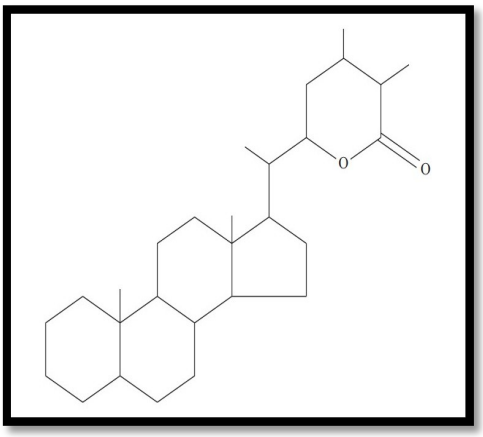
Zangger H, Mottram JC, Fasel N (2002) Cell death in *Leishmania* induced by stress and differentiation: programmed cell death or necrosis? Cell Death Differ 9: 1126-1139.

Zijlstra EE, Ali MS, EI-Hassan AM, EI-Toum I A, Satti M, Ghalib H W, Sandrop E and Winkler A (1991) Kala-azar in displaced people from southern Sudan: epidemiology, clinical and therapeutic findings. Trans R Soc Trop Med Hyg 85: 365-369.

Zuccotto F, Martin AC, Laskowski RA, Thornton JM, Gilbert IH (1998) Dihydrofolate reductase: a potential drug target in *Trypanosomes* and *Leishmania*. J Comput Aided Mol Des 12: 241–257.

Annexure: Publications and Conferences Attended





Publications

Published:

1. **Chandrasekaran S**, Dayakar A, Veronica J, Maurya R. (2013) An *in vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by Withanolides. **Parasitology International** 62: 253-261.
2. **Chandrasekaran S**, Veronica J, Gundampati RK, Sundar S, Maurya R Exploring the inhibitory activity of withaferin A against Pteridine reductase1 of *L. donovani* (**Accepted in Journal of Enzyme Inhibition and Medicinal Chemistry**).
3. Dayakar A*, **Chandrasekaran S***, Prajapati VK, Veronica J, Sundar S, Maurya R. (2012) A rapid method to assess the stage differentiation in *Leishmania donovani* by flow cytometry. **Experimental Parasitology** 132: 495-500**. *Authors contributed equally**
4. Dayakar A, **Chandrasekaran S**, Veronica J, Maurya R. (2011) Role of Leptin in Human Visceral Leishmaniasis? **Medical hypotheses** 77: 416-418.
5. Dayakar A, **Chandrasekaran S**, Veronica J, Sundar S, Maurya R. *In vitro* and *in vivo* evaluation of antileishmanial and immunomodulatory activity of neem leaf extract in *Leishmania donovani* infection. **Experimental Parasitology** 153: 45-54.
6. Gundampati RK, Sahu S, Srivastava AK, **Chandrasekaran S**, Vuddanda PR, Pandey RK, Maurya R, Singh S, Jagannadham MV. (2013) *In-silico* and *in-vitro* studies: Tryparedoxin peroxidase inhibitor activity of methotrexate for antileishmanial activity. **American Journal of Infectious Diseases** 9: 117-129.

Under Review:

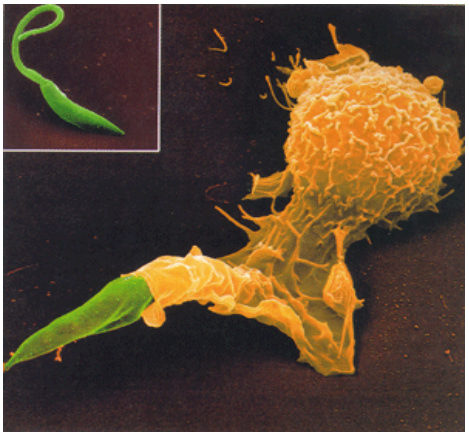
7. Dayakar A, **Chandrasekaran S**, Veronica J, Maurya R. Leptin induces phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and PBMCs. (**Manuscript under review**).
8. Gul MZ, **Chandrasekaran S**, Manjulatha K, Maurya R, Qureshi IA, Ghazi IA. Evaluation of the antiproliferative activity of leaves from *Abrus precatorius* by Bioassay-guided fractionation (**Manuscript under review, Plos One**).
9. Gul MZ, **Chandrasekaran S**, Manjulatha K, Maurya R, Qureshi IA, Ghazi IA. Bioassay-guided fractionation and in vitro antiproliferative effects of fractions of Artemisia nilagirica on THP-1 cell line. (**Manuscript under review, Nutrition and Cancer**).

