LEISHMANICIDAL ACTIVITY EXPRESSED *IN VITRO* BY SOME NEPALESE MEDICINAL PLANTS AGAINST *LEISHMANIA DONOVANI*

M.Sc. Thesis

(2013)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement for the
Master of Science in Biotechnology

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Acronyms

ABCD	Amphotericin B colloidal dispersion
ABLC	Amphotericin B lipid complex
ADP	Adenosine diphosphate
AmB	Amphotericin B
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
BCG	Bacillus Calmette Guerin
CC_{50}	Cytotoxic Concentration of drug for killing 50% cells
CDC	Centre for disease control
CFU	Colony forming unit
CL	Cutaneous leishmaniasis
CMI	Cell-mediated immune response
DALYs	Disability adjusted life years
DAT	Direct agglutination test
DCL	Diffused cutaneous leishmaniasis
DDT	Dichloro-dephenyl-trichlorethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC	Inhibitory concentration
IC_{50}	Concentration inhibition 50% of parasites
IC_{90}	Concentration inhibition 90% of parasites
ICT	Immunochromatographic test
IFA	Indirect fluorescence antibody
IFN-γ	Interferon gamma

IL	Interleukin
iNOS	inducible Nitric oxide synthase
IRS	indoor residual spraying
ITN	insecticide treated net
L-AmB	liposomal amphotericin B
LmsTI1	*L. major* stress-inducible protein-1
LPG	lipophosphoglycan
LST	Leishmanin skin test
MCL	Mucocutaneous leishmaniasis
MPL-SE	Monophosphoryl lipid A plus squalene
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NASBA	Nucleic acid sequence based assay
NCBI	National Centre for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PKDL	Post Kala-azar Dermal Leishmaniasis
PSG	Promastigote secretory gel
rK39 ICT	Recombinant Kinesin 39 amino acid immunochromatographic test
RNA	Ribose-nucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
Sb^v	Pentavalent antimony
SI	Selectivity index
SQ	Sitamaquine
SSG	Sodium stibogluconate
TGF	Transforming growth factor
Th1/ Th2	T helper 1 / T helper 2
VL	Visceral leishmaniasis
WHO	World Health Organisation

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Abstract

Leishmanicidal activity expressed *in vitro* by some Nepalese medicinal plants against *Leishmania donovani*

Visceral leishmaniasis (VL), a neglected tropical disease, affects millions of people worldwide especially in the developing nations. Owing to the limited and toxic chemotherapeutic interventions, there is a greater interest in new drug developments against VL, particularly from plants with unparalleled diversity in phytocompounds and bioactivity. The aim of this study was to evaluate the *in vitro* anti-leishmanial activity of five traditionally used Nepalese medicinal plants, namely *Boerhavia diffusa* (Nyctaginaceae), *Calotropis procera* (Asclepiadaceae), *Phyllanthus niruri* (Euphorbiaceae), *Hedychium spicatum* (Zingiberaceae), and *Woodfordia fruticosa* (Lythraceae) selected on the basis of their ethnomedical use in the treatment of various liver diseases and conditions such as hepatospenomegaly, a hallmark of VL. The crude ethanolic extract of *W. fruticosa* flowers displayed the maximum efficacy with IC_{50} values of 35.30 ± 2.43 µg/mL against the promastigotes and 27.25 ± 1.88 µg/mL against the axenic amastigotes of *Leishmania donovani*. The parasites were also significantly inhibited by other three plants namely *P. niruri* (IC_{50} for promastigote = 67.46 ± 3.03 µg/mL and IC_{50} for amastigote = 40.23 ± 2.23 µg/mL), *H. spicatum* (IC_{50} for promastigote = 73.63 ± 4.34 µg/mL and IC_{50} for amastigote = 46.96 ± 2.11 µg/mL) and *C. procera* (IC_{50} for promastigote = 96.32 ± 8.79 µg/mL and IC_{50} for amastigote = 57.96 ± 0.13 µg/mL). Time dependent efficacy evaluation of the extracts resulted in inhibition greater than 99% over 96 hours. The reference drugs (Miltefosine and Amphotericin B) used, though better in activity were found to be highly toxic. On the other hand, cytotoxicity tests revealed the overall safety of the extracts, the extract of *W. fruticosa* being the most safe (selectivity indices of 2.56 and 3.31 against promastigotes and amastigotes respectively). An antibacterial screening conducted in parallel indicated a selective action towards the parasites. Both the antibacterial and cytotoxicity tests suggested that the leishmanicidal efficacy of the extracts was not due to *in vitro* cytotoxicity. It is important to point out that all five investigated plant species have never been evaluated before for their antileishmanial potential and focuses on the need of such screening works.

Keywords: Visceral leishmaniasis, *Leishmania donovani,* medicinal plants, antileishmanial, IC_{50}, Cytotoxicity, Nepal

Chapter I

Introduction

1.1 Leishmaniasis

1.1.1 Causative agents, vectors and reservoir hosts

Leishmaniasis, a subtropical disease of the poor, is caused by parasitic protozoans of the genus *Leishmania* and transmitted by means of infected sandfly bite to vertebrate hosts including man. The disease has been named after British pathologist William Boog Leishman, who first described it in 1903. Leishmaniasis is a clinically heterogeneous group of diseases with visceral and cutaneous manifestations (Croft et al., 2006; Antinori et al., 2012). More than 20 species of *Leishmania* which are spread by about 30 species of sand flies are responsible for causing leishmaniasis (Desjeux, 2004).

Leishmania spp. belong to the kingdom – Protista and subkingdom Protozoa. They are morphologically similar and exist in two forms: the flagellated promastigotes that develop in the sand fly gut and the amastigotes with rudimentary flagella which is an obligatory intracellular form that develops in mammalian macrophages.

Systematic position:

Kingdom: Protista (Haeckel, 1866)
Subkingdom: Protozoa (Goldfuss, 1818)
Phylum: Sarcomastigophora (Honlgber and Balomuth, 1903)
Subphylum: Mastigophora (Diesing, 1943)
Class: Zoomastigophora (Calkins, 1909)
Order: Kinetoplastidae (Honigberg, 1963; Vickerman, 1976)
Suborder: Trypanosomatina (Kent, 1880)
Family: Trypanosomatidae (Doflein, 1901)
Genus: *Leishmania* (Ross, 1903)

i. Promastigotes

Promastigotes exist as elongated (15-20 µm in length and 1.5-3.5 µm in breadth), motile extracellular stage (Fig.1.1). *Leishmania* promastigotes are highly polarized cells that possess a number of single-copy organelles like nucleus, Golgi apparatus, basal body, mitochondrion (which incorporates the kinetoplast), and the flagellum (15-20 µm long) protruding from the cell body via the flagellar pocket (Herwaldt, 1999; Hommel, 1999).

ii. Amastigotes

Amastigotes are ovoid (2.5–5 µm diameter), non motile intracellular stage that are located in the parasitophorous vacuoles of the host's macrophages. Apart from the size and shape, the general structure of both the stages is fairly the same with the presence of single mitochondrion and kinetoplast (Fig.1.1). The kinetoplast is the dense mass of the mitochondrial DNA and characteristic feature of the order Kinetoplastida. It constitutes about 10-20% of the total DNA. It is a network of concatenated circular DNA, divided into two classes: the homogenous maxicircles (25-250 molecules of 30 kb) and the heterogeneous minicircles (5000-10,000 molecules of ~2 kb) (Chen et al., 1995; Banuls et al., 2007).

The major difference between amastigotes and promastigotes is, of course, the length of the flagellum: barely extruding from the cell body in amastigotes, while up to three times the cell body length in some promastigotes. This difference is a result of change in the environment between the hosts- a temperature shift from 25-28°C in sandflies to 35-37°C in the mammal macrophages and a pH change from neutral in sandflies to acidic (~pH 5) in macrophages. This change in the environment is followed by the loss of flagellum, closing of the flagellar pocket, reduction in size and major changes in gene expression that gives rise to the obligatory intracellular amastigote forms of the parasite (Hommel, 1999).

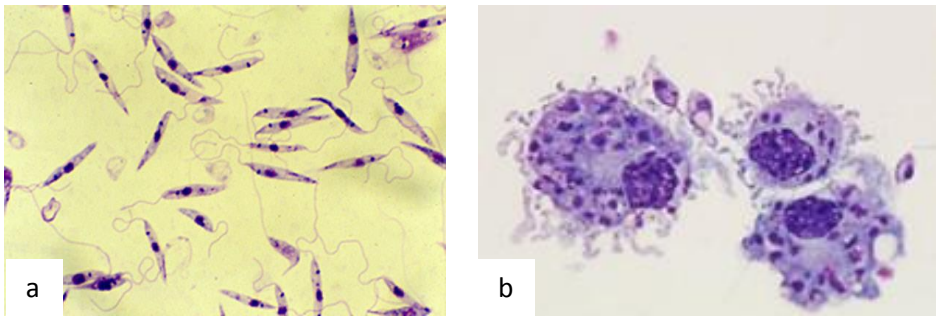


Fig. 1.1: *Leishmania* a. Promastigotes b. Intracellular amastigotes
[Source: a. http://www.uni-tuebingen.de/modeling/Mod_Leish_Intro_en.html;
b. http://bakerinstitute.vet.cornell.edu/faculty/view.php?id=179]

The vectors of *Leishmania* parasites are tiny 2-3mm long female sandflies that belong to Phlebotominae family (Class Insecta) and are distributed throughout the inter-tropical and temperate regions of the world. They are hairy and soundlessly flying insects that belong to two genera: *Phlebotomus* (in the Old World) and *Lutzomia* (in the New World). Only 30 of about 600 sand flies species are important vectors for *Leishmania* and in the Indian subcontinent, *Phlebotomus argentipes* is the one responsible for kala-azar transmission (Sharma and Singh, 2008) (Table1.1).

Epidemiologically, there are two forms of the disease: (1) zoonotic which includes animal reservoir hosts in the transmission cycle and (2) anthroponotic in which man is the sole source of infection for the vector (Desjeux, 2004; Quinnell and Courtenay, 2009). The main reservoir hosts for *Leishmania* are domestic animals (e.g. dogs, cats and horses), peridomestic animals (e.g. mice and rats) and wild animals (e.g. rodents, hyraxes, sloths, bats, opposums, kangaroos, wolves and foxes). Among all these animals, domesticated dogs play the most important role in the transmission as they are closely associated with mankind. In cases of the visceral form of the disease in the Indian subcontinent, it is anthroponotic type whereby the parasite is exclusively maintained in a man-vector-man cycle without the involvement of any animal reservoir (Sharma and Singh, 2008).

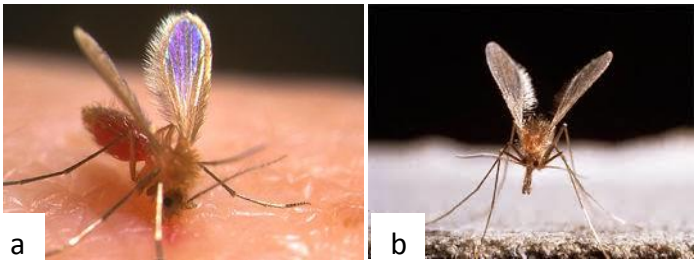


Fig. 1.2: Vectors of Leishmaniasis: a. *Phlebotomus argentipes* in Old World and b. *Lutzomyia longipalpis* in New World. [Source: Sharma and Singh, 2008]

1.1.2 Clinical forms of leishmaniasis

Leishmaniasis is a complex disease. Depending on strain (s) of the parasite involved in pathogenesis and the immune response established by the host it can cause clinical symptoms that range from mild self-limiting cutaneous lesion to fatal visceral disease (Desjeux, 2004).

Visceral leishmaniasis (VL)

Visceral leishmaniasis or kala-azar is the most severe form of leishmaniasis and endemic to many temperate and tropical countries. The clinical picture consists of a protracted course of fever, lymphadenopathy, hepatosplenomegaly, hypergammaglobulinemia, hypoalbulinemia, wasting, and pancytopenia and may be fatal if left untreated (Balasegaram et al., 2012). It is caused by *L. donovani* species complex (i.e. *L. donovani, L. infantum* and *L. chagasi*) (Sharma and Singh, 2008). After recovery or as a new case, patients may develop a chronic cutaneous form called **Post Kala-azar Dermal Leishmaniasis (PKDL)** characterised by a macular, maculopapular, and nodular rash usually on face, upper arms, trunks and other parts of the body, harboring the parasites (Zijlstra et al., 2003). In the Indian subcontinent, PKDL seems to appear within 2-3 yrs in

5-10% of all past VL cases whereas the PKDL cases account to 50% in Sudan that appear within 6 months (Zijlstra et al., 2003; Uranw et al., 2011) (Fig. 1.3).

Cutaneous leishmaniasis (CL)

Also known as 'Oriental Sore', cutaneous leishmaniasis (CL) accounts for approximately two-thirds of all new cases of leishmaniasis (WHO, 2013). The disease is caused by *Lieshmania tropica*, *L. major* and *L. aethiopica* in old world and *L. mexicana* species complex specially *L. mexicana*, *L. amazonesis* and *L. venzuelensis* and some *Viannia* subgenus of *Leishmania* in new world (Herwaldt, 1999). CL is characterised by skin lesions that typically develop over weeks to months from papules to nodules to ulcers with raised indurated borders (Fig. 1.3). The numbers of skin ulcers may be large, as many as 200 in some cases but the lesions eventually heal with scarring. Ninety percent of cutaneous leishmaniasis infections develop in Afghanistan, Pakistan, Syria, Saudi Arabia, Algeria, Iran, Brazil, and Peru.

Mucocutaneous leishmaniasis (MCL)

Also known as 'espundia', mucocutaneous leishmaniasis (MCL) produces lesions that lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities (Fig. 1.3). These lesions are not self-healing and are usually seen months or years after a first episode of cutaneous leishmaniasis, when the macrophages of the naso-oropharyngeal mucosa become colonized. The etiological agent of the disease is *Viannia* subgenus of *Leishmania*, typically *L. (V). braziliensis* and also *L. (V.) panamensis, L. (V.) guyuanesnsis and L. (V.) amazonesis* (Herwaldt, 1999).

Diffuse cutaneous leishmaniasis (DCL)

Diffuse cutaneous leishmaniasis (DCL) is a rare but severe form of cutaneous leishmaniasis with wide spread lesions all over the body. Its clinical hallmark is excessive parasitic growth in the lesions accompanied by profound impairments in host immune responses to the parasites (Soong, 2012).

Fig. 1.3: Clinical spectrum of Leishmaniasis. (a) Visceral leishmaniasis; (b) Cutaneous leishmaniasis); (c) Diffuse cutaneous leishmaniasis (d) Mucocutaneous leishmaniasis ; and (e) Post kala-azar dermal leishmaniasis (PKDL) [Source: (Chappuis et al., 2007; WHO, 2013)]

Table 1.1: Geographical distributions of Leishmaniasis worldwide and their pathogenic species, vector and reservoir. [Source: Lainson, 1996]

Clinical manifestations	Geographical distribution	Pathogenic species	Vector	Reservoir
VL	Northeast India, Nepal, Bangladesh, Burma	*L.donovani* (Asia)	*Phlebotomus argentipes*	Human
	Mediterranean basin, Middle East, China, Central Asia	*L. infantum*	*P. pernicious, P. ariasi*	Dogs, Foxes, Jackals
	Sudan, Kenya, Horn of Africa	*L. donovani* (Africa)	*P. orientalis, P. martini*	Rodents, Canines,Humans
	Central America, Northern South America, esp. Brazil, Venezuela	*L. chagasi*	*Lutzomyia longipalpis*	Foxes, Dogs, Opossums
CL	Semi deserts in Middle East, North India, Pakistan, North Africa, Central Asia	*L. major*	*P. papatassi*	Gerbils
	Sub-Saharan Savannah, Sudan	*L. major*	*P. duboscqi*	Rodents
	Towns in Middle East,Mediterranean basin, central Asia	*L. tropica*	*P. sergenti*	Humans
	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*	Hyraxes
	Yucatan, Belize, Guatemala	*L. mexicana*	*L. olmeca*	Forest rodents
	Tropica forests of South America	*L. amazonensis*	*L. flaviscutellata*	Forest rodents
MCL	Tropical forest of South and Central America	*L. braziliensis*	*Lutzomyia spp., L. umbratilis*	Forest rodents, peridomestic animals
	Guyana, Surinam	*L. guyanensi*	*L. umbratilis*	Sloths, Arboreal Anteaters
	Panama, Costa Rica, Colombia	*L. panamensis*	*L. trapidoi*	Sloths
	West Andes of Peru, Argentine highlands	*L. peruviana*	*L. verrucarum, L. peruenis*	Dogs

1.1.3 Epidemiology and geographical distribution of leishmaniasis

1.1.3.1 Global scenario

Vector-borne diseases represent a major public health concern in most tropical and subtropical areas, and an emerging threat for more developed countries (Murray et al., 2005). And of more serious issues are the neglected diseases that cause significant morbidity among the world's poorest people but have not been targeted for intensive drug development- Leishmaniasis being one of them.

The history of leishmaniasis dates ages back. Oriental sore, commonly known as Old World cutaneous leishmaniasis these days, existed from the 7th century BC itself (Cox, 2002). Similarly, Old World Visceral leishmaniasis or Kala-azar was first noticed in Jessore (now in Bangladesh) in India in 1824 and had turned into an epidemic by 1862. Currently, visceral leishmaniasis is endemic in 88 countries, with a total of 12 million people infected and 350 million at risk. The majority of VL cases (90%) occur in only six countries: Bangladesh, India, Nepal, Sudan, Ethiopia and Brazil (Chappuis et al., 2007) (Fig.1.4). With an estimated 0.7-1.2 million new cases of CL and 0.2-0.4 million new cases of VL, leishmaniasis is responsible for the second-highest burden of disease after malaria: 2,357,000 disability adjusted life years (DALYs) (Alvar et al., 2012; Balasegaram et al., 2012). Numerous cases are undiagnosed, misdiagnosed or unreported and so there is probably an even greater difference between the number of cases actually occurring and the number reported. One of the major threats to control of visceral leishmaniasis (VL) is its emergence as an important opportunistic infection associated with HIV. Leishmania-HIV co-infections have been reported in 35 out of 88 countries in which leishmaniasis is endemic and are a growing concern in Brazil, eastern Africa and the Indian subcontinent, where both diseases co-exist (Cruz et al., 2006).

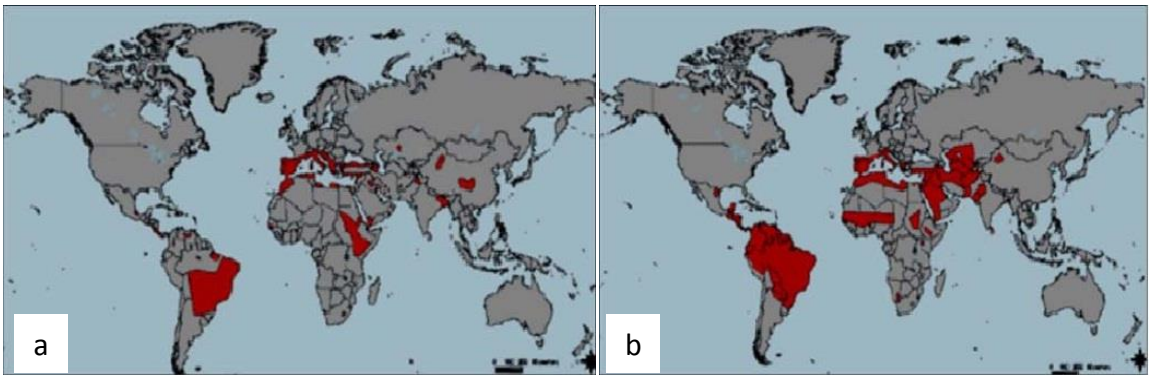


Fig. 1.4: Geographical distribution of (a) Visceral leishmaniasis; and (b) Cutaneous leishmaniasis [Source: Santos et al., 2008]

1.1.3.2 Nepalese scenario

In context of Nepal, not much information is available about the epidemiology of leishmaniasis. Visceral leishmaniasis is endemic in the country whereas there are only few cases reported of the cutaneous form. The first case of VL was recorded from Dhanusha district in 1980 (Rijal et al., 2010). At present, kala-azar is prevalent in southern plains of eastern and central regions of Nepal especially in 13 districts bordering with Bihar. Six million people residing in this region are estimated to be at risk with 1341 cases reported annually. Owing to underreporting of the cases, the actual figures are sure to be higher. On the other hand number of case reports from non-endemic areas is on the rise adding to this national health problem (Joshi et al., 2008; Pun et al., 2013). A national kala-azar control programme has been operational in Nepal since 1993 that aims at early diagnosis and treatment of the disease along with vector control using indoor residual spraying (IRS) (Rijal et al., 2010; Pun et al., 2011).

An estimated 67% of the global VL disease occurs only in three countries in the Indian sub continent - Nepal, India, and Bangladesh. This has led the governments of these countries to launch a regional VL elimination programme that targets to eliminate VL as a public health problem in these countries by 2015. The proposition is to use a local approach to reduce the annual incidence of VL to less than 1 case per 10,000 individuals (Joshi et al., 2009).