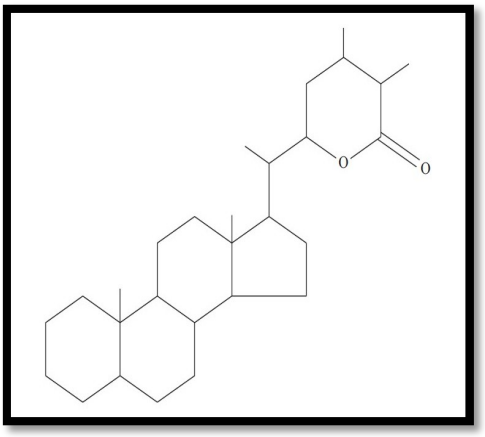
Conference Presentations

Poster presentations:

1. "Apoptotic-like death induced by Withanolides in *Leishmania donovani* promastigotes" presented at **International Symposium on Chemistry and Chemical Biology of Natural Products, 2012, Hyderabad, India.**
2. "Withaferin A: Evaluation of antileishmanial and immunomodulatory properties and *in-silico* docking with Trypanothione reductase" presented at **6th International Conference on Drug Discovery and Therapy, 2014, Dubai, UAE.**
3. "Exploring the antileishmanial potential of withanolides from *Withania somnifera* leaves" presented **at DRILS Science Cafe-2015 young scholar's science meet held at Dr Reddy's Institute of Life Sciences, University of Hyderabad campus on 10th August 2015.**

Reprints of Published Articles





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An *in vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by withanolides

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ABSTRACT

The aim of this study was to isolate and evaluate the withanolides in inducing apoptotic like death in *Leishmania donovani in vitro*. Withanolides were fractionated and isolated from the leaves of *Withania somnifera* and LC-MS/MS analysis of two fractions namely, F5 and F6 of ethanolic extracts, obtained through column chromatography with silica gel, was performed. The antileishmanial effect of withanolides on *L. donovani* promastigotes was assessed *in vitro* using PI dye exclusion test. The effect of withanolides on promastigote morphology was determined by scanning electron microscopy. To understand their mode of action against *L. donovani*, DNA fragmentation, quantification of parasites at sub G_0/G_1 phase, determination of phosphatidylserine externalization, measurement of reactive oxygen species (ROS) and mitochondrial membrane potential (Ψ_m) were done. Results showed that LC–MS/MS analysis confirmed the presence of withanolides in isolated fractions. Treatment with withanolides resulted in morphological alterations from spindle to round shape and loss of flagella/cell integrity in promastigotes. Moreover, it induced DNA nicks, cell cycle arrest at sub G_0/G_1 phase and externalization of phosphatidylserine in dose and time dependent manner via increase in ROS and decrease in Ψ_m. Results of this study indicate that withanolides induce apoptotic like death through the production of ROS from mitochondria and disruption of Ψ_m in promastigotes of *L donovani*.

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1. Introduction

Leishmaniasis is an endemic disease in 98 countries worldwide with 200 to 400 thousands new cases each year [1]. It is caused by the various species of the genus *Leishmania*. *L. donovani* is a causative agent of visceral leishmaniasis (VL) or "Kala-azar" in India. The parasite completes its life cycle in two hosts, namely sand fly and humans [2]. VL is the most severe form of leishmaniasis which is fatal, if left untreated. Approximately, world's 50% VL cases are from Indian subcontinent [3]. Till date, there has been no effective vaccine against leishmaniasis and the treatment relies exclusively on chemotherapy. Pentavalent antimonials have been the mainstay of therapy for all forms of leishmaniasis for last seven decades, however, its efficacy has declined in recent years with the result that only about one third of patients respond to it [4,5]. Efficacy of miltefosine, the drug chosen for the Elimination programme in the Indian subcontinent, has declined over the years as well [6]. Hence, there is an urgent need for the development of effective, cheaper and safer drugs to combat this disease.