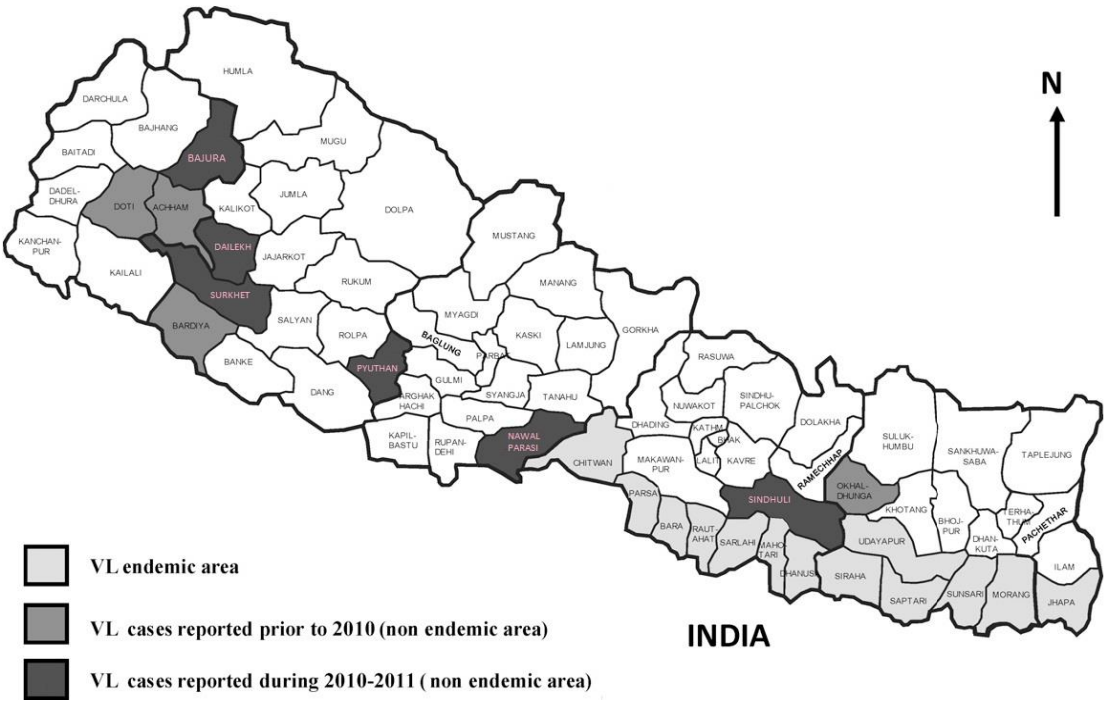


Fig. 1.5: Geographical distribution of visceral leishmaniasis (VL) cases reported prior and during September 2010–October 2011 in Nepal. [Source: Pun et al., 2013]

1.1.4 Transmission, pathogenesis and life cycle of leishmaniasis

Leishmania is transmitted primarily by the bite of the infected sandflies. However, non sandfly transmission by transfer of infected body fluids (e.g. by biting, blood transfusion, needles or sexual contact) or congenitally along with human organ transplantation have also been suggested (Cruz et al., 2002; Antinori et al., 2008; Quinnell and Courtenay, 2009).

Life cycle of *Leishmania spp* is quite complex. These parasites are digenetic with two basic life cycle stages: one extracellular stage within an invertebrate host (phlebotomine sand fly) and one intracellular stage within a vertebrate host. Thus, the parasites exist in two main morphological forms, promastigotes in invertebrate hosts and amastigotes in vertebrate hosts.

Promastigotes undergo different stages of development (within 8-20 days) to transform into infective stages (Fig. 1.6). Once ingested by the sandflies, these transform into the procyclic promastigotes within the sandfly gut. The procyclic promastigotes then differentiate into nectomonad promastigotes, which are a non-dividing migratory stage moving from the posterior to the anterior midgut. These nectomonads at the anterior midgut differentiate into leptomonad forms that are the developmental precursors of the metacyclic promastigotes, the mammal-infective stages. Leptomonad forms also produce promastigote secretory gel (PSG), a substance that plays a key role in transmission. PSG is thought to form a physical obstruction in the gut, forcing the sandfly to regurgitate metacyclic promastigotes during bloodfeeding (Kamhawi, 2006; Bates, 2007).

As the parasitized female sand fly takes a blood meal from a vertebrate host, infective promastigote forms (metacyclic promastigotes) enter the vertebrate host via the insect's proboscis. The promastigotes are then phagocytosed by macrophages which fuse with lysosomes to form phagolysosomes in which the promastigotes metamorphose into amastigote forms and reproduce by binary fission. They increase in number until the cell eventually bursts and then infect other phagocytic cells to continue the cycle (Fig. 1.7). The parasites then disseminate through the lymphatic and vascular systems and infect other monocytes and macrophages in the reticulo-endothelial system, resulting in infiltration of the bone marrow, hepatosplenomegaly and sometimes enlarged lymph nodes (lymphadenopathy) (Banuls et al., 2007; Chappuis et al., 2007).

The pathogenesis of the disease is dependent on both the parasite factors and the host mechanisms. The parasites are equipped to evade the digestive enzymes present in the vacuole and they also have a membrane bound molecule, lipophosphoglycan (LPG) that ensures intracellular survival of parasite. At the same time, the host specific cell-mediated immune (CMI) response has an important role in determining the infection. T-

helper cell-type 1 (Th1) response involves secretion of pro-inflammatory cytokines, including interleukin 12 (IL-12), interferon gamma (IFN-γ), and tumour necrosis factor which are responsible for activating macrophages for leishmanicidal action. On the other hand interleukin 4 (IL-4), interleukin 10 (IL-10), and interleukin 13 (IL-13) (Th2 cell-associated cytokines) and transforming growth factor (TGF) are capable of derailing Th1-type responses and deactivating macrophages, thereby promoting intracellular infection (Murray et al., 2005; Chappuis et al., 2007).

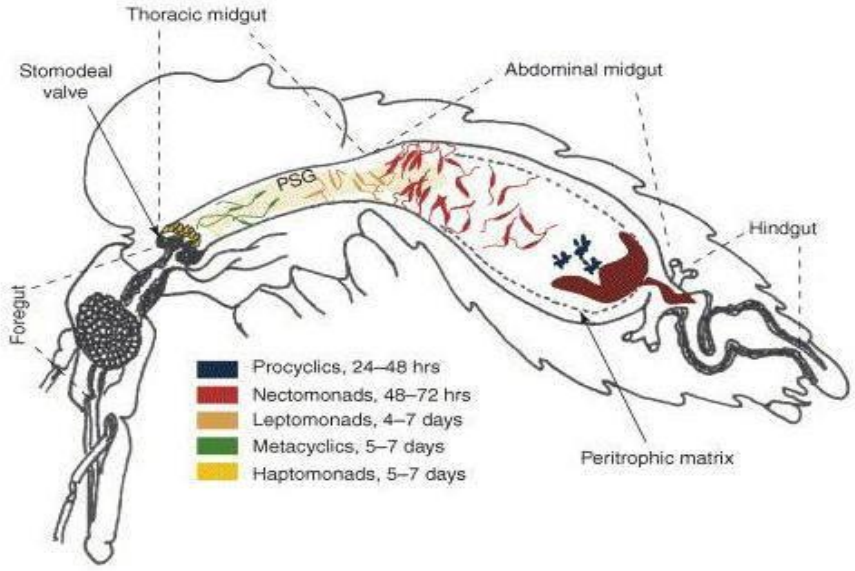


Fig. 1.6: Development of *Leishmania* species in the sand fly vector. [Source: Kamhawi, 2006]

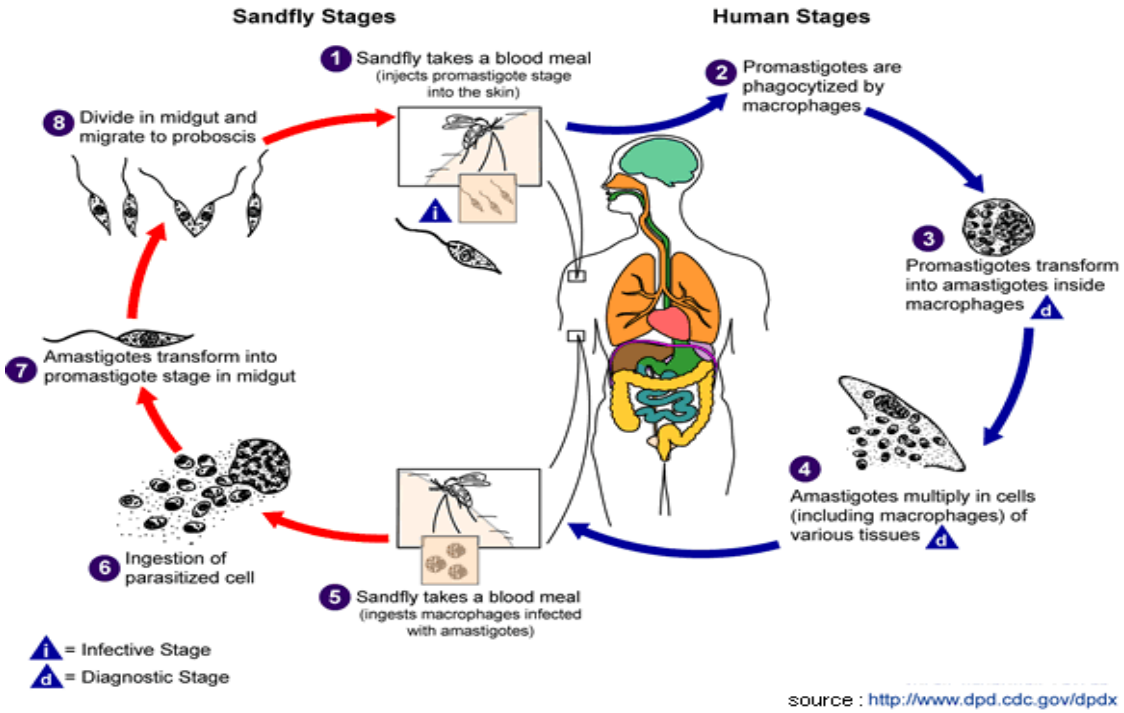


Fig. 1.7: Life cycle of *Leishmania donovani* [Source: CDC website, laboratory identification of parasite]

1.1.5 Diagnosis of leishmaniasis

The broad clinical spectrum of leishmaniasis makes the diagnosis of the cases difficult. Diseases of other etiologies with a clinical spectrum similar to that of leishmaniasis (e.g., leprosy, skin cancers, cutaneous mycoses and tuberculosis for CL, and malaria, African trypanosomiasis, brucellosis, toxoplasmosis, amoebiasis and schistosomiasis for VL) are often present in areas of endemicity rendering the clinical diagnosis to be difficult (Reithinger and Dujardin, 2007).

Ideally, all cases of leishmaniasis should be confirmed by direct detection and identification of parasite species in stained microscopic preparations, in culture medium or in animal inoculation. Microscopy is probably still the standard diagnostic approach at tertiary, secondary, or even primary health levels in areas of endemicity that studies the clinical specimens such as infected skin biopsies (for CL and MCL) or aspirates from spleen, bone marrow or lymph nodes (for VL). The sensitivity is highest for splenic aspiration (as high as 98% compared with <90% for other organs), but so is the risk (Herwaldt, 1999; Zijlstra et al., 2001). Although parasitological diagnosis still remains the method of choice to confirm leishmaniasis, it is time-consuming and not feasible under field conditions. Moreover, the sensitivity of this technique can be low in sub-clinical infections depending on the clinical material, the sampling procedure and the skills of the technical staff.

On the other hand there are serological techniques of diagnosis that are based on the antibodies and antigens. These are simple, non-invasive, fast, and sensitive and require minimal technological expertise or laboratory setup tests to be used best in areas of endemicity. Serological tests include tests such as indirect fluorescence antibody (IFA), enzyme-linked immunosorbent assay (ELISA), western blot, direct agglutination test (DAT), latex bead agglutination test (Katex), rK39-based immunochromatographic test (ICT) etc. In particular, freeze-dried antigen-based direct agglutination tests and commercially available immunochromatographic dipstick tests have increasingly become reference tests since they have high sensitivity and specificity (Chappuis et al., 2006). However these tests are not free of faults. These tests lack in when it comes to identifying asymptomatic cases and the cases of relapses. Serum antibody levels are detectable up to several years after cure therefore; VL relapses cannot be diagnosed by serology. Also a significant proportion of healthy individuals living in endemic areas with no history of VL are detected as positive for anti-leishmanial antibodies owing to asymptomatic infections which are not differentiated by the serological techniques.

Recently, the use of molecular biology techniques is becoming increasingly relevant to the diagnosis of leishmaniasis. These molecular techniques are capable of detecting DNA or RNA unique to the parasite, with a high degree of specificity and sensitivity just a few

weeks after the appearance of the first clinical symptoms (Herwaldt, 1999; Silvestre et al., 2009). Molecular tests such as the polymerase chain reaction (PCR) or nucleic acid sequence based assay (NASBA) are powerful techniques for accurate detection of the parasite in clinical specimens. However, complexity of the procedures, lack of standardisation, high costs and the fact that such techniques are not easily available hampers their broad use (Saad et al., 2010).

Disease prevalence of leishmaniasis is very difficult to define and to measure, particularly since asymptomatic and sub-clinical infections have been estimated to be 5-20 times more frequent than clinical forms. And so a test should be able to make the distinction between acute disease and asymptomatic infection, because none of the drugs currently available is safe enough to treat asymptomatic infections. Moreover, such tests should be simple, affordable and easily available to the local people (Hommel, 1999; Chappuis et al., 2007).

1.1.6 Treatment of leishmaniasis

1.1.6.1 Chemotherapy

Currently, chemotherapy remains the only option for treatment of leishmaniasis. The pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) are the first line of treatment for all forms of leishmaniasis. However, adverse side effects like cardiac arrhythmia and acute pancreatitis as well as increasing clinical resistance are observed in patients treated with antimonials (Sundar and Chakravarty, 2013). In recent years, the use of second line of drugs such as the polyene antibiotic amphotericin B (AmB) and its liposomal formulation (AmBisome) has gained popularity. Liposomal amphoterin B is considered as the best existing drug against VL but cases of infusion reactions, nephrotoxicity, hypokalemia and myocarditis are also reported. Moreover the need of close monitoring and hospitalization for 4 - 5 weeks raises the cost of therapy (Croft et al., 2006; Chappuis et al., 2007; Garcia-Hernandez et al., 2012). On the other hand use of pentamidine as second line therapy has been discouraged due to decreased cure rates and serious toxic effects associated with its use.

Miltefosine (hexadecylphosphocholine), originally developed as an anticancer agent, has now been approved as the first oral drug for VL. Although it shows good efficacy this drug has a long half life (~150 hours) and non-adherence to the recommended regimen could lead to widespread parasite resistance. Miltefosine is also a teratogenic drug and its use is therefore strictly forbidden in pregnant women (Haldar et al., 2011).

Paromomycin is an aminoglycoside antibiotic with good anti-leishmanial activity but only in combination with other drugs (Croft et al., 2006; Chappuis et al., 2007). Due to increasing unresponsiveness to most of the monotherapeutic regimens, the combination

therapy has found new scope in the treatment of VL. These are safe, effective and require shorter periods of hospitalization. But growing resistance remains the problem to deal with. It has been reported that *L. donovani* can easily develop resistance to drug combinations mainly miltefosine/paromomycin and SbIII/paromomycin (Garcia-Hernandez et al., 2012).

Currently, the drugs used in leishmaniasis treatment present several problems, including high toxicity and many adverse effects, leading to patients withdrawing from treatment and emergence of resistant strains (Santos et al., 2008). And so although several drugs are in use for treating leishmaniasis, a novel drug is yet to come.

1.1.6.2 Immunotherapy

Host immune response to the parasite is an important factor in the pathogenesis of leishmaniasis. Various researches have shown that leishmaniasis can be controlled by boosting the immunity of the individuals. It was a common practice to deliberately expose the healthy individuals to sandflies bites or scratched tissues from active lesions from CL patients in order to get them immunized, a process called Leishmanization (McCall et al., 2013). However, due to occurrence of severe or persistent cutaneous lesions and difficulty in standardizing the virulence of vaccine this method has been abandoned and is currently in use only in Uzbekistan. As of now no effective vaccine has yet emerged for both CL and VL (Chappuis et al., 2007). Yet several first generation vaccines (consisting fractions/whole of the parasite) and second generation vaccines (from recombinant molecules, *Leishmania* antigens and sandfly salivary immunomodulators) are under study. The clinical and experimental evidences indicate that leishmaniasis should be preventable by vaccination someday (Murray et al., 2005).

Another important aspect in the immunological studies is the role of host immune components- to be more precise, the pathogenetic role of Th2 cytokines and role of Th1 cytokines in suppressing the disease. Both experimental and clinical data support the fact that clinical outcome and responses to treatment is affected by the cytokine balance (e.g., INF-γ to IL 10 ratio)(Murray et al., 2005). The fact that individuals, after cure, are resistant to re-infection suggests a strong immune build up. The individuals were also found to be leishmanin skin test (LST) positive and mount antigen-specific IFN-γ responses *in vitro* (Maurya et al., 2010). So, direct manipulation of the immune response, either alone or in combination with drugs, may be a useful strategy for improving treatment regimens for VL (Nylen et al., 2007). The first successful use of human recombinant IFN- γ as an adjunct antimony therapy for visceral leishmaniasis was reported by Badaro et al. (Badaro et al., 1990; Singh and Sivakumar, 2004). Role of immunotherapy along with IFN- γ for anti-leishmanial therapy have been considered and

studied time and again. However, cost, safety, and duration of treatment still remain the major concerns.

1.1.7 Prevention and control of leishmaniasis

Prevention and control measures of leishmaniasis at present are targeted at breaking one or more elements in the parasite lifecycle. Since anti-leishmanial vaccines are still under development, the current control strategies rely on case management (case detection and treatment), vector and reservoir control.

The current vector control strategy is based on indoor residual spraying (IRS) of insecticides. DDT still remains the insecticide of choice because of its low cost, high efficacy, long residual action and relative safety when used for IRS. Other insecticides include products such as organochlorines (DDT and dieldrin), organophosphates (Malathion), carbamates (propoxur) and synythetic pyrethroids (permethrin and deltamethrin). The effectiveness of spraying is the main issue of concern which is being tackled with the trials of new chemicals. Other problems of IRS are the side effects on human health and environment and their sustainability. Bed nets treated with insecticide have been proposed as an alternative to indoor residual spraying and are one of the most effective methods of reducing man-vector contact (Murray et al., 2005; Sharma and Singh, 2008). Since man is the only reservoir in case of kala-azar in Indian subcontinent, active and passive case detection and treatment of those found to be infected (including PKDL), is the important way to reduce the human reservoir and control the disease.

VL affects poor communities, generally in remote rural areas and with risk factors such as malnutrition, poor housing conditions, lack of preventive measures in the form of sanitation and bed nets, massive migration, treatment failure and HIV co-infections; the disease is bound to increase. Condition under which man becomes *Leishmania* infected is dependent on ecology and varies with time and place. Therefore, protection from sandfly bite is the best one can do to prevent the disease. This can be achieved by avoiding outdoor activities, using protective clothing and insecticide treated net (ITN) and insect repellents.

1.2 Natural products in medical practices: historical perspective, current status and future trends

The development of various drugs for the treatment of leishmaniasis has emerged as an advantage to drug discovery. But despite these advances in the parasitological and biochemical researches using various species of *Leishmania,* the treatment options available against leishmaniasis are far from satisfactory. The emerging drug resistance in various species of *Leishmania* as well as the frequent toxicities associated with the existing therapy has stimulated the interest of researchers in the development of compounds with even more significant antileishmanial activity and reduced side effects. Screening of various natural products and phytotherapy has emerged as a progressive step in this regard.

The history of herbal medicine is rather old and dates back to the time when the early man became conscious of his environment. It is estimated that about 250,000 to 500,000 species of higher plants are present on earth. But only small proportions (1-10%) of these are currently used either as food or medicine both by humans and other animals. And only few (<10%) of these has been systematically investigated for the presence of bioactive compounds (Cowan, 1999). Ancient records as well as recent literature reports have established the effectiveness of natural products as potentially rich sources of new agents for the treatment of important tropical diseases caused by protozoans and other parasites. Some of the major classes of anti-protozoal drugs today originated from the plants used in the past in traditional medical systems - Quinine (antimalarial) from barks of *Cinchona*, Emetine (amoebicidal) from *Cephaelis ipecacuanha* and Artemisin (antimalarial) from *Artemisia annua* (Iwu et al., 1994). And there is no doubt these are only minor portion of what the nature has to offer.

In recent years many researchers have examined the effects of plants used traditionally by indigenous healers to support liver function and treat diseases of the liver and spleen. Some of such plants found effective are *Phyllanthus niruri, Aloe vera, Solanum xanthocarpum, Boerhavia diffusa, Tephrosia purpurea, Capparis deciduas, Eclipta alba, Calotropis procera, Azadirachta indica, Euphorbia neriifolia, Lagenaria siceraria, Tinospora cordifolia, Lawsonia intermis, Tecomella undulate, Curculigo orchiodes, Peganum harmala, Silybum marianum*, *Picrorhiza kurroa*, *Curcuma longa*, *Camellia sinensis*, and *Allium sativa* (Scott Luper, 1998; Sharma et al., 2011). This is encouraging as visceral leishmaniasis disturbs the liver and spleen functions primarily. Various natural plant products have been screened for antileishmanial properties. Many of them are leishmanicidal and found to be highly discriminatory in their mode of action (Iwu et al., 1994; Rocha et al., 2005; Tiuman et al., 2011; Alviano et al., 2012). And so, these herbal products could be seen as future natural anti leishmanial drugs.

1.3 Research Plan

1.3.1 Research Hypothesis

1. Medicinal plants have a long history of being used in traditional medicine for various ailments, so there should be some medicinal plants that works competently in case of leishmaniasis.
2. Plants that are used traditionally for signs and symptoms similar to leishmaniasis such as enlargement of abdominal viscera and hepatic ailments should be effective against leishmaniasis.

1.3.2 Research objectives

General-

Assessment and evaluation of some selected medicinal plants of Nepal for their *in vitro* anti-leishmanial activity.

Specific-

1. Ethanolic extraction of phytochemical compounds from selected medicinal plants.
2. *in vitro* efficacy evaluation of the extracts on *Leishmania donovani* promastigotes and axenic amastigotes.
3. Assessment of cytotoxicity of the extracts on primary mouse peritoneal macrophages.
4. Screening of the extracts for their antibacterial potential against some standard strains.
5. Comparative analysis of efficacy among the extracts and reference drugs using statistical tools and techniques.

1.3.3 Rationale

Due to limited chemotherapeutic drugs in use, toxicity of drugs used, increasing drug resistance and high cost; the treatment options available today against leishmaniasis are not satisfactory. Moreover, lack of effective vaccination programmes, co-infections with HIV and rising cases from non endemic areas worsen the situation endangering millions of lives. This calls for the development of alternative medicines that are highly effective against leishmaniasis with no or minimal side effects comparatively. A number of medicinal plants are traditionally used to treat infectious conditions in endemic areas, thus providing promising sources for finding new anti infectious leads. An ideal drug, in case of leishmaniasis, should have both direct and selective leishmanicidal effect as well as the ability to properly activate the patient's immune system. Immunostimulatory activities in addition to anti infective potential have been reported for a number of plant extracts and natural products, providing a rational explanation for their medicinal application. Nepal has great botanical diversity and a practice of traditional medicine using these herbs that dates long back in the history. This huge diversity and the knowledge of traditional medicine could be explored for the possibilities of new medications against *Leishmania*. The current research work is an attempt to validate the traditional uses of some medicinal plants in laboratory set up.

1.3.4 Scope

1. The initial screening results could pave a way for further isolation of active compounds and their analysis on a large scale.
2. Further, studies on immunomodulatory activities of the plants can be carried out.
3. This study could open a door for discovery of a new drug against leishmaniasis.
4. This research work will also document the importance of the plants under study and promote their conservation.
5. The techniques used in the study can be applicable to other similar anti-parasitic and antimicrobial studies.
6. This research work will focus on exploring many more medicinal plants native to Nepal for their medical values.

1.3.5 Work Plan

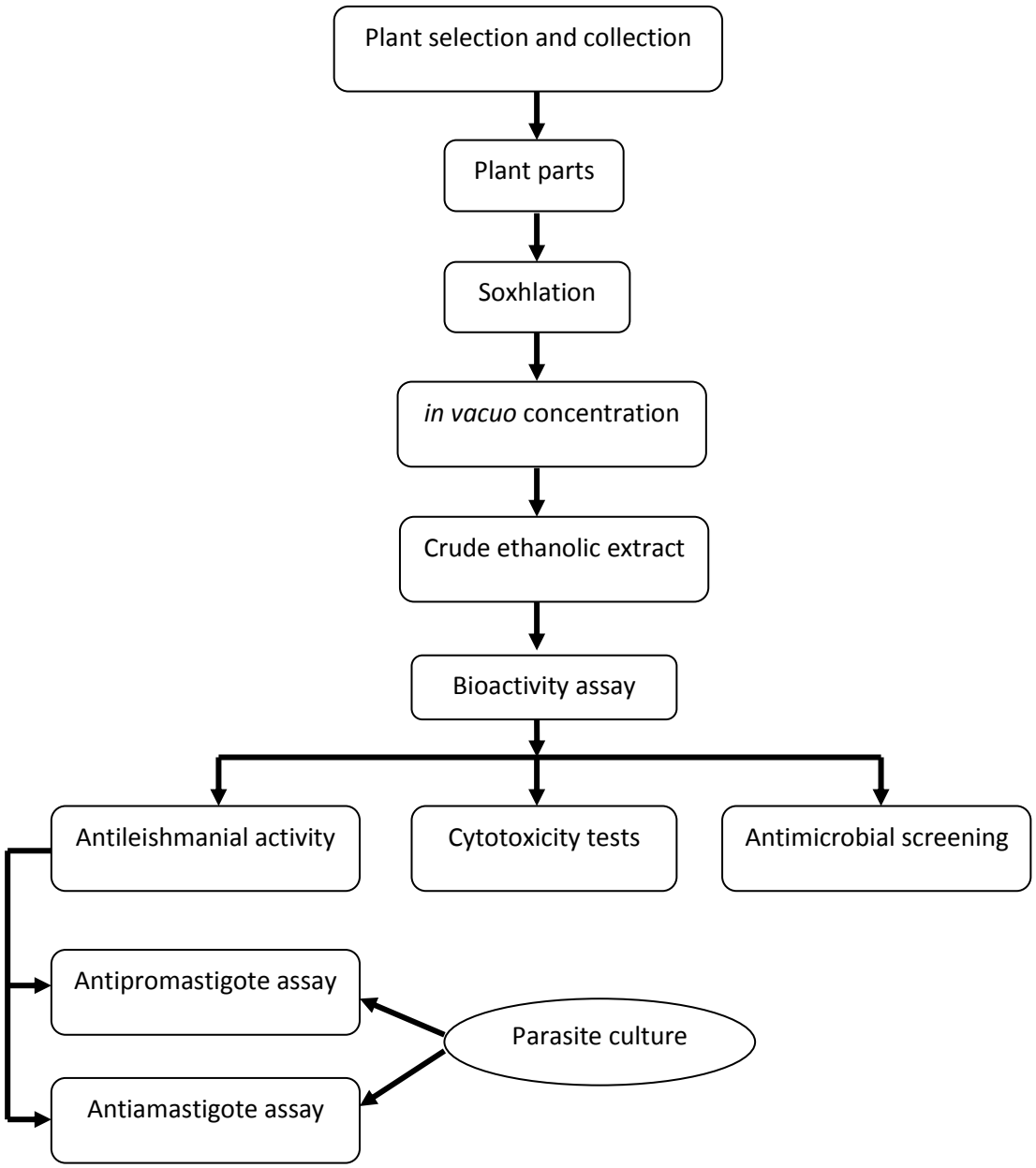


Fig. 1.8: Flow chart: Preparation of plant extract and Evaluation of the antileishmanial, antimicrobial activity of the extracts and accessing their cytotoxicity.

Chapter II

Literature Review

2.1 Synthetic chemotherapy for leishmaniasis and its therapeutic limitations

Chemotherapy of leishmaniasis is aimed at minimizing morbidity and mortality associated with the disease. It is primarily based on toxic antimony compounds. But when this agents lack efficacy, other second-line drugs are used. Several other drugs are also in the pipeline.

2.1.1 Drugs approved for treatment of leishmaniasis

i. Pentavalent Antimonials (Sb^{v})

Organic pentavalent antimonials [Sb^{v}] have been the first-line drugs for the treatment of leishmaniasis for the last six decades. Two major pentavalent antimonials are currently used: Sodium stibogluconate [Pentostam®; Albert-David, UK] and Meglumine antimoniate [Glucantime®; Aventis, France]. World Health Organisation (WHO) recommends a dose of 15-20mg Sb^{v}/kg of body weight per day for 21-28 days, injected intramuscularly or intravenously. Mean total apparent volume of distribution is 0.22 ± 0.057 L/kg of body weight and its half-life is 2 hours (Singh and Sivakumar, 2004; Mishra et al., 2007)

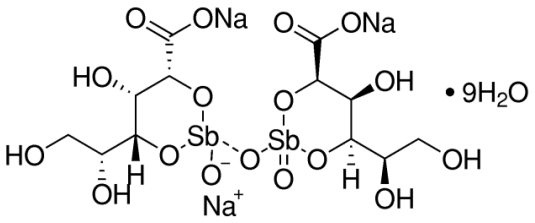


Fig. 2.1: Chemical structure of sodium stibogluconate

Pentavalent antimonials have been the standard treatment for VL in most parts of the world. However, their molecular and cellular mechanisms of action are not yet well understood. Although the precise mechanism of action is not fully known, the antimonials are known to inhibit glycolytic enzymes and other metabolic pathways such as fatty acid oxidation in *Leishmania* amastigotes. Since ADP phosphorylates to ATP using NADH generated by glycolysis and citric acid cycle, the intracellular ATP levels

essential for the survival of *Leishmania* are depleted. Another potential target for the drug is the DNA Topoisomerase I resulting in inhibition of topoisomerization catalysed by the enzyme. In addition, pentavalent antimony is also found to bind the ribose moiety and form stable complexes with adenine nucleosides, which act as inhibitors of parasite purine transporters (Demicheli et al., 2002; Mishra et al., 2007). Yet another model describes the drug as a prodrug which is distributed in high concentrations in the plasma, liver and spleen. In liver, it is reduced to its active leishmanicidal trivalent state (SbIII) (Berman et al., 1989). The reduction is carried out by thiol compounds such as GSH (reduced glutathione) and the parasite specific thiol compound trypanothione. Sb (III) targets the trypanothione reductase (TR) that is essential to maintain the oxidoreductive balance in *Leishmania* parasites and protect the parasites from oxidative damage and toxic heavy metals (Frezard et al., 2009; Haldar et al., 2011). It also affects the zinc-finger protein in the parasites that are likely to be involved in DNA replication, structure and repair (Webb and McMaster, 1993).

Several limitations have, however, discouraged the use of antimonials for past few years. These include the parenteral mode of administration, the long duration of therapy and the adverse reactions. These highly water-soluble compounds are considered inactive when given enterally thus, requiring a parenteral administration. Also, the drug is subjected to rapid renal clearance after parenteral administration, requiring a multiple-dosing regimen (Demicheli et al., 2004). Besides, toxic side-effects have also been observed in patients which are believed to be an outcome of the presence of residual trivalent antimony, Sb(III) (Salaun and Frezard, 2013). Though severe adverse events are rare, secondary effects (such as fatigue, bodyache, nausea, vomiting, myalgia, abdominal colic, diarrhoea, skin rashes, hepatotoxicity, electro-cardiographic abnormalities, raised aminotransferase levels and chemical pancreatitis) are frequent, though usually reversible (Sundar and Chakravarty, 2013). However, sudden death due to arrhythmia has been reported in a patient receiving high doses for a prolonged period. Fatal acute pancreatitis has also been reported in HIV-infected patients (Gasser et al., 1994). The major factor that this drug has been discontinued in various regions however is resistance. Clinical resistance to these drugs has emerged as a primary obstacle to successful treatment and control of leishmaniasis (Croft et al., 2006; Ashutosh et al., 2007; Maltezou, 2010).

ii. Amphotericin B (Polyene antibiotics)

Amphotericin B is a polyene antifungal antibiotic agent, discovered in 1956, from a bacterium *Streptomyces nodosus* and currently recommended as a second-line treatment for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). This drug is seen most effective to treat VL- HIV coinfections and in cases of growing

resistance to pentavalent antimonials (Laguna et al., 2003). Amphotericin B is a hydrophobic molecule, characterized by a hydrophilic polyhydroxyl head and a hydrophobic polyene tail. It is nearly insoluble in water and shows poor solubility in most organic solvents. Since Amphotericin B is poorly absorbed by the gastrointestinal tract, the preferred route of administration is intravenous with a dosage regimen of 1mg/kg either daily for 20 days or on alternate days (15 infusions of the same sample over 30 days). In the body, Amphotericin B can be found in low concentrations in aqueous humor, pleural, pericardial, peritoneal and synovial fluids exhibiting multicompartmental distribution. It takes the drug approximately 24 hours to be eliminated from the body while in cases of discontinuation of intravenous therapy, it can be found in blood for upto 4 weeks and in urine for 4-8 weeks (Mishra et al., 2007).

The mode of action of this drug is based on selective inhibition. Amphotericin B binds to cell wall sterols that too, preferentially to ergosterol, which is the major cell membrane sterol of fungi as well as *Leishmania*, but not mammalian cell membranes. Therefore, it selectively inhibits the membrane synthesis of the parasite and causes holes in the membrane, leading to leakage of a variety of intracellular constituents and parasite death (Singh and Sivakumar, 2004).

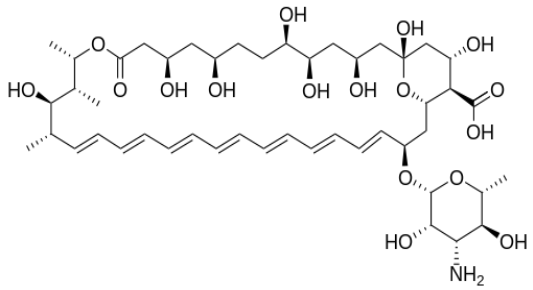


Fig. 2.2: Chemical structure of Amphotericin B

Fungizone, the lyophilized form of sodium deoxycholate - AmB complex, was the first commercial preparation of AmB. However, this drug has many adverse effects including infusion reactions, high fever, nausea, nephrotoxicity, hypokalemia and myocarditis, etc. Also, there is a need of close monitoring and hospitalization for 4 - 5 weeks which ultimately burdens the cost of therapy (Sundar and Chakravarty, 2013). To minimize the adverse events of amphotericin B, various lipid formulations have been introduced where deoxycholate is replaced with other lipids leading to less exposure of the free drug to organs. These formulations include liposomal amphotericin B [L-AmB (Ambisome)], amphotericin B, colloidal dispersion [ABCD (Amphocil)], and amphotericin B lipid complex [ABLC (Abelcet)].

Liposomal Amphotericin B

AmBisome, the liposomal formulation of amphotericin B produced by Gilead, is one of the very few liposomal formulations to have been registered by the US FDA since the potential of liposome as a drug delivery system was discovered in the 1970s. A liposome is a microvesicle composed of a bilayer of lipid amphipathic molecules enclosing an aqueous compartment consisting of hydrophilic, as well as lipophilic drugs. Ambisome consists of small unilamellar vesicles (60 – 70 nm in size) of phospholipids such as phosphatidylcholine or distearoyl phosphatidylglycerol that are stabilized by cholesterol. The liposome acts as a masking system for the drug so that it is isolated from the environment and protected from metabolism and inactivation in the plasma. The versatility of a liposome's design (composition, morphology, size and surface characteristics) enables more targeted distribution and improved therapy (Adler-Moore and Proffitt, 2002; Balasegaram et al., 2012). Food and Drug Administration of USA recommends a total dose of 21 mg/kg in immunocompetent patients (Meyerhoff, 1999). The safety profile of AmBisome is superior to that of all other formulations of amphotericin B; therefore, this is the best (and, unfortunately, most expensive) antileishmanial compound that is available (Sundar et al., 2006).

Amphotericin B Lipid Complex (ABLC)

ABLC (Abelcet) is the most recent formulation studied for the treatment of kala-azar. It is a ribbon-like molecular structure obtained by combination of dimyristoylphosphatidyl choline and dimyristoylphosphatidyl glycerol (molar ratio of 7:3) with AmB (Gupta et al., 2010). A dose of 3mg/kg administered every other day for five injections was 100% successful to cure patients with antimony-resistant kala-azar (Sundar and Murray, 1996). Different workers state that ABLC might to be a better alternative than using no therapy at all or using meglumine antimoniate in cases of VL–HIV coinfection (Laguna et al., 2003; Lopez-Velez et al., 2004). ABLC is a drug with low nephrotoxicity, even when administered to patients with pre-existing renal insufficiency (Aguado et al., 2004). Infusion related toxicity occurred with this formulation also, and 50% of patients had chills and fever during the last infusion.

Amphotericin B Colloidal Dispersion (Amphocil)

Amphocil or Amphotec, a disc-like structure (~ 15 nm in diameter), is complex of cholestryl sulfate with AmB in the ratio 1:1. It is administered by intravenous route at a rate of 1 - 2 mg/kg/hour. Although ABCD was the first lipid formulation, only a few patients have received this formulation. In spite of its low dose for a short period and 100% success rate, the high incidence of infusion-related side effects such as chills, fever, and increased respiratory rate has limited its wider use (Singh and Sivakumar, 2004).

Lipid formulations are rapidly concentrated into organs such as liver, spleen while their concentration remains fairly low in the kidneys. This explains the low toxicity of lipid formulations of AMB and stands out to be the best option for the treatment of HIV-VL coinfection. A significant drawback, however, to the newer, less toxic, commercial lipid-based formulations is their cost. The use of nanoparticles and microspheres for the delivery of conventional amphotericin B also increased its efficacy against experimental VL (Vyas and Gupta, 2006; Manandhar et al., 2008). Since cost is the main limiting factor, there is a need to develop more affordable lipid-based formulations of amphotericin B to fight against this global disease of the poor (Sundar et al., 2004).

iii. Pentamidine (Aromatic diamidine)

Pentamidine was discovered in 1937 and originally used in the treatment of African Trypanosomiasis (Mishra et al., 2007). It is an aromatic diamidine used previously as a hypoglycaemic compound. It is also toxic to large number of protozoa and some fungi. Its anti leishmanial activity, is due possibly to polyamine biosynthesis and its effect on mitochondrial membrane potential. Polyamines are substituted at nucleic acid binding sites, which preferentially bind to kinetoplast DNA. Pentamidine thus acts on the parasite genome by hampering replication and transcription at the mitochondrial level (Singh and Sivakumar, 2004). The recommended regimen is 4 mg/Kg intravenously or intramuscularly slowly on alternate days for 15 injections and has a present cure rate of about 70%. It has a half-life of 5 to 15 minutes and 54 minutes respectively, in above two routes of administration. However, because of multiple toxic effects like anaphylactic shock, nephrotoxicity, myalgias, pain at the injection site, nausea, headache, diabetes mellitus (in about 10% of the cases) and reversible hypoglycemia (in about 2% of cases), its use has been restricted and has been discarded of late (Pandey et al., 2009).

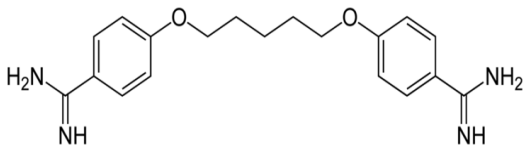


Fig. 2.3: Chemical structure of pentamidine

iv. Paromomycin (Aminosidine)

Paromomycin (Aminosidine®) was originally used in oral treatment of amoebiasis, cryptosporidiosis and giardiasis and topically for trichomoniasis. It is an aminoglycoside-

aminocyclitol antibiotic used in the treatment of VL in a parenteral formulation and CL in both topical and parenteral formulations. It is administered parenterally at dose of 16-20 mg/kg daily for 21 days or 12–20 mg/kg/d combined with antimonials for 20 days. Aminoglycoside act primarily by impairing the macromolecular synthesis and altering the membrane properties of *Leishmania*. It specifically inhibits protein synthesis binding to polysomes and causing misreading and premature termination of translation. Paromomycin in combination with either cycloheximide or chloramphenicol interferes with the dissociation of mitochondrial and cytoplasmic ribosomes thereby inhibiting the protein synthesis (Maarouf et al., 1997; Mishra et al., 2007). Toxic effects such as nephrotoxicity and ototoxicity as well as eighth cranial nerve toxicity are associated with paromomycin (Scott et al., 1992). Paromomycin in combination with stibogluconate has also been attempted successfully. It was observed that paromomycin plus stibogluconate for 21 days at either 12 or 18 mg/kg daily was significantly more effective than stibogluconate alone for 30 days (Thakur et al., 2000).

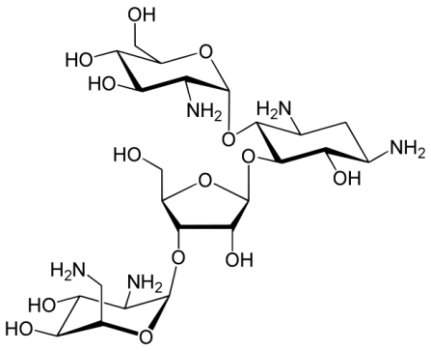


Fig. 2.4: Chemical structure of Paramomycin

v. Miltefosine (Alkyllysophospholipid)

Miltefosine (hexadecylphosphocholine), an alkyl phospholipid compound, was originally intended for breast cancer and other solid tumors. The clinical assessment of miltefosine in human visceral leishmaniasis was initiated in 1996 (Sundar and Olliaro, 2007). It is the first oral drug to be registered for use against leishmaniasis. Recommended regimen of miltefosine is 2.5 mg/kg/day for 28 days given orally. It has low therapeutic ratio, but with 90% yield cure rate (Mishra et al., 2007).

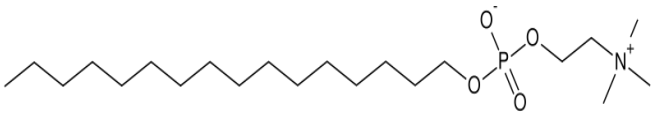


Fig. 2.5: Chemical structure of Miltefosine

Various mode of action have been reported for this drug. It has been observed to induce apoptosis-like death in *L. donovani.* Phenomena such as nuclear DNA condensation, DNA fragmentation, cell shrinkage, DNA oligonucleosomal digestion was seen in the parasites when treated with this drug. During miltefosine treatment, translocation of phosphatidylcholine residues to the outer layer of plasma membrane, one of the most peculiar characteristic features of programmed cell death, was observed in dying promastigotes (Verma and Dey, 2004; Khademvatan et al., 2011). Furthermore, Na+, K+-ATPase has been reported to be inhibited by miltefosine (Berkovic et al., 1992).

Limitations of miltefosine are its relatively high cost, need for monitoring of gastrointestinal side effects and occasional hepatic toxicity and nephrotoxicity. As miltefosine is teratogenic, it is contraindicated in pregnant women. Also women of child-bearing potential have to observe contraception for the duration of treatment and for an additional three months, due to its long half life (Sundar et al., 2012). This drug has a long half life (~150 hours) which makes it vulnerable to the rapid development of drug resistance. It is observed that parasite resistance is easily induced *in vitro* and so non-adherence to the recommended regimen could lead to widespread parasite resistance (Perez-Victoria et al., 2003).