Traditionally, plants have been used for the treatment of protozoan diseases [7] and plant products may play a significant role in the search of a new antileishmanial compounds. *Withania somnifera* (WS), also known as Ashwagandha is being used as an important medicinal herb for over 3000 years [8]. It has been used for various purposes ranging from an antioxidant to antibacterial [9–13]. The biological activity of WS leaves is mainly attributed to withanolides which comprises withaferin A, withanolides A-y, withanone, *etc.*, which are C_{28} steroidal lactone triterpenoids with ergosterane framework [14,15]. Mainly, much of the pharmacological activities are attributed to the two withanolides namely, withaferin A and withanolide D.

In this study, we evaluated the antileishmanial role of the withanolides isolated from the ethanolic extract of WS leaves through column chromatography with silica gel. Two fractions named as F5 and F6, at a dosage of 60 μg/ml and 15 μg/ml respectively, induce apoptotic like death in the promastigote stage of the *L. donovani in vitro*. The antileishmanial effect was found to be exerted through morphological alterations like cell shrinkage, DNA fragmentation, externalization of phosphatidylserine, induction of ROS ultimately leading to the loss of mitochondrial membrane potential. These observations clearly indicate that withanolides induce apoptotic like death in the promastigote stage of the parasite.

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A rapid method to assess the stage differentiation in *Leishmania donovani* by flow cytometry

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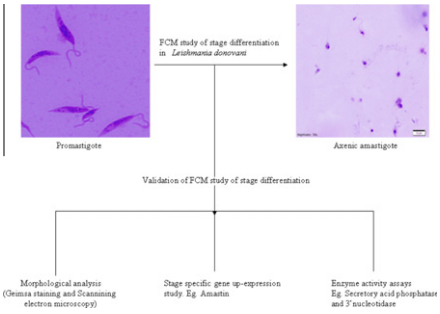
HIGHLIGHTS

- FCM is a non-flourimetric tool to study stage differentiation in *Leishmania donovani*.
- FCM was validated using different molecular and enzymatic assays.
- Partial CDS sequence (472 bp) of amastin gene was submitted in GenBank (HQ840734).
- Successful differentiation and propagation of LEM 138 were established.

GRAPHICAL ABSTRACT



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Amastin gene
ABCA3
ATP binding cassette A3
SAcP
Secretory acid phosphatase
3′-Nucleotidase.

ABSTRACT

In this study we describe a rapid and novel method to assess the morphological stage differentiation in *Leishmania donovani* by flow cytometry (FCM). FCM is fast, accurate, and inexpensive to study the stage differentiation of promastigote into *L. donovani* axenic amastigote (LdAxAm). The non-flourimetric FCM method is easy to perform; with requirement of little expertise, and provides unambiguous results. It is an advanced tool, requires minimal time, and no fluorescent dyes. The gradual increase of differentiation and reduction in size from promastigote stage to LdAxAm leads to peak shifting from right to left on histogram. Earlier reports assessed the stage differentiation of *Leishmania* by studying the expression of stage specific markers like surface or secretory proteins and genes. For validation, conventional methods like microscopic analysis are used. These methods are quite expensive, laborious and time consuming. Non-flourimetric morphological parameters were further validated by conventional methods like optical and scanning electron microscopy. Additionally, differential expression of stage specific genes (e.g. upregulation of amastin and ATP binding cassette A3 (*ABCA3*) transporter gene transcripts) and differential activity of enzymes (down regulation of secretory acid phosphatase (SAcP) and 3′-nucleotidase enzyme activity) in LdAxAm suggest stage differentiation. Therefore, we believe that our method is an alternative tool for high reproducibility and reliability in assessment of stage differentiation.

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Role of leptin in human visceral leishmaniasis? ☆

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ABSTRACT

Human visceral leishmaniasis (VL) is frequently found in poor population who are suffering from malnutrition in endemic areas. Therefore, obviously they may have reduced levels of leptin due to reduction in number of adipocytes which are major source of leptin production. Human pathogenesis of VL and reduced levels of leptin both are associated with increase in Th2 type immune response, characterized by secretion of cytokines such as IL-4 and IL-10. Whereas, the protective immune response during visceral leishmaniasis is associated with effective Th1 type immune response characterized by secretion of IFN-γ, IL-2 and IL-12, which correlates with leptin induction of T cells polarizing to Th1 population and secretion of proinflammatory cytokines, and also inhibition of Th2 type response. Therefore, we hypothesized that leptin might be effective in treatment of visceral leishmaniasis alone or VL patients who have co-infection with other immune deficiency syndromes such as AIDS/diabetes/autoimmune disorders by regulation of Th1/Th2 homeostasis.

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Introduction

Kala-azar or human visceral leishmaniasis (HVL) is caused by protozoa belongs to the genus *Leishmania* which infect host macrophages. In India, *L. donovani* is responsible for this disease which is characterized by parasite invasion of lymphoid tissues without causing clinical symptoms [16]. The disease is spread by the bite of certain species of sand fly (subfamily–Phlebotominae). The genera that transmit this disease to humans are *Lutzomyia* in the New World and *Phlebotomus* in the Old World [1]. HVL is mainly affects poor people who are suffering from malnutrition, and is also a serious public health problem in many tropical and subtropical regions of the world. It is endemic in 88 countries of the world including tropics, sub-tropics and the Mediterranean basin (WHO, 1984) among which 16 are developed countries and 72 are developing countries. HVL is one of the top 10 diseases of concern to the Office International des Epizooties (OIE) and top 2 tropical parasitic diseases according to World Health Organization, is rapidly spreading, often in association with HIV infection, especially in sub-Saharan Africa and South America. Recently, it was estimated that 12 million people were infected and 350 millions were at risk of acquiring infection. HVL has emerged as an opportunistic infection in HIV-positive patients. The incidence of HIV infection has been rapidly increasing, which may lead to increasing number of Kala-azar patients. Signs and symptoms include fever, weight loss, mucosal ulcers, fatigue, anemia and substantial swelling of the liver and spleen.

Leptin is a non-glycosylated, 167 amino acids containing protein with molecular weight of 16-kDa, encoded by the obese (*ob*) gene, which is located on human chromosome 7. White adipose tissue is the major source of leptin production [2]. Leptin functions as a hormone as well as a cytokine (adipokine). As a cytokine, it attributes to various functions such as hematopoiesis, angiogenesis, and innate and adaptive immunity [2–6]. Leptin belongs to a family of class I cytokines, which are characterized by a four α-helix bundles [7].

Th1/Th2 polarization in HVL

During visceral leishmaniasis in humans the immune response is predominantly Th2 type, with absence of IFN-γ to *Leishmania* antigens [17,18] and the production of IL-10 and IL-4. IL-10 producing $CD25^-$ T cells were recently implicated in the pathogenesis of HVL in India [9]. Protective immunity in *Leishmania* infections is generally attributed to a Th1 immune response that generates IFN-γ and IL-2 producing $CD4^+$ cells. IL-12 plays an important role in the activation of Th1 cells to secret IFN-γ and IL-2 [21,22].

Leptin induces protective Th1 response

Studies on *Ob/ob* (leptin deficient) mice appeared to show an incredible role of leptin in inflammation and autoimmunity. Leptin deficient mice have reduced secretion of IL-2, IFN-γ, and IL-18 and increased production of Th2 cytokines (IL-4 and IL-10). Leptin

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A RISK OF VISCERAL LEISHMANIASIS IN CASE OF HELMINTHS CO-INFECTION IN ENDEMIC REGIONS.

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ABSTRACT

People in the endemic region are more prone to helminth infections due to the poor hygienic conditions and their genetic susceptibility may be a factor for *Leishmania* infection. The acute worm infections may not cause fatal outcome to host but it may facilitates the infection of other intracellular pathogens which cause higher rate of morbidity. In case of *Leishmania donovani* infection, helminth infections mainly elicit Th2 type protective host immune response characterized by secretion of IL-4, IL-13, IL-5, IL-9 and IL-10, which may suppress the Th1 protective host immune response. Pre-immune polarized (Th2/Th1) individuals due to the helminth infections and their recovery from infection after treatment may have higher levels of serum IL-4 and IL-13 are highly susceptible for visceral leishmaniasis in endemic regions. These Th2 type cytokines, IL-4, IL-13 and IL-10 have immunosuppressive activity, help in the parasite survival by inhibiting the macrophage induced IFN-γ production and oxidative burst mechanism, thereby enhancing disease progression in chronic visceral leishmaniasis. In addition, IL-5 dependent eosinophilia in helminth infections may cause inflammation in visceral organs leading to tissue damage hence these individuals might be susceptible for parasite attack. Therefore, we hypothesize that the Th2 type cytokine milieu of helminth infection might be increase rate of susceptibility to VL occurrence in endemic regions.