This was the first oral antileishmanial agent registered for use in India in March 2002. Similarly, miltefosine was chosen for the elimination program in India, Nepal and Bangladesh for its ease of use and applicability in the control program. In Nepal, Miltefosine treatment has been available at district hospitals since 2007 and has replaced sodium stibogluconate (SSG) as 1st line therapy for VL. The drug is delivered free of charge through the public health system and is not available in private pharmacies (Uranw et al., 2013).

Due to the high prevalence of gastrointestinal side effects on one hand and the relatively fast resolution of clinical VL symptoms on the other, patients may lose the motivation to complete the 28 days of treatment. Therefore, monitoring for the adherence to treatment is essential for successful control of the disease through miltefosine.

2.1.2 Anti-leishmanial drugs in clinical trial

i. Sitamaquine (Primaquine analogue)

Sitamaquine (SQ) is an 8-aminoquinoline currently undergoing phase IIb clinical trials for the treatment of visceral leishmaniasis. This drug was found to be effective for VL in many trials and was well tolerated in Indian VL as well (Jha et al., 2005). However, no activity was observed on the experimental cutaneous leishmaniasis models (Loiseau et al., 2011). SQ caused a dose-dependent inhibition of complex II (succinate dehydrogenase) of the respiratory chain in promastigotes, together with a drop in intracellular ATP levels and a decrease in the mitochondrial electrochemical potential. This suggested a lethal mechanism for SQ that involves inhibition of the respiratory chain complex II, which in turn triggers oxidative stress and finally leads to an apoptosis-like death of *Leishmania* parasites (Carvalho et al., 2011). Some data also suggest that the combination of sitamaquine with miltefosine or antimony could avoid the appearance of resistance in *Leishmania* (Perez-Victoria et al., 2011). The most common adverse events during the active treatment phase were vomiting, dyspepsia, cyanosis, nephrotic syndrome and glomerulonephritis.

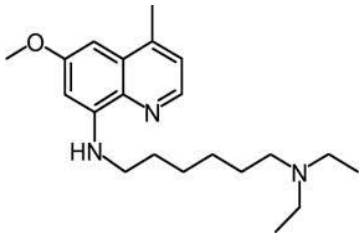


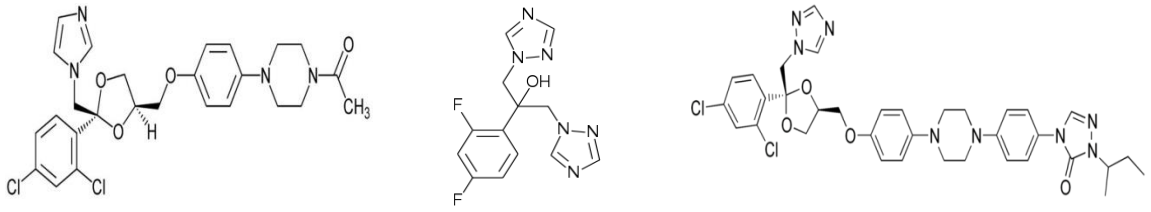
Fig. 2.6: Chemical structure of Sitamaquine

ii. Azoles

Other agents under clinical trial include several antifungal azoles, ketoconazole, itraconalzole and fluconazole for CL and VL. *Leishmania* and fungi share features of sterol biosynthesis i.e., both contain ergosterol rather than the mammalian cholesterol. Azoles block ergosterol synthesis thereby causing killing of *Leishmania* parasites. A number of clinical reports on Ketoconazole and the newer antifungal azoles/triazoles such as Fluconazole and Itraconazole have been obtained (Berman, 2005; Sundar and Chakravarty, 2013).

Ketoconazole (600 and 800 mg/day for 6 weeks) cured all of the 21 patients with CL in Kuwait (Alsaleh et al., 1995) while only 4 of 19 patients with presumed *L. tropica* disease in India were cured with 400 mg/day for 10 weeks (Singh et al., 1995). For visceral disease in India, only 33% of the patients responded to 600 mg for 4 weeks versus 82% of antimony cases (Wali et al., 1997). In India, kala-azar patients using a dose of 6

mg/kg/day of Fluconazole for 30 days showed apparent clinical and parasitological cure in only 50% of cases, but relapsed within 2 months (Sundar et al., 1996). The fact that fluconazole is 10 times more concentrated in the skin compared with plasma, makes it appropriate to be tested against cutaneous forms. There has been case reports of CL treated with fluconazole in Old World. In contrast to fluconazole, Itraconazole has not been effective for cutaneous leishmaniasis but many trials are being carried out (Berman, 2005).



i. ii. iii.

Fig. 2.7: Chemical structures of i. Ketoconazole ii. Fluconazole iii. Itranazole

iii. Allopurinol

Allopurinol (4-hydroxypyrazolo [3, 4-d] pyrimidine), the first oral drug to be used against leishmaniasis, is a structural analogue of hypoxanthine. It is well absorbed in gastrointestinal tract and its half-life is about 1-2 hours (Mishra et al., 2007). Allopurinol and its major metabolic product oxipurinol (alloxanthine) inhibit the enzyme adenylosuccinate synthetase, which mediates the conversion of inosonic acid to adenosine monophosphate. Allopurinol hydrolyses to allopurinol ribosides, an analogue of inosine and gets incorporated instead of ATP into leishmanial RNA, and thereby interfering in the normal protein synthesis (Marr, 1991). Unfortunately the clinical trials are not supporting enough to consider allopurinol effective enough. But fluconazole plus allopurinol shows good activity in treatment of visceral leishmaniasis as a combination therapy (Torrus et al., 1996)

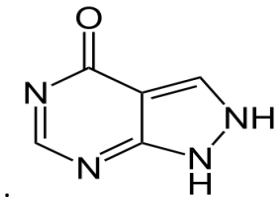


Fig. 2.8: Chemical structure of Allopurinol

iv. Immunotherapy of leishmaniasis

Ability of *Leishmania* to survive and establish infection in humans is by impairment of host macrophage signalling pathways and modulation of macrophage cytokine production. It has been shown that Th1 cytokines such as IFN- γ activates macrophages to express iNOS2, the enzyme catalysing the formation of nitric oxide and oxygen metabolites responsible to kill the intracellular amastigotes (Awasthi et al., 2004). An immunomodulatory drug can therefore be rationally used in the treatment of leishmaniasis.

Badaro et al. first reported use of human recombinant interferon-γ as an adjunct antimony therapy for visceral leishmaniasis. These investigators found that seven of nine cases of Sb-resistant kala-azar could be cured with the combination of interferon-γ given for 28 days (Badaro et al., 1990). It has been specifically mentioned that the addition of IFN- γ to standard therapy such as pentavalent antimony could reduce the cumulative dose of antimonial drugs, shorten the treatment period and probably reduce the number of relapses (van Lunzen et al., 1993). Similarly, it was also observed that stimulation of Th1-cell-associated immune responses, mediated by interleukin 12 (IL-12) and gamma interferon (IFN- γ), enhanced the antileishmanial effect of amphotericin B (AMB) (Murray et al., 2003).

Successful cure of VL depends on the immune status of the host in combination with the effects of the antileishmanial drugs. Hence, the rationale approach towards eradication of this disease would be to potentiate the immune functioning of the host in addition to parasite killing (Bhattacharya and Ali, 2013).

2.2 Vaccines

The feasibility of preventing *Leishmania* infection through vaccination is supported by the fact that individuals who recover from a primary infection are resistant to clinical manifestations upon reinfection. Vaccination against leishmaniasis has been studied using different strategies, ranging from inoculation of virulent parasites (Leishmanization) to immunization with killed parasite preparations, live attenuated parasites, or with recombinant proteins or plasmid DNA coding for defined *Leishmania* antigens (Duthie et al., 2012).

First-generation *Leishmania* vaccines that consisted of killed parasites have gradually replaced Leishmanization. With new findings second-generation vaccines were also introduced. These were based on either live, genetically modified *Leishmania* spp. designed to cause abortive infection in man or recombinant bacteria or viruses carrying *Leishmania* antigen genes, defined synthetic or recombinant subunits (Yang et al., 1990; Skeiky et al., 1995; Gomes et al., 2012). Also the native fractions purified from parasites

are used (White and McMahon-Pratt, 1990; Passero et al., 2012). The use of third-generation vaccines that include genes coding for a protective antigen, cloned into a vector containing eukaryotic promoter, is the more recent approach (Stober, 2004).

Though first generation vaccines of whole killed parasite vaccines have been developed and tested against CL and VL, their average clinical efficacy was low (54%) (Palatnik-de-Sousa, 2008). While in case of field trials against human VL, the only performed was in Sudan with an autoclaved *L. major* vaccine with BCG which achieved 43.3% of vaccine efficacy (Khalil et al., 2000). A recent metanalysis indicated that the whole-parasite vaccine candidates tested do not confer significant protection against human leishmaniasis (Noazin et al., 2009). Only a single product (Leish-111f), a fusion protein of three relatively conserved *L. major* proteins (thiol-specific antioxidant, stress inducible protein1, and elongation initiation factor) formulated with MPL-SE is entering phase II clinical testing in humans, including human VL as a therapeutic vaccine (Nascimento et al., 2010).

Several vaccine preparations are in more or less advanced stages of testing but, to date, there is no vaccine against *Leishmania* in routine use anywhere in the world (Duthie et al., 2012).

2.3 Natural anti-leishmanial agents

2.3.1 Screening of medicinal plants worldwide

The tremendous chemical diversity present in natural products and the promising leads could be a source of new chemotherapeutic compounds for leishmaniasis. Various metabolites have been extracted from natural plants and investigated in this matter. Bioactive phytocompounds such as quinones, alkaloids, terpenes, saponins, phenolic derivatives and other metabolites present in the crude extracts and essential oils of medicinal plants are found to be leishmanicidal in action (Fournet and Munoz, 2002; Alviano et al., 2012).

Plant-screening program for potential leishmanicides was initiated in 1984 in French Guiana, based on the ethnomedical knowledge of the local population (Rocha et al., 2005). Since then such studies have been undertaken time and again. Many plants from different locations all over the world have been studied for their possible leishmanial action. In one of such studies, the leishmanicidal activities of methanolic extracts of some Israeli plants have been evaluated *in vitro*, against the free-living promastigotes and intracellular amastigotes of *Leishmania major.* The extract of *Nuphar lutea* particularly was found to be as effective as paromomycin while all other 41 extracts of various plants showed good efficacies (El-On et al., 2009). Thirty one extracts of thirteen medicinal plants from the Brazilian Cerrado was accessed for anti leishmanial activity

against promastigotes of *L. donovani* by Mesquita et al. Fifteen extracts particularly those of *Annona crassiflora* (Annonaceae), *Himatanthus obovatus* (Apocynaceae), *Guarea kunthiana* (Meliaceae), *Cupania vernalis* (Sapindaceae), and *Serjania lethalis* (Sapindaceae) were active with IC_{50} values between 0.1-10 µg/mL (Mesquita et al., 2005). Similar studies have been carried out to evaluate the antileishmanial property of Bolivian plants (Fournet et al., 1994), Sudanese plants (Fatima et al., 2005), Kenyan plants (Kigondu et al., 2009), Colombian plants (Osorio et al., 2007), Cameroonian plants (Ndjakou Lenta et al., 2007), Peruvian plants (Kvist et al., 2006), Iranian plants (Kheiri Manjili et al., 2012) and many more and the results have been encouraging.

The eugenol-rich essential oil of *Ocimum gratissimum* progressively inhibited *L. amazonensis* growth at concentrations ranging from 100 to 1000 µg/mL (Ueda-Nakamura et al., 2006). Anti-leishmanial activity of *Nuphar lutea* was reported by Ozer et al., 2010 whereby the anti-leishmanial activity was shown to be mediated through the activation of NF-kβ and increased iNOS production.

Piper species have been reported to have a very good activity against *Leishmania* parasites. A related study showed that the leaf essential oil from *Piper claussenianum* was able to inhibit the growth of *L. amazonensis* promastigotes with an IC_{50} of 0.0038% (Marques et al., 2010). Similarly, Monzote et al. described the anti-leishmanial activity of the essential oil from *Piper auritum*. In that study, essential oil inhibited the growth of promastigotes in all species of *Leishmania* used, with IC_{50} values between 12.8 and 63.3 µg/mL. In addition, piper-oil inhibited the growth of intracellular amastigotes of *L. donovani* at non-toxic concentrations (Monzote et al., 2010).

In another study, extract of *Tinospora sinensis Linn* and its fractions were tested *in vitro* against promastigotes and intracellular amastigotes and also *in vivo* in *L. donovani* infected hamsters. Ethanolic extract exhibited an appreciable activity against promastigotes (IC_{50}: 37.6 ± 6.2 µg/mL) and intracellular amastigotes (IC_{50}: 29.8 ± 3.4 µg/mL). In hamsters, it resulted in 76.2 ± 9.2% inhibition at 500 mg/kg/day x 5 oral dose level. Among fractions, n-butanol imparted highest *in vitro* and *in vivo* activities. It was also found that ethanolic extract and butanol fraction enhanced reactive oxygen species (ROS) and nitric oxide (NO) release adding to the anti leishmanial activity (Singh et al., 2008).

Another study was carried out to evaluate the *in vitro* anti-leishmanial activity of various extracts from ten traditionally used Indian medicinal plants. The results showed that the methanolic extract from two plants, *Withania somnifera* (ashwagandha) and *Allium sativum Linn.* (Garlic), were active against *L. donovani*. Further active compounds from these two plants were isolated and purified based on bioactivity-guided fractionation. HPLC-purified fraction A6 of ashwagandha and G3 of garlic showed consistently high

activity with IC_{50} of 12.5 ± 4 and 18.6 ± 3 µg/mL against promastigotes and IC_{50} of 9.5 ± 3 and 13.5 ± 2 µg/mL against amastigote form, respectively. The fraction A6 of ashwagandha was identified as withaferin A while fraction G3 of garlic is yet to be identified, and the work is in progress. The results indicate that fraction A6 of ashwagandha and fraction G3 of garlic might be potential sources of new anti-leishmanial compounds (Sharma et al., 2009).

In most cases, the traditional therapy consists of the administration of plant extracts orally for the systemic forms of the disease and as topical preparations for the cutaneous infection (Iwu et al., 1994). While many studies involve random selection of medicinal plants present in a specific geographical area, some are based on the traditional knowledge of local people. One such study was the evaluation of medicinal plants in use by the Yanesha, an Amazonian Peruvian ethnic group, for affections related to leishmaniasis and malaria. Ninety-four ethanolic extracts of plants used were screened *in vitro* against *L. amazonensis* amastigotes. Eight species displayed interesting leishmanicidal activities (IC_{50} < 10 µg/mL), namely *Carica papaya L.* (Caricaceae), *Piper dennisii* Trel (Piperaceae), *Hedychium coronarium* J. Konig (Zingiberaceae), *Cestrum racemosum* Ruiz & Pav. (Solanaceae), *Renealmia alpinia* (Rottb.) (Zingiberaceae), *Lantana sp.* (Verbenaceae), *Hyptis lacustris* A. St.-Hil. ex Benth. (Lamiaceae) and *Calea montana* Klat. (Asteraceae) (Valadeau et al., 2009). A similar study documents the traditional plants of Chayahuita, another ethnic group in Peru. Thirty-seven extracts corresponding to 31 species designated useful against CL and /or MCL were screened *in vitro* against *L. amazonensis* axenic amastigotes. Six species displayed a good activity (10 µg/mL <IC_{50}<20 µg/mL) (Odonne et al., 2009).

Several other works of similar nature has been recorded and still being carried out all over the world. A summary of plant crude extracts, fractions, isolated compounds, and essential oils evaluated against the *Leishmania* genus has been illustrated in the table 2.1. These efforts are now validating natural products as genuine sources for drug discovery and emphasizing the importance of phytoscience in the search for novel anti-leishmanial therapeutic agents (Tiuman et al., 2011; Alviano et al., 2012).

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

Family **Plant species**	**Extracts/ compounds**	***Leishmania* species**	**IC $_{50}$ (µg/mL)**	
			PRO	**AMA**
Aloeaceae				
Aloe nyeriensis	Methanolic extract	*L. major*	68.4	ND
	Aqueous extract		53.3	ND
Annonaceae				
Annona coriacea	Total alkaloids extract	*L. chagasi*	41.6	ND
Annona crassiflora	Total alkaloids extract	*L. chagasi*	24.9	ND
Annona muricata	Ethyl acetate extract	*L. amazonensis*	25.0	NT
Guatteria australis	Total alkaloids extract	*L. chagasi*	37.9	ND
Polyalthia suaveolens	Methanolic extract	*L. infantum*	1.8	8.6
Pseudomalmea boyacana	Ethyl acetate extract	*L. amazonensis*	48.9	NT
Rollinia exsucca	Hexane extract	*L. amazonensis*	20.8	NT
Rollinia pittieri	Hexane extract	*L. amazonensis*	12.6	NT
Xylopia aromatica	Methanolic extract	*L. amazonensis*	20.8	NT
Apocynaceae				
Himatanthus sucuuba	Ethanolic extract	*L. amazonensis*	20.0	5.0
Pagiantha cerifera	Dichloromethane extract		25.0	12.5
Asteraceae				
Achillea millefolium	Essential oil	*L. amazonensis*	7.8	6.5
Anthemis auriculata	Anthecotulide	*L. donovani*	NT	8.18
	4 Hydroxyanthecotulide	*L. donovani*	NT	3.27
	4-Acetoxyanthecotulide	*L. donovani*	NT	12.5
Baccharis dracunculifolia	Crude extract	*L. donovani*	45.0	NT
	Hautriwaic acid lactone	*L. donovani*	7.0	NT
	Ursolic acid	*L. donovani*	3.7	NT
	Uvaol	*L. donovani*	15.0	NT
	2a-Hydroxyursolic acid	*L. donovani*	19.9	NT
Calea montana	Ethanolic extract	*L. amazonensis*	NT	10.0
Elephantopus mollis	Dichloromethane extract	*L. donovani*	NT	0.6

Tanacetum parthenium	Plant powder	*L. amazonensis*	490	74.8
	Dichloromethane extract	*L. amazonensis*	3.6	2.7
	Parthenolide	*L. amazonensis*	0.37	0.81
	Guaianolide	*L. amazonensis*	2.6	ND
Vernonia polyanthes	Methanolic extract	*L. amazonensis*	4.0	NT
Caricaceae				
Carica papaya	Ethanolic extract	*L. amazonensis*	NT	11.0
Celastraceae				
Maytenus putterlickoides	Methanolic extract	*L. major*	60.0	ND
Clusiaceae				
Calophyllum brasiliense	(-) Mammea A/BB	*L. amazonensis*	3.0	0.88
Crassulaceae				
Kalanchoe pinnata	Quercetin diglycoside	*L. amazonensis*	NT	45.0
Fabaceae				
Acacia tortilis	Aqueous extract	*L. major*	52.9	ND
Albizia coriaria	Aqueous extract	*L. major*	66.7	ND
Copaifera reticulata	Oleoresin	*L. amazonensis*	5.0	15.0
Flacourtiaceae				
Laetia procera	Casearlucine A	*L. amazonensis*	11.1	5.98
	Caseamembrol A	*L. amazonensis*	11.0	10.5
	Laetiaprocerine A	*L. amazonensis*	10.9	47.4
	Laetiaprocerine D	*L. amazonensis*	50.9	30.3
	Butanolide	*L. amazonensis*	111.0	129.0
Ginkgoaceae				
Ginkgo biloba	Isoginkgetin	*L. amazonensis*	NT	1.9
Goodeniaceae				
Scaevola balansae	Dichloromethane extract	*L. amazonensis*	8.7	NT
Lamiaceae				
Hyptis lacustris	Ethanolic extract	*L. amazonensis*	NT	10.0
Ocimum gratissimum	Essential oil	*L. amazonensis*	135.0	100.0
	Eugenol	*L. amazonensis*	80.0	NT
	Methanolic extract	*L. chagasi*	71.0	NT
Premna serratifolia	Dichloromethane extract	*L. amazonensis*	4.4	NT
Lecythidaceae				
Careya arborea	Arborenin	*L. donovani*	15.0	12.5
Liliaceae				
Asparagus racemosus	Methanolic extract	*L. major*	58.8	ND
	Aqueous extract	*L. major*	56.8	ND

Malpighiaceae				
Lophanthera lactescens	LLD3	*L. amazonensis*	NT	0.41
Meliaceae				
Dysoxylum binectariferum	Chloroform fraction	*L. donovani*	50.0	ND
	Rohitukine	*L. donovani*	100.0	ND
Menispermaceae				
Cissampelos ovalifolia	Total alkaloids extract	*L. chagasi*	63.9	ND
Olacaceae				
Minquartia guianensis	Dichloromethane extract	*L. donovani*	NT	2.8
Papaveraceae				
Bocconia integrifolia	n-Hexane extract	*L. donovani*	NT	1.8
	Dichloromethane extract	*L. donovani*	NT	0.5
	Methanol extract	*L. donovani*	NT	0.7
Piperaceae				
Piper auritum	Essential oil	*L. donovani*	12.8	22.3
Piper dennisii	Ethanolic extract	*L. amazonensis*	NT	10.0
Piper hispidum	Ethanolic extract	*L. amazonensis*	69.0	5.0
Piper regnellii	Eupomatenoid-5	*L. amazonensis*	9.0	5.0
Piper strigosum	Ethanolic extract	*L. amazonensis*	>100	7.8
Piper sp	Dichloromethane extract	*L. donovani*	NT	2.2
Poaceae				
Cymbopogon citratus	Essential oil	*L. amazonensis*	1.7	3.2
	Citral	*L. amazonensis*	8.0	25
Rhamnaceae				
Gouania lupuloides	Dichloromethane extract	*L. donovani*	NT	1.9
	Methanol extract	*L. donovani*	NT	2.9
Rutaceae				
Galipea panamensis	Coumarin compound 1	*L. panamensis*	NT	9.9
	Coumarin compound 2	*L. panamensis*	NT	10.5
	Phebalosin	*L. panamensis*	NT	14.1
	Artifact murralongin	*L. panamensis*		>100
	Murrangatin acetonide	*L. panamensis*	NT	NT
Scrophulariaceae				
Scoparia dulcis	Dichloromethane extract	*L. donovani*	NT	1.8
Scrophularia cryptophila	Crypthophilic acid A	*L. donovani*	NT	12.8
	Crypthophilic acid C	*L. donovani*	NT	5.8
	Harpagide	*L. donovani*	NT	2.0
	Acetylharpagide	*L. donovani*	NT	6.9
	Buddle jasaponin III	*L. donovani*	NT	6.2

Solanaceae				
Brugmansia sp	Dichloromethane extract	*L. donovani*	NT	3.0
Umbelliferae				
Ferula szowitsiana	Auraptene	*L. major*	5.1	NT
	Umbelliprenin	*L. major*	4.9	NT
Verbenaceae				
Lantana sp	Ethanolic extract	*L. amazonensis*	NT	10.0
Zingiberaceae				
Hedychium coronarium	Ethanolic extract	*L. amazonensis*	NT	10.0

[Source: Tiuman et al., 2011].