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INTRODUCTION

Billions of people and their domesticated animals worldwide are constantly affected by the parasitic helminths, or worms, which come under a diverse group of metazoan organisms [1] causing great morbidity, increased susceptibility to other infectious agents and, in some cases, it may leads to death. Majority of individuals infected with parasitic worms are usually asymptomatic or less symptoms compared to those infected with acute viral or bacterial infections and very few will have life-threatening consequences. Good hygienic conditions and health care can minimize the helminthic parasite infections, but in endemic regions immunological intervention can be an effective option of treatment. Till date, there have been no vaccines or other effective immunotherapies for helminth infections and the understanding of the immune response to these important pathogens remains at a very early stage.

Visceral leishmaniasis (VL), also known as kala-azar, black fever, and Dumdum fever [2], is the most severe form of leishmaniasis. It is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 500,000 cases each year worldwide, with 90% of cases caused by the transmission of *Leishmania donovani* in India, Sudan, Nepal, and Bangladesh. [3]. Every year, more than 100,000 cases of VL occur in India alone and in the state of Bihar accounting for more than 90% of these cases, followed by West Bengal and Eastern Uttar Pradesh [5]. It is endemic in 88 countries of the world including tropics, sub-tropics and the Mediterranean basin (WHO, 1984) among which 16 are developed countries and 72 are developing countries. The parasite migrates to the internal organs such as liver, spleen (hence '*visceral*') and bone marrow without causing clinical symptoms [4].

Host immune response to helminth infection

The primary cause of disease in many helminth infections is due to the under development of immune system after encountering the pathogens which lives longer and causes chronic infections. Helminth pathogens differ based on the presence of different glycoconjugates, which contain unusual sugars [15, 21, 24], thought play a role in Th2 response development [22]. These features of helminth Ag are likely to be recognized by Toll receptors and/or other pattern recognition receptors [6]. This extensive organismal complexity, in the majority of cases evokes Th2-like immune response against the worms with the production of a significant quantity of IL-4, IL-5, IL-9, IL-10, and IL-13 and thereby developing a strong immunoglobulin E (IgE), eosinophil, and mast cell responses. Among these cytokines, IL-4 and IL-10 may play a crucial role in reducing the severity of acute disease and

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Full length article

In vitro and *in vivo* evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection

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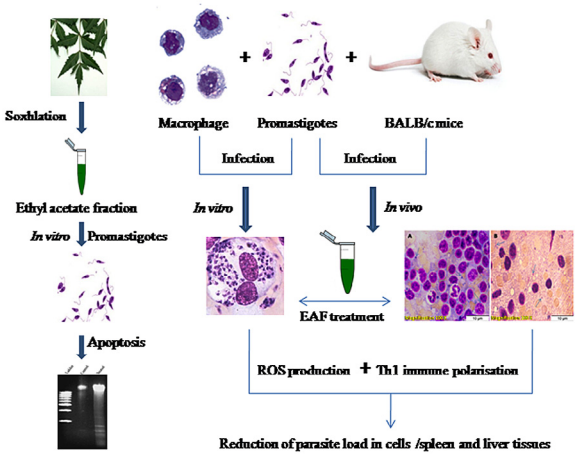
HIGHLIGHTS

- Anti-leishmanial activity of Neem leaf ethyl acetate fraction on promastigotes.
- Anti-proliferation activity and apoptosis like death in promastigotes.
- Anti-leishmanial activity on intracellular amastigotes both *in vitro* and *in vivo*.
- Th1 polarization and Th2 downregulation at m-RNA level by real-time PCR analysis.
- Increased production of reactive oxygen and nitrogen species from macrophages with extract treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity and emergence of resistance to available chemical drugs against visceral leishmaniasis is evoking to explore herbal treatment. One such attempt with the Neem is being reported here. The current study is primarily focused to evaluate the anti-leishmanial effects of Neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC_{50} to be 52.4 μg/ml on the promastigote stage. Propidium iodide (PI) staining of dead cells substantiated the aforementioned activity. Carboxy fluorescein–diaceate succinimidyl ester (CFSE) staining of promastigotes has affirmed its anti-proliferation activity. The characteristic features such as DNA fragmentation, reduced mitochondrial membrane potential, increased sub G_0/G_1 phase parasites and increased reactive oxygen species (ROS) production in EAF treated promastigotes indicate the apoptosis like death. In addition, the reduced parasite burden both *in vitro* (*viz.* ~45% in human monocytic leukemia cell line (THP-1) and ~50% in peripheral blood mononuclear cells) and *in vivo* (spleen and liver) provides the evidence for its anti-leishmanial activity on amastigote stage. The increase of ROS levels

Abbreviations: EAF, ethyl acetate fraction; VL, visceral leishmaniasis; THP-1, human monocytic leukemia cells; PBMCs, peripheral blood mononuclear cells; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ROS, reactive oxygen species; Ψ_m, mitochondria membrane potential; MFI, mean fluorescence intensity; LDU, Leishman–Donovan units; NO, nitric oxide.

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IN SILICO AND *IN VITRO* STUDIES: TRYPAREDOXIN PEROXIDASE INHIBITOR ACTIVITY OF METHOTREXATE FOR ANTILEISHMANIAL ACTIVITY

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ABSTRACT

In order to understand the mechanism of molecular interactions at the active site of Tryparedoxin Peroxidase (Try P), homology modeling and docking studies were performed. We generated a Three-Dimensional (3D) model of target protein based on the Crystal structure of *Leishmania Major* Try PI (PDB ID: 3TUE) using modeler software. Docking analysis was carried out to study the effects of methotrexate on Tryparedoxin Peroxidase (Try P). Inhibition of the Tryparedoxin peroxidase interaction has become a new therapeutic strategy in treating leishmaniasis. Docking analysis was carried out to study the effects of methotrexate on Tryparedoxin Peroxidase (TryP). Tryparedoxin peroxidase of Trypanosomatidae family functions as antioxidant through their peroxidase and peroxynitrite reductase activities. The theoretical docking study, conducted on a sample previously reported for anti-cancer properties of Methotrexate at the binding site of 3D models of Tryparedoxin Peroxidase of *Leishmania braziliensis* (*L. braziliensis* Try P) examine interaction energy. Our studies indicate that Methotrexate displays potent activity against Try P with lowest binding energy and RMSD values to be -14.5879 Kcal/Mol and 2.0 A. The results of the present study clearly demonstrated the Tryparedoxin Peroxidase inhibitory activity by methotrexate in *in silico* docking analysis and *in vitro* assay which contributes towards understanding the mechanism of antileishmanial activity.

Keywords: Homology Modeling, Molecular Docking, *Leishmania Braziliensis*, Tryparedoxin Peroxidase, Methotrexate

1. INTRODUCTION

Over the past 60 years very limited number of drugs has been developed for the treatment of Leishmaniasis and the use of available drugs has been hampered by high cost, adverse side effects, development of resistance by the parasite and also due to low efficacy (Croft *et al*., 2006). Some experimental as well as *in silico* attempts have been made to identify inhibitors or subversive substrates for various molecular targets (Krauth-Siegel and Inhoff, 2003; Perez-Pineiro *et al*., 2009). The enzyme Tryparedoxin Peroxidase (Try P) of *Leishmania braziliensis* is a 199 amino acid enzyme with a molecular weight of approximately 22.5 kDa. Try P belongs to the protein family of peroxiredoxins was selected in this investigation as molecular target for *in-silico* screening of anti-leishmanial activity. This enzyme cascade involves Trypanothione Reductase (Try R), Tryparedoxin (TXN) and trypanotione (N^1, N^8-bis (glutathionyl)-spermidine) serving as a mediator for

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