[PRO: promastigote; AMA: amastigote; IC50, concentration in µg/mL that inhibits growth of 50% of the cells; NT: not tested; ND: not determined.]

2.3.2 Antileishmanial screening of Nepalese medicinal plants

Spread over an area of 147,181 Km^2, Nepal lies on the central region of the great Himalayan range. The altitudinal variation starting from almost sea level (~70 meter) to the top of the world (8,848 meter) and the resulting climatic differences, varied topography and abundant ecological habitats offer rich flora and fauna life (Gewali, 2008). More than 900 types of valuable medicinal plants among 7000 medicinal plants present all over the world are found in Nepal (Manandhar, 2000). However, only few of this prosperous biodiversity has been explored for their therapeutic potentials and it is even fewer in case of leishmaniasis.

Amongst the few studies is the one by Choudhary et al., 2010. In this study, secondary metabolites from root extract of a medicinal plant *Sarcococca coriacea* from Nepal was found to possess anti leishmanial properties (IC_{50}: 13.20 ± 0.5 µM and 24.76 ± 0.01 µM) (Choudhary et al., 2010). Another Nepalese medicinal plant, Tulsi (*Ocimum sanctum* L.), showed strong leishmanicidal activity from the ethyl acetate soluble fraction of the plant (Suzuki et al., 2009). More recently, the antileishmanial potential of flowers of *Bombax ceiba* was also studied. The methanol fraction showed a greater inhibitory effect against *L. donovani* promastigotes (IC_{50} of 89.62 ± 0.55 µg/mL) as well as amastigotes (IC_{50} of 58.73 ± 1.89 µg/mL) (Basukala, 2011).

There are several medicinal plants native to Nepal that has been implicated traditionally in various ailments as documented by Adhikari et al. (2007). Some of them typically those that are being used in symptoms similar to kala-azar, or ailments with similar pathogenesis have been listed in Table 2.2.

Table 2.2: Some Nepalese medicinal plants with their scientific name, distribution and uses

Family **Plant name** **[Scientific Name]**	**Distribution in Nepal**	**Parts used**	**Uses**
Asclepiadaceae Aank [*Calotropis gigantea* (L.) Dryand, *Asclepias gigantean*]	100-1200 m, east to west	Roots, root bark, leaves, latex and flowers	febrifuge, antihelmintic, expectorant, useful in cutaneous diseases, dysentery, asthma, cough, and enlargement of abdominal viscera. Latex is used in leprosy, dropsy, rheumatism. Flowers are used as tonic and stomachic. Leaves are applied in painful joints, swellings and to heal wounds. Milk is used in scabies, ringworm, and eruptions on the body.
Bombacaceae Simal [*Bombax ceiba, Salmalia malabaricum*]	200-900 m, east to west	Roots, gums, bark, leaves , flowers and seeds	Gum used in diarrhoea, dysentery, influenza, haemoptysis of pulmonary tuberculosis, and menorrhagia. Root useful in dysentery. Bark used in healing wounds. Leaves are good for skin eruptions. Flowers are good in skin troubles, splenomegaly and haemorrhoids. Young fruit useful in calculus affections and ulcerations of the bladder and kidney. Seeds useful in treating gonorrhoea and chronic cystitis.
Flacourtiaceae Talishpatree, Maran, Paneru [*Flacourtia jangomas Stigmarota jangomas*]	900-1200 m west to central	Bark, leaves and fruits	Bark and leaves are astringent, refrigerant and diaphoretic. Decoction of bark used in biliousness. Leaves used in diarrhoea. Fruits are astringent, digestive and liver tonic.
Labiatae Tiyaangku, priyangku [*Dracocephalum tanguticum, D.hookeri*]	4600-5000 m, central, near Tibetan borders.	Vegetative parts with flower	Young leaves are used in dysentery and fever of child. The plant of mature stage is used in liver diseases, cough, cuts, and wounds and bleeding.
Loranthaceae Hadchoor, Aijeru [Viscum album, V. stellatum]	1000-2300m, west to central.	Plant and berries	Plant is given in the enlargement of of spleen, wounds, tumors and ear diseases. Paste is applied on the broken limb as a plaster. Berries are laxative, tonic, cardio-tonic and aphrodisiac.
Lauraceae Kapoor [*Cinnamomum camphora*]	1300-1500m, east to west	whole plant	Plant is anodyne, antispasmodic, diaphoretic, antihelmintic, stimulant, carminative and used in insecticidal preparation.
Leguminosae Koiraalo [*Bauhinia variegata* L.*, B. candida* Aiton]	150-1900 m, east to west	root and bark	The bark is tonic and blood purifier. It is useful in diarrhoea, dysentery, piles and liver complaints. The decoction of root is valuable drink for reducing corpulence. Fresh flowers used as laxative.

Lythaceae Dhataki [*Woodfordia fruticosa*]	200-1800m, east to west	flowers	Antibacterial, useful in leprosy, burning sensation, skin diseases, diarrhoea, dysentery, liver disorders, menorrhagia, ulcers, wounds
Myrsinaceae Chapraa, seteekath [*Myrsine africana* L.*, M. bifaria*]	900-1800 m, west to central	leaves and fruits	Decoction of leaves is used as a blood purifier and gums in dysmenorrhoea. Fruits are antihelmintic, especially for tapeworms and are laxative in dropsy and colic.
Nyctaginaceae Punarnavaa [*Boerhavia diffusa* L. *Boerhavia repens* L.]	300-1200 m, east to west.	whole plant	The plant is used in all types of inflammations, leucorrhoea, scabies, cardiac disorders, anemia, cough, bronchitis and constipation. Root is diuretic and promotes urination in dropsy. Leaf juice is given in jaundice and other liver complaints.
Phyallanthaceae Bhuee-Amala [*Phyllanthus niruri*]	470-900 m, east to west	leaves, root	anti-hepatotoxic, anti-lithic, anti-hypertensive, anti-HIV and anti-hepatitis B
Ranunculaceae Atees [*Aconitum heterophyllum* Wall. Ex Royale, *Aconitum* L. *atees* Royale]	3200-3700 m, central	roots	Roots are antiperiodic, aphrodisiac, astringent, liver tonic, and useful in diarrhoea, dyspepsia, and cough.
Umbelliferae Ghodtapre [*Centella asiatica* (L.) Urb.]	100-1800 m, east to west	whole plant	The leaves are useful remedy for syphilis, leprosy and skin diseases. The leaf powder is given with milk in small doses in mental weakness to improve memory, also used as blood purifier, diuretic, and insecticide. Leaf stalk is used in toothache. It is given in indigestion and nervousness.
Zingiberaceae Pankhaaphool, Paaneesaro [*Hedychium spicatum, Hedychium album*]	1500-2100 m, east to west	Rhizome	Rhizome is astringent, fragrant, stomachic, carminative, tonic, stimulant, expectorant and good in liver complaints, vomiting, diarrhoea, inflammation, pains and also used in snake-bite. The rhizomes possess strong aromatic with camphoraceous odor. The rhizomes are considered to have insect repellant properties and are used for preserving clothes.

[Source: Adhikari et al., 2007]

2.3.3 Review of medicinal plants included in this study

2.3.3.1 *Boerhavia diffusa*

Boerhavia is a genus of 40 species, almost all of which are widely distributed in tropical and sub-tropical areas of Asia, Africa, America and Australia (Chaudhary and Dantu, 2011). Among all the species, *Boerhavia diffusa* Linn is the most widely studied plant and has a long history of uses by the indigenous and tribal people. This is a perennial much branched creeping and climbing herb. It bears small reddish or pink flowers in small clusters (umbel) and oval fruits. The flowering and fruiting takes place from May to August (Adhikari et al., 2007).

Taxonomic Position:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Caryophyllales
Family: Nyctaginaceae
Genus: *Boerhavia*
Species: *diffusa*
Botanical name: *Boerhavia diffusa* Linn.
Common name: Punarnava, Sothagni (Sans.)

Fig.2.9: *Boerhavia diffusa.* a.leaves b. Flower c. Fruits. d. Whole plant

Distribution in Nepal: 300-1200 m, east to west

B. diffusa has pleiotropic medicinal properties and is also a main ingredient of many Ayurvedic and Unani medicines and ethnomedical formulations. Different parts of this plant such as seeds, roots, leaves has been used for treatment of ailments like jaundice, inflammation, oedema, hypertension etc. This plant is also being used as a green leafy vegetable in different parts of Asia and Africa due to its nutraceutical properties (Vineetha et al., 2013). In countries like Brazil and Iran, the plant as a whole is being used for conditions like liver disorders and enlarged spleen (a hallmark of VL) (Chaudhary and Dantu, 2011). Pharmacological studies have demonstrated that *B. diffusa* possesses anticonvulsant (Kaur and Goel, 2011), diuretic and antiurolithiatic (Yasir and Waqar, 2011), anti-inflammatory, antibacterial (Gopal et al., 1999), antifungal (Agrawal et al., 2003), antihelmintic, antipyretic, antilymphoproliferative (Mehrotra et al., 2002), antimetastatic (Manu and Kuttan, 2009), immunosuppressive (Pandey et al., 2005), antidiabetic (Pari and Amarnath Satheesh, 2004), antioxidant (Satheesh and Pari, 2004), immunomodulating (Mehrotra et al., 2002), nephroprotective and antiurethritis

(Singh et al., 2011) activities. Various works have also defined this plant to be hepatoprotective (Chandan et al., 1991; Rawat et al., 1997).

Phytochemical studies of different parts of the plant have revealed several groups of compounds. The main chemical ingredients of this plant include alkaloids, rotenoids and flavones. The alkaloid present in the plant is typically called Punarnavine that are responsible for the anti metastatic and immunomodulatory properties (Manu and Kuttan, 2007). Different analyses have deduced the presence of total 10 rotenoids (boeravinones A to J) (Borrelli et al., 2005; Bhope et al., 2013). It also contains a large number of compounds such as steroids, triterpenoids and phenol glycosides (Maurya et al., 2007). A major glycoside - Punarnavoside is known to have anti fibrinolytic affect. Other phytochemicals present are lipids, lignins, carbohydrates, proteins, glycoproteins, potassium nitrate, hypoxanthine 9- L-arabinofuranoside, ursolic acid, caffeoyl tartaric acid, quercetin and kaempferol (Ferreres et al., 2005; Pereira et al., 2009).

2.3.3.2 *Calotropis procera*

It is a small shrub about 2 m tall growing on dry waste land. Stem exudes milky latex when excised. Leaves are long (10-20 cm) and broad (2.5-7.5) and the flowers are lilac, rosy or purple in umbellate lateral cymes. The flowering and fruiting time of the plant lasts from March till October. Different parts of *Calotropis* such as root, root bark, leaves, latex and flowers are used in traditional medicine for the treatment of leprosy, ulcers, tumors, diseases of the spleen and liver, enlargement of abdominal viscera and piles (Adhikari et al., 2007).

Taxonomic Position:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Gentianales
Family: Asclepiadaceae
Genus: *Calotropis*
Species: *procera*
Botanical name: *Calotropis procera* Aiton
Asclepias procera Aiton
Common name: Aank, Mandara (sans),
Giant milk weed
Distribution in Nepal: 100-1200 m,
east to west

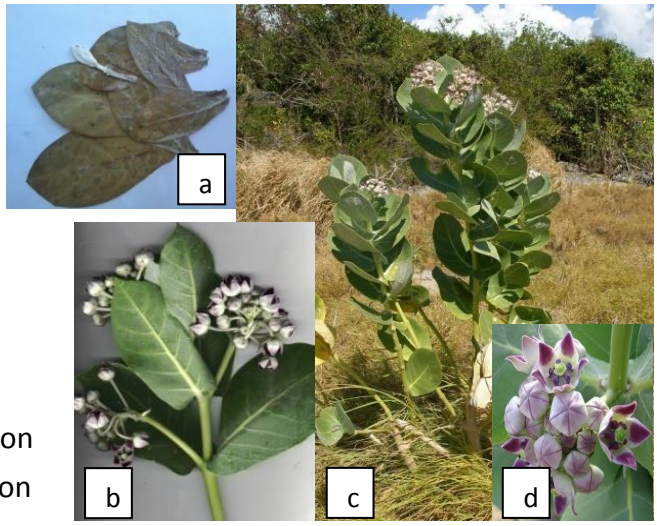


Fig.2.10: *Calotropis procera* Aiton. a. dried leaves, b. leaf, c. whole plant, and d. flower.

Calotropis acts as purgative, anticoagulant, antipyretic, analgesic, anti inflammatory, and antimicrobial, and as a palliative in problems with respiration and blood pressure. It also has a neuromuscular blocking activity (Alencar et al., 2006; Seddek et al., 2009; Oliveira et al., 2012; Nenaah, 2013). Besides, hepatoprotective activity of the flowers, leaves and latex are also reported (Padhy et al., 2007; Qureshi et al., 2007; Ramachandra Setty et al., 2007). The antihelmintic property of this medicinal plant has also been widely studied (Al-Qarawi et al., 2001; Iqbal et al., 2005) along with the insecticidal potentials (Moursy, 1997; Shahi et al., 2010). Members of this family are rich in cardiac glycosides and also are regarded as promising sources for antitumor agents (Mathur et al., 2009; Magalhaes et al., 2010). A study showed that the aqueous suspension of dried latex of *C. procera* conferred protection against the complications associated with diabetes exhibiting its antioxidant and anti-hyperglycaemic property (Kumar and Padhy, 2011).

The phytochemical investigation of *Calotropis procera* revealed the presence of cardenolides, flavonoids, and saponins as major components (Moustafa et al., 2010). The plant has the highest percentage of cardenolides, viz., uzarigenin, syriogenin, calotropagenin, proceroside, calotropin, calactin, frugoside, coroglaucigenin and corotoxigenin, calotoxin, uscharidin, uscharin, voruscharin, and 3-episarmentogenin (Bruschweiler et al., 1969; Erdman, 1983; Mossa et al., 1991; de Freitas et al., 2011). Various cysteine proteases are also reported (Dubey and Jagannadham, 2003).

2.3.3.3 *Hedychium spicatum*

Hedychium spicatum is a perennial rhizomatous herb, 0.9-1.5 m tall that grows in subtropical and temperate regions. It is also one of the important medicinal herbs that have been used in traditional medicine since ages. Flowering and fruiting period is short from July to August. The rhizome is the main part that has been used in ethnomedicine which is light brown in colour and has a strong camphorous odour and bitter taste (Ghildiyal et al., 2012).

The rhizome is stomachic, carminative, a bronchodilator, a stimulant and tonic, and traditionally used in dyspepsia, nausea, vomiting, and liver complaints (Adhikari et al., 2007). The medicinal abilities of this herb are accredited to its numerous phytocompounds.

Taxonomic Position:

Kingdom: Plantae
Division: Magnoliophyta
Class: Liliopsida
Order: Zingiberales
Family: Zingiberaceae
Genus: *Hedychium*
Species: *spicatum*
Botanical name: *Hedychium spicatum* Sm.
Hedychium album Buch.
Common name: Pankhaphool, Paaneesaro, Kapurkachari (Sans), Ginger lily

Distribution in Nepal: 1500-2100 m, east to west

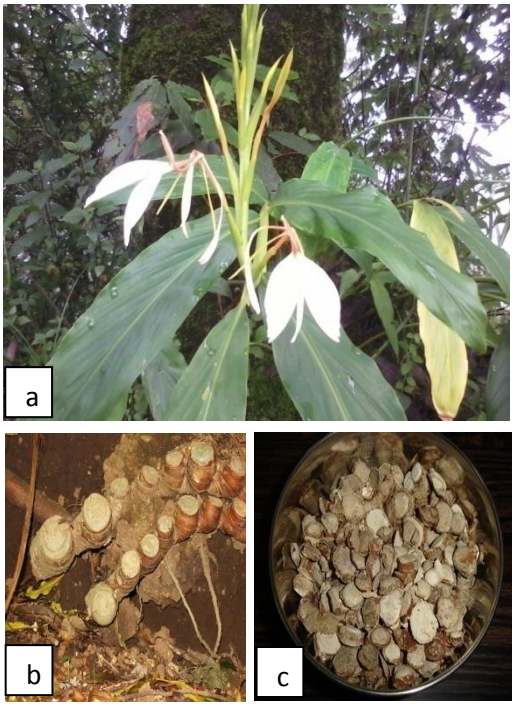


Fig. 2.11: *Hedychium spicatum* Sm., a. whole plant , b. rhizome, c. cut and dried rhizome

Alkaloids, carbohydrates, proteins, resins, saponins, steroid, tannin, starch, glycosides, flavonoids and triterpenoids have been isolated from various extracts of the plant (Ghildiyal et al., 2012). The rhizome has been reported to contain sitosterol and its glucosides, furanoid diterpene-hedychenone, 7- hydroxy hedychenone and Metoxycinnamate, Eudesma-4(15)-ene-β-11diol , crytomeridiol , β-udesmol ,3-hydroxy-β-eudesmol, mucrolidin, oplapanone, α-terpineol, elemol, Opladiol, hydroxycryptomeridiol, β caryophyllene oxide, coniferaldehyde and ethylferulate (Sharma et al., 1975; Tandon et al., 1984; Reddy et al., 2009). The presence of Labdane-type diterpenes has been reported to be responsible for a variety of biological activities such as anti algal, antibacterial, antifungal, antiprotozoal, enzyme inducing, anti-inflammatory, immunomodulatory as well as antitumor activity (Prabhakar Reddy et al., 2009). The efficacy of anti-asthmatic property of a herb were indicated by the results whereby the drug demonstrated a potent histamine antagonism property with significant mast cell stabilizing and spasmolytic activity in the experimental animals (Kajaria et al., 2012).

Essential oil present in rhizome has cineole, terpinene, limonene, phellandrene, p-cymene, linalool and terpeneol as major constituents. This essential oil is reported to have antimicrobial and antioxidant activities (Sakhanokho et al., 2013).

2.3.3.4 *Phyllanthus niruri*

Phyllanthus niruri Linn. is an annual herb, 30-60 cm tall. Leaves are small arranged like leaflets alternately on lateral branches with single female flower or a group of 1-3 male flowers at the base of leaves. The flowering and fruiting takes place between June and August. An aqueous infusion of the whole plant, which is a typical preparation, is employed as a stomachic, aperitive, antispasmodic, laxative, diuretic, carminative, against constipation, fever including malaria, hepatitis B, dysentery, gonorrhoea, syphilis, tuberculosis, cough, diarrhoea and vaginitis (Adhikari et al., 2007). It is used in Brazilian folk medicine as an effective remedy to eliminate gallstones, kidney stones and other kidney disorders (Freitas et al., 2002).

Taxonomic Position:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida

Order: Euphorbiales
Family: Euphorbiaceae
Genus: *Phyllanthus*
Species: *niruri*
Botanical name: *Phyllanthus niruri* Linn.
Phyllanthus amarus Schum. & Thonn.
Common name: Bhuinamalaa, Tamalaki, BhumyamLaki (Sans)

Distribution in Nepal: 470-900 m, east to west

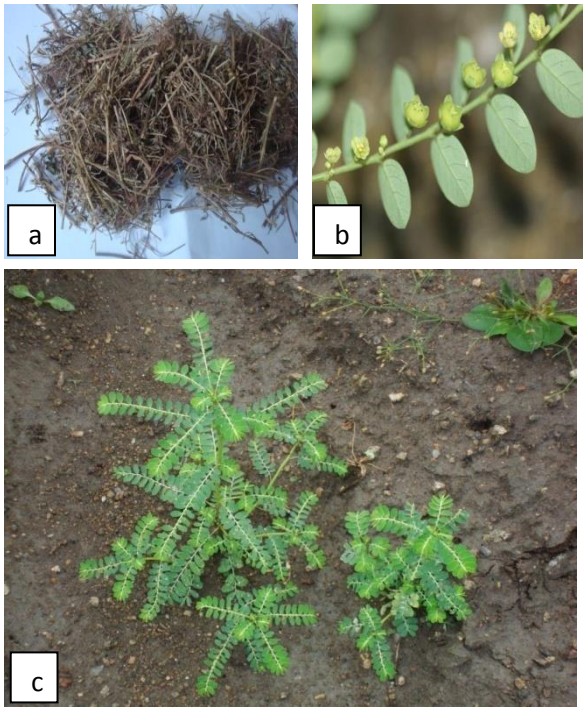


Fig.2.12: *Phyllanthus niruri* Linn., a. dried whole plant , b. fruiting body, c. whole plant.

Various works have claimed its importance time and again. The medical potential of this plant is numerous. The plant extract is found to possess antioxidant and hepatoprotective qualities (Bhattacharjee and Sil, 2006). The anti-hepatotoxic activity of *P. niruri* has been attributed to two novel lignans, phyllanthin and hypophyllanthin (Syamasundar et al., 1985). Similarly, the *in-vitro* and *in-vivo* anti-plasmodial activities of the ethanolic and dichloromethane extracts, as well as the toxicity of the lyophilized aqueous extract, from *P. niruri* were also reported (Tona et al., 2001). Research on *P. niruri* revealed that its antiviral activity extends to human immunodeficiency virus (HIV)

and a simple aqueous extract of the plant inhibited HIV-1 reverse transcriptase (Ogata et al., 1992; Naik and Juvekar, 2003). The lipid-lowering activity of *P. niruri* was also studied in triton and cholesterol-fed hyperlipaemic rats (Khanna et al., 2002).

These medicinal effects are attributed to the active components present in *Phyllanthus* niruri, such as lignans (Murugaiyah and Chan, 2007), phenols (De Souza et al., 2002), flavonoids (Shakil et al., 2008), xylans (Mellinger et al., 2005), terpenoids and in particular, tannins (Thakur et al., 2011). Other detailed works have identified several phytochemicals such as Rutin (flavonol glycoside), Quercetin, Quercitrin, Astragalin, Gallocatechin, Nirurin Limonene, Ellagic acid, Phyllanthin, Hypophyllanthin, Niranthin, Repandusinic acid, Norsecurinine, Saponins etc (Bagalkotkar et al., 2006; Elfahmi et al., 2006; Mellinger et al., 2008; Wei et al., 2012).

2.3.3.5 *Woodfordia fruticosa*

The full-grown leafy shrub of *Woodfordia fruticosa* is about 3.5m high, having long and spreading branches with fluted stems. The leaves are opposite or sub-opposite in nature. Flowers are brilliant red, innumerable, arranged in dense axillary paniculate-cymose clusters. The fruits are small capsules, ellipsoid and membranous and are irregularly dehiscent. Flowering and fruiting takes place between February and April (Das et al., 2007).

Although all parts of this plant possess valuable medicinal properties, there is a heavy demand for the flowers, both in domestic and international markets specialized in the preparation of herbal medicines. The flower is pungent, acrid, cooling, alexiteric, uterine sedative, and antihelmintic and is useful in thirst, leprosy, blood diseases, leucorrhoea, menorrhagia and toothache. The dried flowers are astringent to bowel complaints and useful in dysentery and diarrhoea and safe stimulant in pregnancy (Adhikari et al., 2007). Many of these ethnomedical therapies are supported by various research works. A study demonstrated the immunomodulatory activity of the Ayurvedic drug '*Nimba Aristha*', which contains *Woodfordia fruticosa* flowers. It was specifically demonstrated that the increased biological activity of human complement system and leukocytes was due to immunoactive constituents released from the flowers of *W. fruticosa* (Kroes et al., 1993). Similarly, the immunostimulatory activity was also observed with ethanol extract of *W. fruticosa* flowers that stimulated non-specific immune responses (Shah and Juvekar, 2010). The hepatoprotective activity of the flower extract has also been reported against chemical induced hepatotoxicity (Chandan et al., 2008; Baravalia et al., 2011). Woodfordin C, an essential compound of this plant was found to exhibit antitumor activity (Yoshida et al., 1990). Another study demonstrated that *W. fruticosa* possess potential antihyperglycemic effect and antioxidant efficacy (Verma et al., 2012). This plant also showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus*

aureus, *Salmonella typhi*, *Salmonella paratyphi*, *Citrobacter fruendii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Shigella dysenteriae, Enterobacter* spp. and *Acenitobacter spp.* (Bhattarai and Bhuju, 2011).

Taxonomic Position:

Kingdom: Plantae
Division: Mangnoliophyta
Class: Malvida

Order: Myrtales
Family: Lythraceae
Genus: *Woodfordia*
Species: *fruticosa*
Botanical name: *Woodfordia fruticosa* (Linn.) Kurz

Common name: Dhayaanro, Dhataki, Fire flame bush, Agnijwala(Sans)

Fig.2.13: *Woodfordia fruticosa* (Linn.) Kurz. a. leaves, b. dried flower, c. whole plant, d. fresh flower

Distribution in Nepal: 200-1800m, east to west

The phytochemical studies have been undertaken time and again to study the essential compounds responsible for their medicinal value. The compounds identified are predominantly phenolics, particularly hydrolysable tannins and flavonoids like octacosanol and B-sitosterol. The non-phenolic constituents reported include the steroid sapogenin, hecogenin and meso-inositol from the flowers along with naringenin 7-glucoside, kaempferol 3-glucoside and quercetin glycosides (Chauhan et al., 1979). Besides the flavonoids or tannins, the major phenolic constituents so far known to occur in the plant include gallic acid in leaves and stems (Kadota et al., 1990). A large number tannins have been isolated from the flowers and other structures like tellimagrandin, gemin D, heterophylliin A, oenothein B and woodfordins (Yoshida et al., 1990). The notable structural feature of the macrocyclic structures of many of these oligomeric tannins is the presence of a novel constituent, the woodfordinoyl group, linking the monomers. Many other such works are being carried out to reveal the underlying phytochemicals (Das et al., 2007).

2.3.4 Antimicrobial Screening: An Integrated Approach

Medicinal plants have been long used in our society due to their wide therapeutic uses. The vast array of secondary metabolites has lot to offer when it comes to medicine. The anti microbial properties are one of those many drawing attention from researchers all over the world. The antimicrobial activity of plants and their metabolites are greatly seeked out due to the challenge of growing incidences of drug-resistant pathogens. Some plants have shown the ability to overcome resistance in some organisms and this has led the researchers to investigate their mechanisms of action and isolate the active compounds (Ncube et al., 2008). Bioactive constituents like tannis, terpenoids, flavonoids, polyphenols, and alkaloids have been the chemistry behind the antimicrobial potentials of medicinal plants (Cowan, 1999).

Too often, natural products are tested only against a single species or class of micro-organisms, mostly selected based on ethnomedical use data. When the product exhibits high activity, it is then identified as a potential 'hit'. However, this narrows the research and overlooks the incidence of false-positives. Such findings also suffer from the lack of discrimination from aspecific cell toxicity. This should be solved by inclusion of a parallel evaluation on other microbial strains of bacteria, fungi, parasites and viruses (Maes et al., 2004). Total extracts and derived fractions exhibiting strong non-selective action in the panel of *in vitro* screens can only be properly evaluated in animal models. An integrated screening concept for anti-infective activity should therefore be carefully regarded in a research work (Cos et al., 2006).

Chapter III

Materials and Methods

3.1 Selection of Medicinal plants

Plant materials used for this study are the medicinal plants of Nepalese origin. A review of literature on medicinal plants, native to Nepal was carried out using the reference source, Medicinal Plants of Nepal by Adhikari et al. (2007) published by Government of Nepal, Ministry of Forests and Soil conservation, Department of Plant Resources, Thapathali, Kathmandu, Nepal. Those plants that are being used traditionally for liver and spleen ailments and signs and symptoms as that of visceral leishmaniasis such as enlargement of abdominal viscera were listed out (Table 2.2). Amongst the many listed, five medicinal plants that were found within Kathmandu valley were chosen for studying the anti leishmanial activity. Besides, plants that have never been reported to have antileishmanial activity were considered while choosing the plants to be accessed for antileishmanial activity. The absence of records for antileishmanial activity were confirmed by reference sources: PubMed NCBI (NCBI, 2012) and ISI Citation Indexes at Web of Science (ISI) using Endnote X4 software (Reuters, 2010) using the search query "(Plant name)" "and/or" "antileishmanial activity", "leishmanicidal activity".

3.2 Collection of Plant Materials

The rhizome of *H. spicatum* and *C. procera* leaves were collected from Phulchowki and Koteshor respectively. The remaining plants were not at their flowering stages at the time of collection and so they were collected from local herbs market at Nardevi, Kathmandu, Nepal. The plants were verified by Dr. Deepak Raj Pant, Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal.They were also authenticated with Annoted Checklist of Flowering Plants in Nepal (Press et al., 2000).

3.3 Phytochemical extraction

Plant parts were air dried and crushed into fine powder in a metallic miller. The powdered material (upto 25 gms) was filled in a cellulose thimble and was subjected to soxhlation with ethanol (250 mL) as solvent for 36 hrs at 78^{O}C. Ethanolic extract was *in vacuo* concentrated at reduced pressure using rotatory vacuum evaporator (Hanshin Scientific Co., Korea) and the solvent free extracts were stored at -20°C until use. The percentage yield was also calculated using the following formula:

$$\text{Percentage yield } (\%) = \frac{\text{Dry wt. of extract}}{\text{Dry wt. of plant material}} \times 100$$

3.4 Parasite culture

Promastigotes: A culture of *Leishmania donovani* (JKP01/2011) was maintained *in vitro* in Roswell Park Memorial Institute (RPMI, Himedia) supplemented with 15% FBS (Invitrogen, USA), and antibiotics (gentamycin 20 µg/mL, streptomycin 100 µg/mL, penicillin 100 U/mL) throughout the study. The promastigotes were maintained in a BOD incubator at 26°C.

Amastigotes: For axenic amastigotes, the promastigotes were cultured in the same medium acidified to pH 5.5 and temperature at 37°C in a humidified CO_2 incubator with 5% CO_2. Axenic amastigotes were characterized by their round and oval shape without flagella under phase contrast microscope.

Passaging: For regular maintenance and availability of the parasites, they were routinely passaged. Briefly, 100 µL of stationary phase parasite culture (generally >1.5 x 10^7 par/mL) was transferred aseptically to a new flask with 5 mL fresh medium (cRPMI-1640). The culture flask was incubated at 26°C in BOD incubator for 7 days to harvest stationary phage promastigotes.

Cryopreservation: For cryopreservation, equal volume of stationary phage promastigotes with count 1-2 x 10^7 promastigotes/mL was mixed well with 3% DMSO (Merck) prepared in heat inactivated FBS. The resulting solution was divided into aliquots of 1ml in cryovials and the vials were immediately transferred to Mc. Frosty Can containing chilled isopropanol. Then the vials were kept at -20^{o}C for overnight and finally, stored at -80^{o}C until reviving.

3.4.1 Determination of parasite count

The parasites were counted in a haemocytometer (Neubauer's chamber). A haemocytometer is a thick glass microscope slide with a rectangular indentation engraved with a laser-etched grid of perpendicular lines consisting 9 large squares (further divided in 16 smaller squares) each measuring 1mm x 1 mm in area and 0.1 mm in depth equating to a volume of 1 mm^3. For determining the parasite count 10 µL of promastigote suspension was loaded in the counting chamber and covered with a coverslip and observed under a light microscope. The non-motile, distorted and dead parasites were excluded and the parasite count was determined using the following formula:

$$\text{Parasite number} = \frac{\text{total no. of parasites counted in 4 corner squares}}{4} \times DF \times 10^4$$

3.5 Primary mice peritoneal macrophage cell culture

The cytotoxicity tests were carried out using mice peritoneal macrophages. Mice was first injected with 2% starch solution, to stimulate macrophage proliferation, and sacrificed after 2 days. The body surface was disinfected and the skin was torn dorso-ventrally to expose the peritoneum. With the help of a sterile syringe, about 4-5 mL of phosphate buffer saline (PBS) was injected into the stimulated peritoneum and mouse peritoneal macrophages were harvested by withdrawing the fluid. The cell suspension was collected in sterile centrifuge tubes and centrifuged at 2000 rpm for 10 min. The pellet was re-suspended in 5 mL of complete RPMI 1640 medium. For execution of cytotoxicity experiments, cells (3×10^4 /well) were seeded in 96-well tissue culture plates (Nunc, Demark).

3.6 Reconstitution of antileishmanial reference drugs and plant extracts

Miltefosine and Amphotericin B were used as the reference drugs and dissolved in double distilled de-ionised water to obtain a stock solution of 2 mg/mL and 50 mg/mL respectively. Crude ethanolic extracts of the selected medicinal plants were dissolved in 100% of dimethyl sulfoxide (DMSO, Merck) at 50 mg/mL and syringe filtered (0.22 µm). Stock solutions of the reference drugs and extracts were stored in dark at 4°C until use. For working solutions, drugs were further diluted in complete RPMI-1640 medium in required concentration for *in vitro* tests.

3.7 *in vitro* antileishmanial assay on *L. donovani* promastigotes and axenic amastigotes

3.7.1 Preparation of working solutions

For *in vitro* anti-leishmanial assay, working solutions of reference drugs and crude ethanolic extracts of all five medicinal plants were prepared. A 4-fold dilution of the drugs and extracts was setup in 96-well plate (NUNC, Denmark). For which 120 µL of miltefosine, Amphotericin B and the crude ethanolic extracts were dispensed in triplicate in the row of a 96-well plate. 90 µL of cRPMI was dispensed in rest of the wells. From the drug/extracts well, 30 µL of the drug/extracts was aspirated and transferred to the successive well of the second row and so on to obtain a fourfold drug/extracts dilution (Table 3.1). The last row was used as control well.

Table 3.1: Drug/extract dilution series for *in vitro* antileishmanial assay.

Drugs/Extracts	For promastigotes		For amastigotes	
	dispensing conc (µg/mL)	Final conc. (µg/mL)	dispensing conc (µg/mL)	Final conc. (µg/mL)
Miltefosine	250 to 3.9	125 to 1.95	200 to 3.125	100 to 1.5625
AMB	2 to 0.03	1 to 0.015	2 to 0.03	1 to 0.015
Crude ethanolic extracts	2000 to 31.25	1000 to 15.625	2000 to 31.25	1000 to 15.625

3.7.2 Anti promastigote assay

For anti-promastigote assay, 90 µL of the promastigote suspension at 10^6/mL was dispensed in all the drug rows, extract and control rows to obtain miltefosine concentrations from 125 to 1.95 µg/mL; amphotericin concentrations from 1 to 0.015 µg/mL; crude ethanolic extract concentrations from 1000 to 15.625 µg /mL (detailed in Table 3.1) respectively in triplicate. The plates were incubated at 26°C in a BOD incubator. After incubation live promastigotes were counted in Neubaurs counting chamber excluding non-motile, distorted or dead parasites at 24, 48, 72 and 96 hours.

3.7.3 Anti amastigote assay

Axenic amastigote suspension in 90 µL at 10^6/mL was dispensed in all the drug rows, extract and control rows to obtain drug miltefosine concentrations from 100 to 1.5625 µg/mL; Amphotericin B concentrations from 1 to 0.015 µg/mL; crude ethanolic extract concentrations from 1000 to 15.625 µg /mL (Table 3.1) respectively in triplicate. The plates were then incubated at 37°C in humidified CO_2 incubator with 5% CO_2. After 48 hrs, the viability of the amastigotes was accessed by MTT assay. Briefly, 50 µL of 0.5% MTT was added to all the test and control wells and incubated for 4 hours at 37°C, 5% CO_2. Then, 100 µL of DMSO was added in each well to dissolve blue formazan formed by reduction of yellow tetrazolium salt within the cells and absorbance of the plate was read at 540 nm.

3.8 Determination of percentage inhibition, IC_{50}, IC_{90} and absolute inhibition estimation

Percentage inhibition (PI) by the drugs and extracts was determined considering the mean parasite counts of the control wells as 100% survival and converting all the other counts at different treatments into percentages in reference to the control. Calculation of Percentage of inhibition was done by using the formula,

$$\text{Percentage of inhibition (PI)} = \frac{(\text{PC}-\text{PT})}{\text{PC}} \times 100$$

Where, PC is the count of parasites in control well and
PT is the count of parasites in the treatment well

The concentration of treatments inhibiting 50% of the parasites (IC_{50}) was then calculated along with the IC_{90} values which is the concentration of treatment(s) that inhibits 90% of the parasites. Absolute inhibition of the parasites were also noted when the concentration of treatment(s) killed total (100%) of the parasites. IC_{50} and IC_{90} were generated using following formulae in Microsoft Excel 2007 software as described by Prof. Dr. Louis Maes and Prof. Dr. Paul Cos (Louis and Paul, 2010).

$$IC_{50} = \text{EXP}\left[\text{LN}\left(\text{conc} > 50\%\right) - \left(\frac{\text{signal}>50\% - 50}{\text{signal}>50\% - signal<50\%}\right) \times \text{LN}\left(\frac{\text{conc}>50\%}{\text{conc}<50\%}\right)\right]$$

$$IC_{90} = \text{EXP}\left[\text{LN}\left(\text{conc} > 90\%\right) - \left(\frac{\text{signal}>90\% - 90}{\text{signal}>90\% - \text{signal}<90\%}\right) \times \text{LN}\left(\frac{\text{conc}>90\%}{\text{conc}<90\%}\right)\right]$$

EXP: exponential function; LN is natural log function used in Microsoft Excel 2007 software. Signal > 50% refers to PI value just above 50% and signal < 50% refers to PI just below 50%. Conc > 50% refers to corresponding signal greater than 50% and conc < 50% refers to corresponding concentration of signal <50%.

3.9 Assessment of cytotoxicity

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay on primary mouse peritoneal macrophages was performed for assessment of cytotoxicity by determining the percentage reduction in cell viability by the reference drugs and extracts. Briefly, macrophages $3x10^4$ cells/well in complete RPMI-1640 medium were seeded in a 96 well tissue culture plate. The macrophages were allowed to adhere to the wells by incubation in a humidified CO_2 incubator containing 5% CO_2 at 37°C for 4 hours. The cells were then washed with PBS to remove non-adherent cells and 100µl of fresh complete medium was dispensed. Then 100 µl of reference drugs and the extracts at different concentration (in a series of four fold dilution) was added to each well. Miltefosine was used at a concentration from 100µg/mL to 1.5625 µg/mL, Amphotericin B from 15 to 0.23 µg/mL and extracts at different concentration starting from 1000 to 15.625 µg/mL. Plates were then incubated for 72 hours at same parameters of humidity, CO_2 and temperature. After completion of incubation, 50µl of 0.5% MTT was added to

each well and plates were further incubated for 4 hours. Then 100µl of DMSO was added in each well to dissolve blue formazan. The absorbance of the plate was read at 540 nm. Results were expressed as percentage reduction in cell proliferation, compared with controls. Cells without drug served as control and wells with media only was used as blank in the experiment. The experiments were done in triplicate.

$$\% \text{ cell survival} = \frac{(\text{At}-\text{Ab})}{(\text{Ac}-\text{Ab})} \times 100$$

Where, At = absorbance of test
Ab = absorbance of band
Ac = absorbance of control

% reduction in cell proliferation = 100 - % cell survival

The cytotoxic concentration required to inhibit the 50% of cells proliferation (CC_{50}) was determined using a graph plotted against percentage reduction in cell proliferation versus drug concentration taking the absorbance of control well as 100% survival using Microsoft Excel 2007 software.

3.10 Determination of survival index

The cytotoxicity of crude extracts and drugs on the macrophages was compared with the activity against promastigotes and axenic amastigotes of *L. donovani*, by using the selectivity index (SI). SI is the ratio of cytotoxicity (CC_{50}) to inhibitory concentration of the parasite (IC_{50}). A value greater than 1 indicates the treatment to be more selective for the parasite.

3.11 Antibacterial screening

With regard to the wide biological activity of the medicinal plants, an antibacterial screening of the extracts was carried out. For this assay, four strains of Gram negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 14028, and *Pseudomonas aeruginosa* ATCC 27853) and a single strain of Gram positive bacterium (*Staphylococcus aureus* ATCC 25923) were used. All the strains were obtained from National Public Health Laboratory, Teku, Nepal. The antibacterial activity of the extracts was screened by disc diffusion method (Bauer et al., 1966) whereby the bacterial cultures were acted upon by extracts contained in paper discs.

3.11.1 Preparation of discs

Paper discs were prepared by punching out Whatman filter paper. The punched out paper discs were first sterilized and treated with the extracts using a micropipette. The

extracts were dissolved in DMSO and used at a concentration of 500 µg/ disc. Along with the plant extracts, paper disc for the reference drug Miltefosine was also prepared. Thus prepared paper discs were then allowed to dry under aseptic condition before using them for the test.

3.11.2 Antibacterial screening via disc diffusion

Before carrying out the tests, the standard strains of bacteria were subcultured in Nutrient broth for 12-18 hrs at 37°C for inoculum preparation. Then the turbidity of bacterial suspension was adjusted to McFarland standard number 0.5 to obtain a desired inoculum of 1.0-1.5 X 10^8 CFU per mL. With a sterile cotton swab bacterial culture was streaked on Mueller Hinton agar plate (Himedia, India). Then the previously prepared discs were placed at equidistance in a circle on the seeded plate. A standard antibiotic disc of Gentamycin (10mcg/disc) was also placed along with the extracts. Blank disc impregnated with solvent DMSO was used as negative control. These plates were kept for 2-4 hours at low temperature to allow the test materials to diffuse from disc to the surrounding medium. The plates were then incubated at 37°C for 18-24 hours after which they were observed for the formation of halozones. The diameter of zone of inhibition produced by the extracts was then compared with standard antibiotic.

3.12 Statistical analysis

All the experiments were performed in triplicates and the data represent the mean ± standard deviation from three independent assays. The inhibitory concentration values (IC_{50}, IC_{90}, absolute inhibition and CC_{50}) values and dose-responsive curves, graphs and charts were generated using Microsoft Excel 2007 software and GraphPad Prism version 5. GraphPad Prism was used for statistical analysis. An unpaired t-test (two-tailed) was applied to compare the inhibitory concentration between drugs and extracts. P value <0.001 was taken as extremely significant, 0.001 to 0.01 as very significant, 0.01 to 0.05 as significant and >0.05 as non significant.

Chapter IV

Results

4.1 Culture of *Leishmania donovani*

4.1.1 Promastigotes

Promastigotes of *L. donovani* were maintained in Central Department of Biotechnology. They were cultured in cRPMI medium and routinely passaged for regular availability. Every 7^{th} day of passaging, the parasites converted to stationary phase were counted and the counts were found to range from 10^7 to 10^8 per ml in 20 ml culture suspensions.

4.1.2 Amastigotes

The flagellated promastigotes morphologically changed to oval and/or rounded aflagellated amastigote forms with a change in physiological conditions (pH 5.5, temp. 37°C). A giemsa stained slide of the amastigotes was prepared and observed under the inverted microscope to confirm the conversion into amastigote state (Fig 4.1.). Parasite suspensions of count 10^6/ml were used for further experimental works.

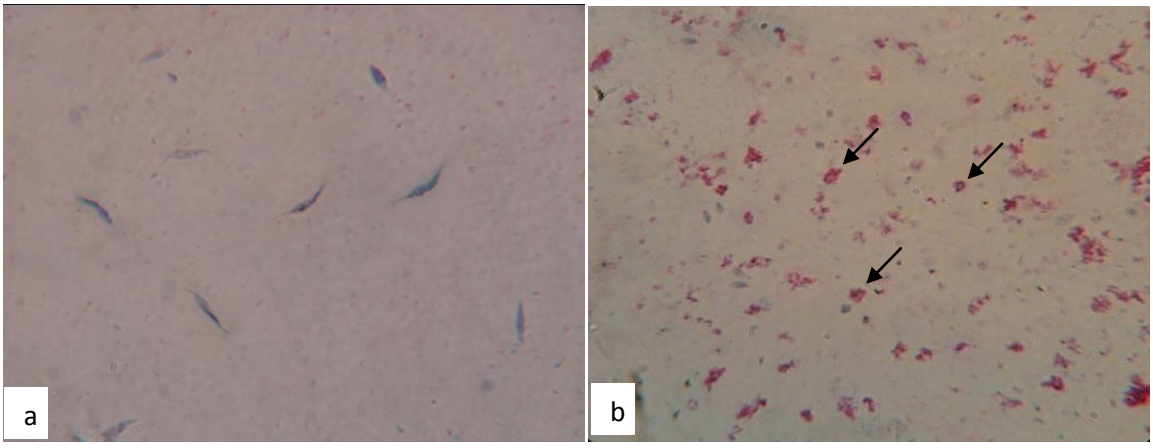


Fig 4.1: Giemsa stained parasites a. Promastigotes b. Axenic amastigote

4.2 Phytochemical extraction

Crude ethanolic extract (after the completion of soxhlation process) obtained in the soxhlet collection flask, differed in colour ranging from red (*Woodfordia*), green (*Calotropis*) and pale yellow (*Hedychium*, *Phyllanthus* and *Boerhavia*) depending upon the parts and plants used. All of the extracts turned dark in colour after *in vacuo* concentration. The extracts were semisolid in consistency except for *Woodfordia* which was solid and crystalline. Similarly, their yield percentages were also different (Table 4.1.)

Table 4.1: Parts used for the extraction process and characterization of the crude ethanolic extracts

Plants	Parts used	Dry wt. used (gm)	Colour of extract	Consistency	Yield (gm)	Yield %
Boerhavia diffusa	Whole plant	21	Greenish black	Semisolid, Sticky	1.62	7.71
Calotropis procera	Leaves	20	Dark brown	Semisolid	3.07	15.35
Hedychium spicatum	Rhizome	20	Reddish brown	Semisolid	2.04	10.02
Phyllanthus niruri	Whole plant	25	Greenish black	Semisolid	2.06	8.24
Woodfordia fruticosa	Flowers	25	Dark brown	Solid, crystalline	8.35	33.4

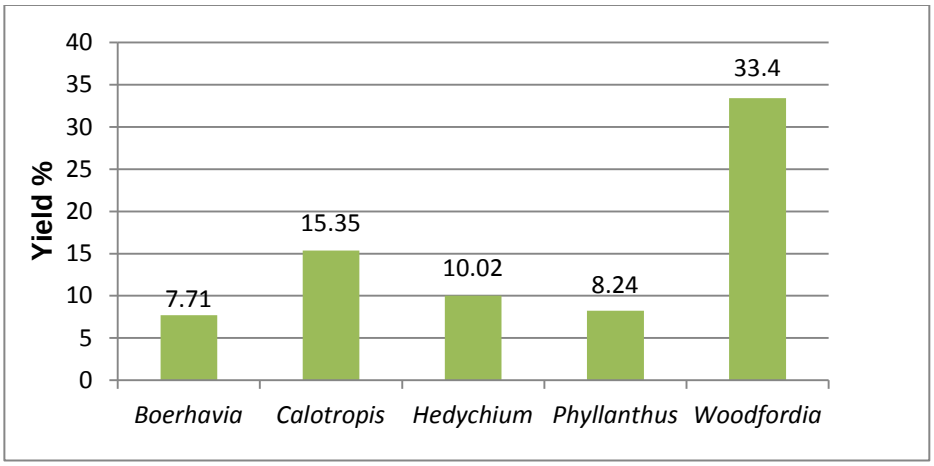


Fig. 4.2: Percentage yield of selected medicinal plants

4.3 *in vitro* antileishmanial assay on *L. donovani* promastigotes

The anti promastigote assay was studied at an interval of 24 hours for 96 hours. After the addition of the crude extracts, various morphological abnormalities like loss of flagella, distorted shape and considerable loss of mobility in the parasites was observed under the microscope. Similar trend was observed with the reference drugs while the control wells contained highly active and morphologically sound parasites.

4.3.1 Determination of IC_{50} values

The half maximal inhibitory concentration (IC_{50}) was determined for all the crude ethanolic extracts as well as the reference drugs. Promastigotes were largely inhibited by the ethanolic extract of *Woodfordia fruticosa* amongst others with IC_{50} value 35.30 ± 2.43 µg/ml. While *Boerhavia diffusa* was found to be least effective with IC_{50} value of 294.7 ± 18.09 µg/ml, all the other remaining plants exhibited good efficacies as indicated

by their IC_{50} values and were below 100 µg/ml. The reference drugs showed lower values of IC_{50}. The value of Miltefosine was calculated as 12.35 ± 0.82 µg/ml and that of Amphotericin B was the minimal with a value of 0.029 ± 0.0007 µg/ml (Table 4.2).

4.3.2 Determination of IC_{90} values and absolute inhibition

At 96 hours the IC_{90} value was the lowest for *W. fruticosa* (113.72 µg/ml). Also three plants extract those of *W. fruticosa, P. niruri* and *C. procera* caused an absolute inhibition of the parasites at a concentration of 1 mg/ml. The efficacies of other two plant extracts were also found to be similar. At a concentration of 1 mg/ml, plants extracts of *H. spicatum* and *B. diffusa* inhibited 99.22% and 98.45% of the parasites respectively (Table 4.2 and Fig 4.3).

Table 4.2: IC_{50}, IC_{90} and absolute inhibition values of crude ethanolic extracts of selected plants and reference drugs against *L. donovani* promastigotes

Extracts/Drugs	[a]IC_{50} (µg/ml)	[b]IC_{90} (µg/ml)	[b]Absolute inhibition (µg/ml)
B. diffusa	294.7 ± 18.09	420.11± 21.8	>1000
C. procera	96.32 ± 8.79	575.57± 12.17	1000
H. spicatum	73.63 ± 4.34	454.12± 17.75	>1000
P. niruri	67.46 ± 3.03	276.9 ± 30.25	1000
W. fruticosa	35.30 ± 2.43	113.72 ± 1.73	1000
Miltefosine	12.35 ± 0.82	21.06 ± 0.13	125
Amphotericin B	0.029 ±0.0007	0.3 ± 0.02	1

Results expressed as mean ± SD of three independent experiments

a– Results noted at the end of 48 hrs

b– Results noted at the end of 96 hrs

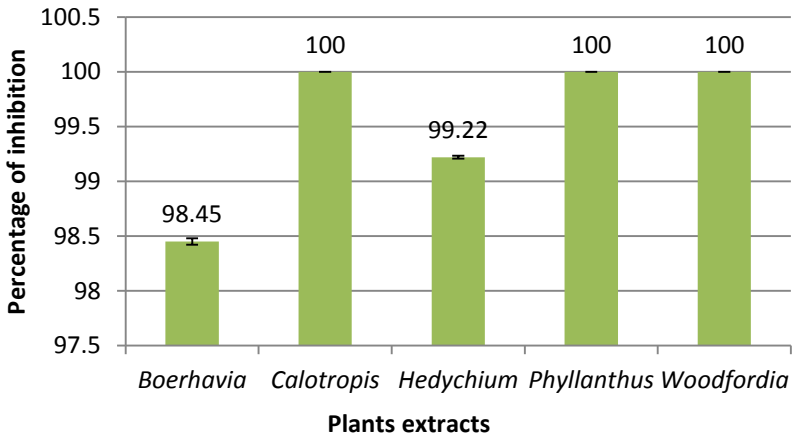


Fig 4.3: Comparative inhibition by the plant extracts at a dose of 1 mg/ml in 96 hours.

4.4 *in vitro* antileishmanial assay on *L. donovani* amastigotes

In case of axenic amastigotes, the OD values of the treatment wells were lower than the OD value of control (0.232) in course of MTT assay (Table 4.3). The lower OD values in the treated wells indicated the lower levels of purple formazan formation suggesting the inhibition of the parasites in the treated wells. The OD values were then converted into the inhibition percentage as described in materials and methods.

Table 4.3: Mean OD values as obtained during MTT assay to determine anti amastigote activity

Extracts/Drugs	Concentration (µg/ml)			
	15.625	**62.5**	**250**	**1000**
B. diffusa	0.198	0.129	0.091	0.022
C. procera	0.177	0.112	0.059	0.014
H. spicatum	0.203	0.093	0.068	0.010
P. niruri	0.193	0.0795	0.053	0.013
W. fruticosa	0.141	0.078	0.023	0.003
	1.5625	**6.25**	**25**	**100**
Miltefosine	0.155	0.103	0.042	0
	0.015	**0.0625**	**0.25**	**1**
Amphotericin B	0.125	0.05	0.026	0.017

4.4.1 Determination of IC_{50} values

A similar trend of efficacy was displayed against the axenic amastigotes by the plant extracts with *W. fruticosa* again being the most effective. It was also observed that the inhibitory concentrations of the extracts and the reference drugs against the axenic amastigotes were lower than that against the promastigotes. *W. fruticosa* showed IC_{50} value of 27.25 ± 1.88 µg/ml against the axenic amastigotes. IC_{50} of miltefosine for axenic amastigotes was found to be 4.55 ± 0.31 µg/ml, which is about 3 fold lower than the IC_{50} value for the promastigotes. Similarly, another reference drug Amphotericin B also showed lower IC_{50} value of 0.017 ± 0.001 µg/ml. The IC_{50} values of all the plants as well as the reference drugs against the axenic amastigotes has been illustrated in Table 4.4.

4.4.2 Determination of IC_{90} values and absolute inhibition

The inhibition against amastigotes was studied at 48 hours. Against the amastigotes, *W. fruticosa* again had the lowest value of IC_{90} of 257.51 µg/ml while none of the extracts were successful in inhibiting the amastigotes completely within 48 hours. The details of IC_{90} values and absolute inhibition of the extracts against promastigotes and amastigotes are given in Table 4.4 below.

Table 4.4: IC_{50}, IC_{90} and absolute inhibition values of crude ethanolic extracts of selected plants and reference drugs against *L. donovani* amastigotes in 48 hours.

Extracts/Drugs	IC_{50} (µg/ml)	IC_{90} (µg/ml)	Absolute inhibition (µg/ml)
B. diffusa	101.75 ± 0.73	975.51 ± 29.65	>1000
C. procera	57.96 ± 0.13	763.46 ± 7.71	>1000
H. spicatum	46.96 ± 2.11	738.30 ± 0.69	>1000
P. niruri	40.23 ± 2.23	722.22 ± 26.25	>1000
W. fruticosa	27.25 ± 1.88	257.51 ± 12.19	>1000
Miltefosine	4.55 ± 0.31	58.13 ± 1.61	100
Amphotericin B	0.017 ± 0.001	0.31 ± 0.13	>1

Results expressed as mean ± SD of three independent experiments in 48 hrs.

4.5 Dose response curves: Percentage of promastigote survival vs concentration of extracts

An integrated survival curve was created (Fig. 4.4a) that depicts the percentages of promastigote survival after 48 hrs of treatment with crude extracts. From the dose response curves, it can be inferred that the crude extracts inhibited promastigotes in a dose dependent manner and only few parasites survived the higher concentration of the treatments. The survival percentages for *Boerhavia*, *Calotropis*, *Hedychium*, *Phyllanthus*, and *Woodfordia* treatment were 15.7, 9.8, 10.3, 7.2 and 6.4% respectively after 48 hours.

4.6 Dose response curves: Percentage of amastigote survival vs concentration of extracts

The anti amastigote activity was also observed at various concentrations of the extracts at 48 hours of treatment. An integrated survival curve was generated as shown in Fig. 4.3b. The dose response curve indicated that axenic amastigotes have been increasingly inhibited by the increasing doses of treatments. Though none of the extracts were successful in inhibiting 100% amastigotes even at 1 mg/ml, all of them however inhibited > 90% of the amastigotes at this concentration. *Woodfordia* extract inhibited most of the parasites with only 1.5% parasites surviving at the end of 48 hours. For rest

of the plants the survival percentages were 4.5% (*Hedychium*), 5.8% (*Phyllanthus*), 6.2% (*Calotropis*) and 9.4% (*Boerhavia*).

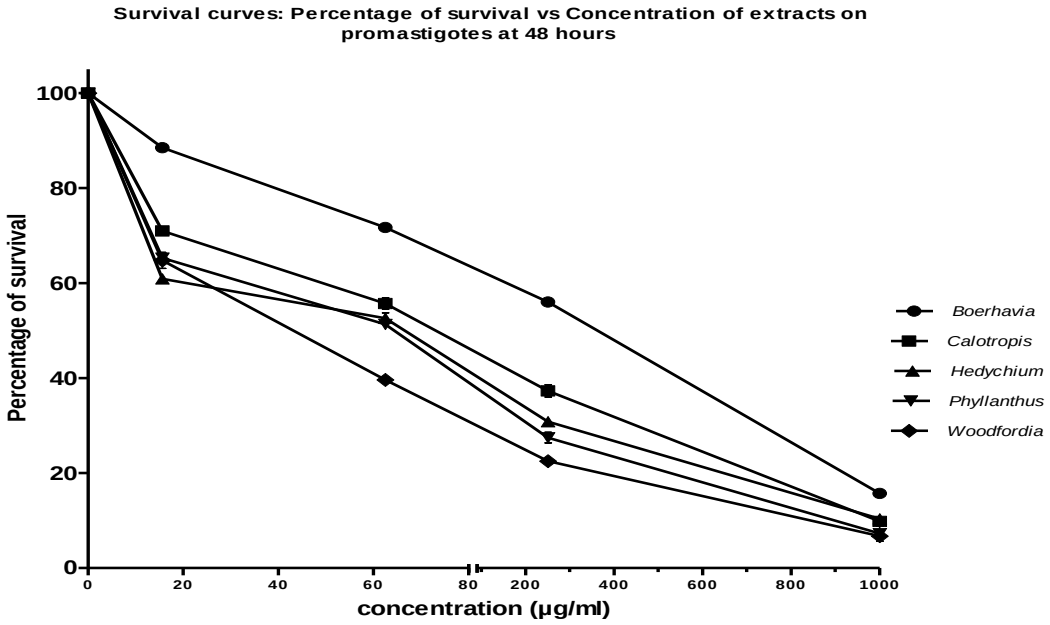


Fig. 4.4a: Dose response curve showing survival percentage of promastigotes at different concentrations upto 1 mg/ml of crude ethanolic extracts in 48 hrs.

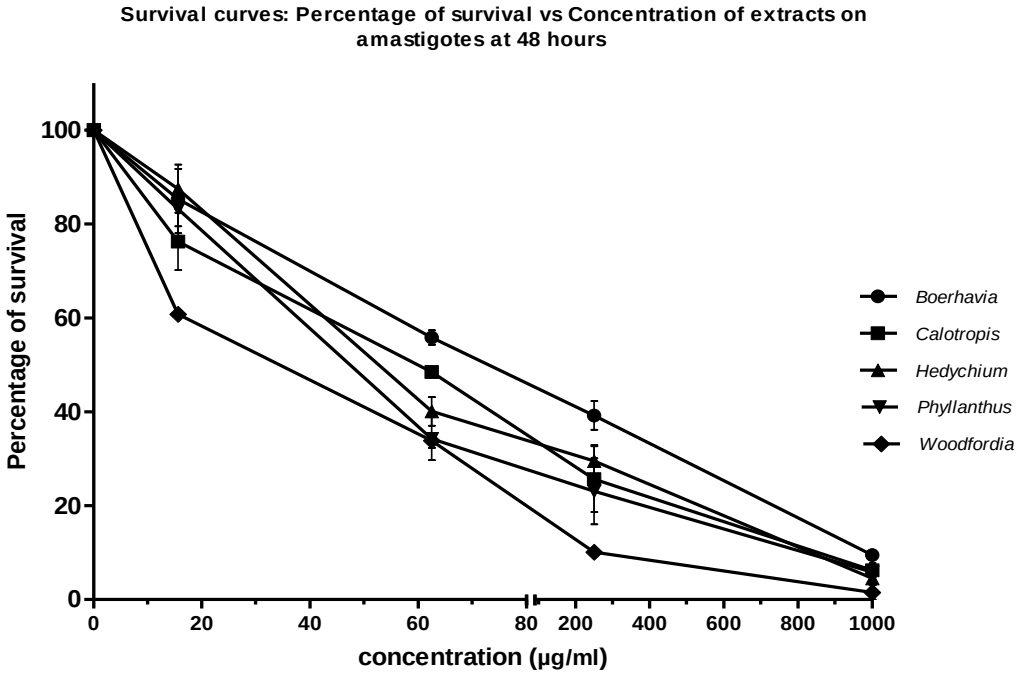


Fig. 4.4b: Dose response curve showing survival percentage of amastigotes at different concentrations upto 1 mg/ml of crude ethanolic extracts in 48 hrs.

4.7 Time dependent efficacy of extracts over 96 hours

The anti promastigote abilities of the plants were studied over a time period of 96 hours. There was an increase in inhibition with increasing time. The time dependent inhibition indicated that *W. fruticosa* extract could completely inhibit the promastigotes at 1 mg/mL within 72 hours (Fig. 4.5a). At this concentration, crude extracts of two other plants namely, *P. niruri* and *C. procera*, caused a total inhibition of the promastigotes in 96 hours (Fig. 4.5b and Fig. 4.5c). The remaining extracts of *H. spicatum* and *B. diffusa* could achieve a maximum inhibition of 99% over 96 hour (Fig. 4.5d and Fig. 4.5e). A summary of percentage of inhibition of *L. donovani* promastigotes by various extracts over 96 hours has been shown in Table 4.5.

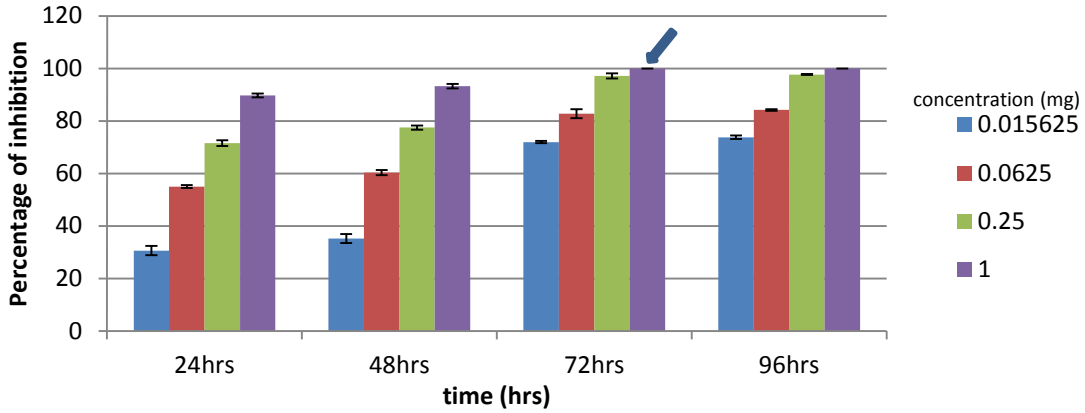


Fig. 4.5a: Effect of *W. fruticosa* ethanolic extract on *L. donovani* promastigotes over 96 hrs. Absolute inhibition was found in 72 hrs at the concentration of 1 mg/ml. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

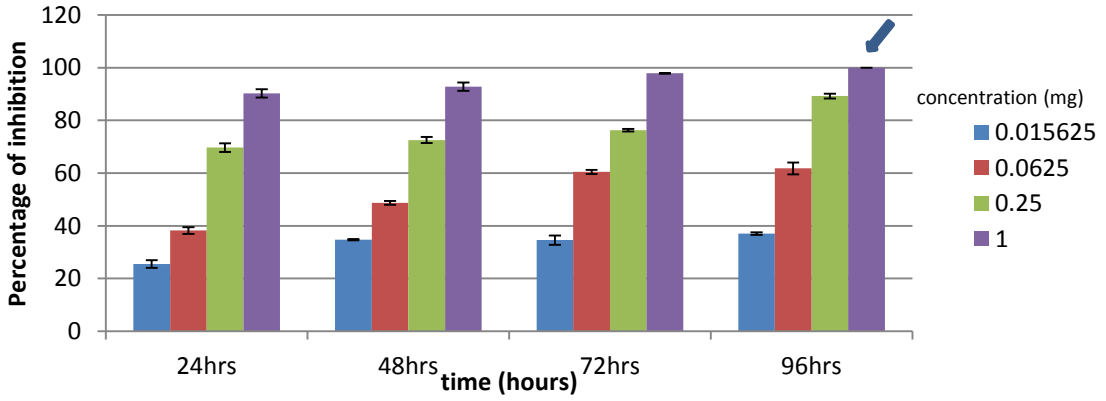


Fig. 4.5b: Effect of *P. niruri* ethanolic extract on *L. donovani* promastigotes over 96 hrs. Absolute inhibition was found in 96 hrs at the concentration of 1 mg/ml. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

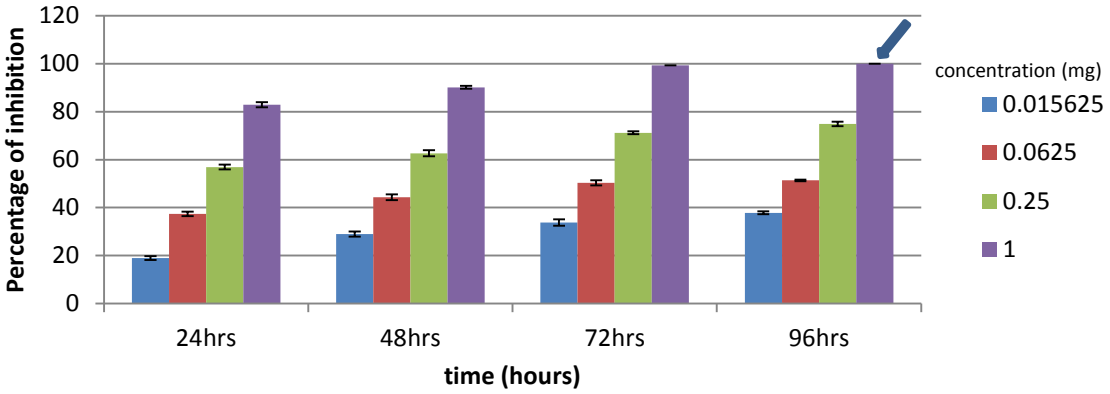


Fig. 4.5c: Effect of *C. procera* ethanolic extract on *L. donovani* promastigotes over 96 hrs. Absolute inhibition was found in 96 hrs at the concentration of 1 mg/ml. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

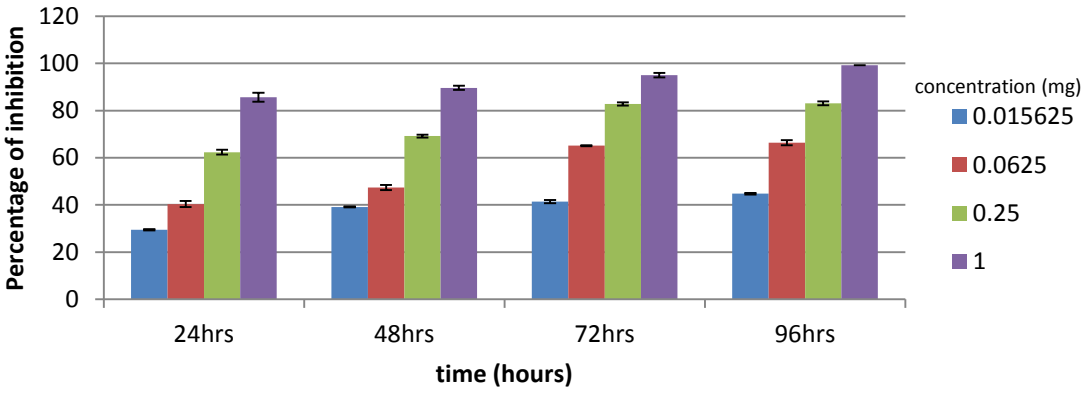


Fig. 4.5d: Effect of *H. spicatum* ethanolic extract on *L. donovani* promastigotes over 96 hrs. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

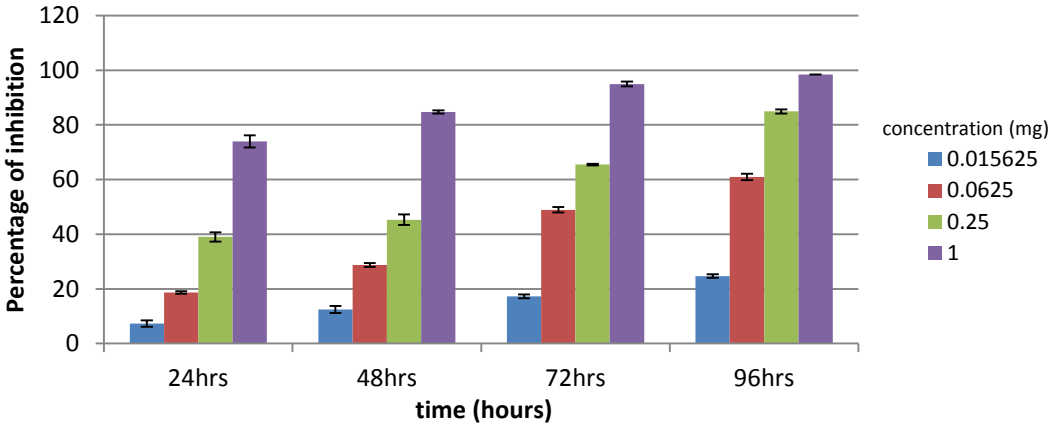


Fig. 4.5e: Effect of *B. diffusa* ethanolic extract on *L. donovani* promastigotes over 96 hrs. Absolute inhibition could not be achieved at any concentration upto 1 mg/ml. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

Table 4.5: Inhibition percentage of *L. donovani* promastigotes by crude ethanolic extracts over 96 hours

Extracts	Time (hrs)	Concentration (mg/ml)			
		0.015625	0.0625	0.25	1
B. diffusa	24	7.32	18.70	39.02	73.99
	48	12.43	28.75	45.32	84.71
	72	17.26	48.93	65.47	94.97
	96	24.70	60.99	84.94	98.46
C. procera	24	18.97	37.4	56.91	82.93
	48	29.01	44.29	62.68	90.16
	72	33.8	50.35	71.22	99.28
	96	37.84	51.35	74.9	100
H. spicatum	24	29.54	40.38	62.33	85.64
	48	39.12	47.40	69.18	89.63
	72	41.36	65.11	82.74	95.62
	96	44.78	66.42	83.02	99.23
P. niruri	24	25.47	38.21	69.64	90.24
	48	34.71	48.70	72.53	92.73
	72	34.55	60.42	76.25	97.84
	96	37.06	61.76	89.20	100
W. fruticosa	24	30.62	55.01	71.55	89.70
	48	35.22	60.36	77.47	93.26
	72	71.95	82.75	97.13	100
	96	73.75	84.17	97.68	100

In case of axenic amastigotes, the inhibition was studied directly at 48 hours. Though none of the plant extract caused a total inhibition, all the extracts however inhibited more than 90 % of the amastigotes within 48 hours. From the observations, it can be inferred that the ethanolic extract of *W. fruticosa* was the best amongst all in inhibiting the amastigotes at lower doses and in less time period (Table 4.6).

Table 4.6: Inhibition percentage of *L. donovani* amastigotes by crude ethanolic extracts in 48 hours

Extracts	Time (hrs)	Concentration (mg/ml)			
		0.015625	0.0625	0.25	1
B. diffusa	48	14.66	44.18	60.78	90.52
C. procera		23.71	51.51	79.31	93.75
H. spicatum		12.50	59.91	72.84	95.47
P .niruri		16.81	65.73	71.98	94.18
W. fruticosa		39.22	66.16	89.65	98.49

4.8 Cytotoxicity of crude extracts and reference drugs

Cytotoxicity tests carried out on primary mice peritoneal macrophages via MTT assay in which mitochondrial reductase reduces the yellow tetrazolium salt – MTT to blue/purple formazan within cells showed the cytotoxicity values of all the crude ethanolic extracts far higher than their anti-leishmanial IC_{50} values (IC_{50}^{a} and IC_{50}^{b}) indicating that they are non toxic to the cells.

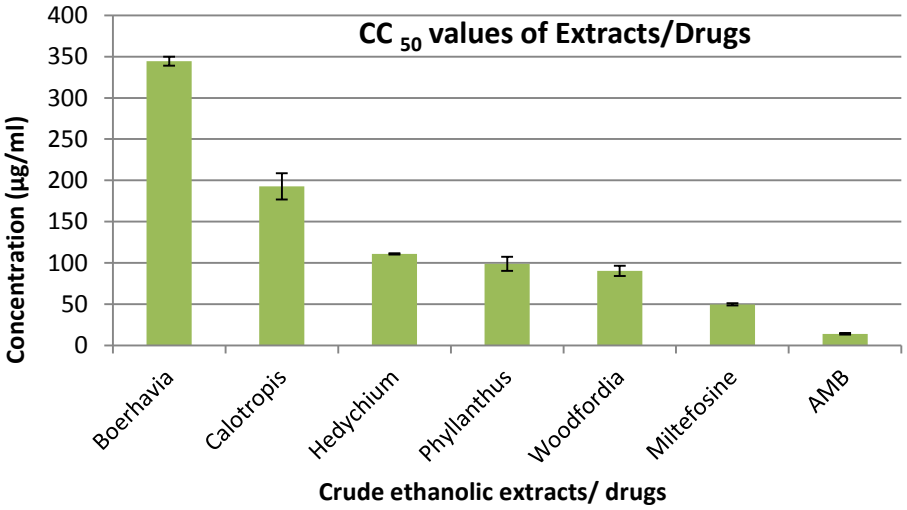


Fig. 4.6: CC_{50} value of crude extracts and drugs on mice peritoneal macrophages after 72 hrs. Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

Cytotoxicity (CC_{50}) of the crude ethanolic extract of *W. fruticosa* was determined to be 79.06 ± 3.9 µg/ml. *B. diffusa* expressed least cytotoxicity with CC_{50} value of 336.4 ± 3.43 µg/ml followed by *C. procera, P. niruri* and *H. spicatum* with CC_{50} values of 175.13 ± 6.13, 102.26 ± 1.68 and 101.99 ± 10.43 µg/ml respectively. The reference drugs - AMB and miltefosine were found to be highly cytotoxic than any of the extracts. CC_{50} of miltefosine was found to be 40.12 ± 0.16 µg/ml and that of AMB was 14.00 ± 0.89 µg/ml. Cytotoxicity values (CC_{50}) of the extract and fractions are shown in Table 4.7 and Fig. 4.6.

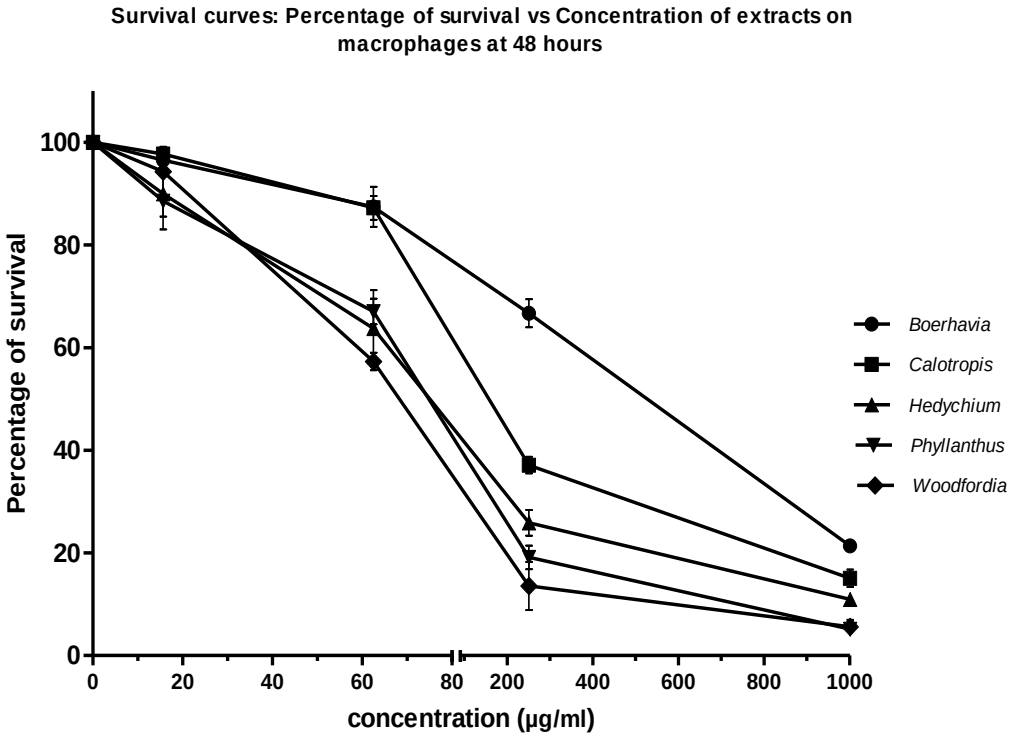


Fig. 4.7: Dose response curve showing survival percentage of macrophages at different concentrations upto 1 mg/ml of crude ethanolic extracts in 48 hrs.

4.9 Efficacy comparison on the basis of selectivity indices (SI)

On the basis of selectivity index (SI^a for promastigote and SI^b for amastigote), among the extracts, *W. fruticosa* extract was found to be the most selective against the promastigote form (SI^a = 2.56). The selectivity index against the amastigotes (SI^b) was 3.31. The extracts of *C. procera*, *P. niruri*, *B. diffusa* and *H. spicatum* were found to be 2.0, 1.46, 1.16 and 1.5 times selective for promastigotes and 3.32, 2.45, 3.38 and 2.36 times selective to amastigotes respectively. All the selectivity indices were higher than 1 indicating their safety/nontoxicity towards the cells. Reference drug miltefosine was 10.92 times more selective for amastigotes than the macrophages and 4.02 times more selective for the promastigotes. The next reference drug Amphotericin B showed very high selectivity for both the forms of the parasites than the cells (SI^a, SI^b => 100).

Table 4.7: *in vitro* antileishmanial activity (IC_{50}) and cytotoxicity (CC_{50}) of crude ethanolic extracts of selected medicinal plants against *L. donovani* promastigotes and axenic amastigotes and their respective selective indices

Extracts/ drugs	Promastigotes IC_{50} (µg/ml)	Axenic amastigotes IC_{50} (µg/ml)	Cytotoxicity CC_{50} (µg/ml)	Selectivity index	
				$(SI)^a$	$(SI)^b$
Boerhavia	294.7 ± 18.09	101.75 ± 0.73	344.63 ± 5.45	1.16	3.38
Calotropis	96.32 ± 8.79	57.96 ± 0.13	192.83 ± 15.82	2.0	3.32
Hedychium	73.63 ± 4.34	46.96 ± 2.11	111.01 ± 0.72	1.5	2.36
Phyllanthus	67.46 ± 3.03	40.23 ± 2.23	98.90 ± 8.60	1.46	2.45
Woodfordia	35.30 ± 2.43	27.25 ± 1.88	90.38 ± 6.09	2.56	3.31
Miltefosine	12.35 ± 0.82	4.55 ± 0.31	49.69 ± 1.42	4.02	10.92
AMB	0.029 ±0.0007	0.017 ± 0.001	14.00 ± 0.89	>100	>100

Results are expressed as mean ± SD of three independent experiments.

$(SI)^a$ – Selectivity Index for promastigotes: CC50 of test sample against macrophages/IC50 of test sample against promastigotes.
$(SI)^b$ – Selectivity Index for amastigotes: CC50 of test sample against macrophages/IC50 of test sample against axenic amastigotes.

4.10 Statistical comparison on antileishmanial effects of crude ethanolic extracts and reference drugs

One way analysis of variance (ANOVA) between the mean IC_{50} values of promastigote and amastigote between different extracts and reference drugs was carried out. The results indicate that their activities are significantly different (P value is < 0.0001). However, an ANOVA test was also carried out to see if there are any significant differences in the inhibition of parasites by different extracts at the concentration of 1 mg/ml in 48 hours. There was no significant differences between them (P value = 0.5061).

An unpaired t-test (two tailed) analysis between the extracts also showed significant differences in their antileishmanial activity. Moreover, a comparative antileishmanial activity of miltefosine vs. crude extracts at their 50% (CC_{50}) cytotoxic concentration was analyzed with unpaired t-test (two tailed) to determine if a significant difference occurs between them (Fig. 4.6a and Fig. 4.6b). All the results showed significant differences with the inhibition percentage (79.23 % of promastigote and 90.18 % of amastigote) of Miltefosine at its CC_{50} concentration.

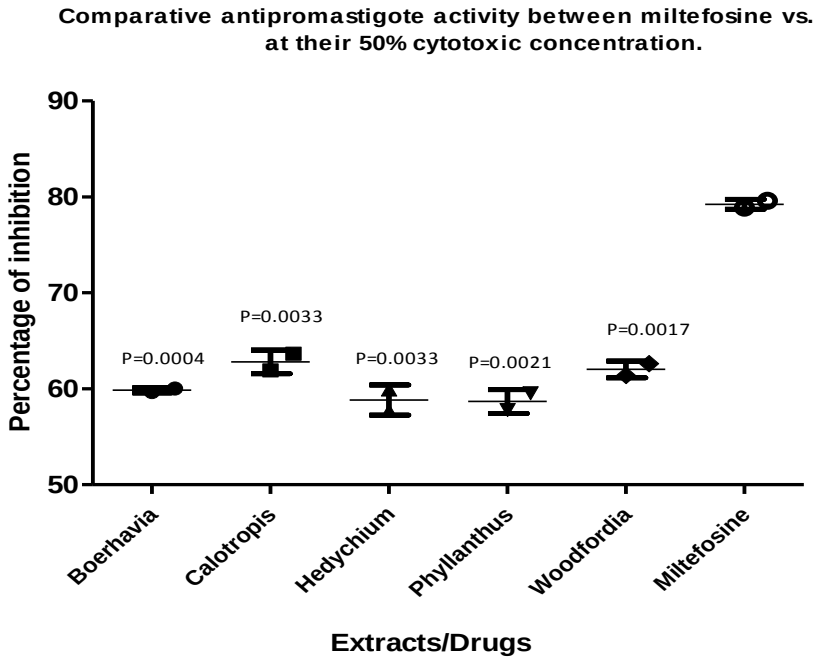


Fig. 4.8a: Comparative antipromastigote activity between miltefosine vs. crude extracts at their 50% cytotoxic concentration

P-value mentioned above the triplicate experiments of each component explains significance between test components with miltefosine.

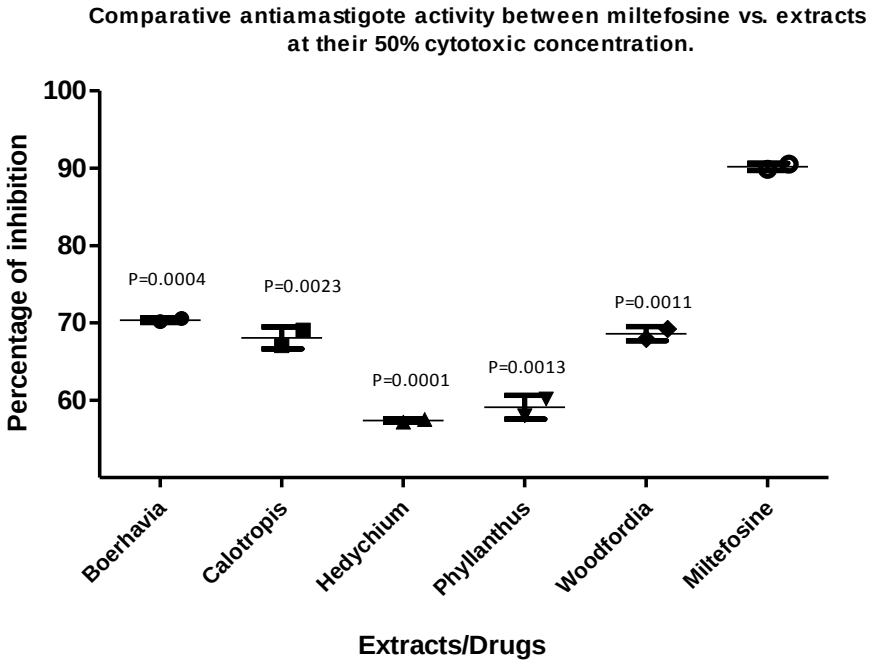


Fig. 4.8b: Comparative antiamastigote activity between miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration

P-value mentioned above the triplicate experiments of each component explains significance between test components with miltefosine.

4.11 Antibacterial Screening

No zone of inhibition was observed with all the extracts against two strains of bacteria used- *K. pneumoniae* and *S. typhimurium.* While the extracts of *Calotropis* and *Boerhavia* had no effects on any of the strains, the other extracts inhibited some strains minimally. Only the extract of *W. fruticosa* showed an inhibition zone of 7 mm diameter for *E. coli*. For other two strains *Ps. aeruginosa* and *S. aureus,* a zone of inhibition of about 7-10 mm diameter was observed with *Hedychium, Phyllanthus* and *Woodfordia* extracts as well as the reference drug Miltefosine (Fig.4.9a and Fig.4.9b). In contrast, the standard antibiotic Gentamycin (10mcg) showed larger zones of inhibition against all the strains (Table 4.8). Thus, it can be inferred that the extracts did not exhibit significant antibacterial activity as compared to the standard antibiotic used. Moreover, the highest in test concentration of the extracts used for antibacterial screening was 500 µg. This value is very higher compared to their respective IC_{50} values. Thus the antibacterial screening indicates the absence of any relevant inhibitory activity of the extracts towards the standard bacterial strains as compared to the parasites.

Table 4.8: Zones of inhibition (diameter) as produced by the extracts and drugs against standard strains during antibacterial screening

Extracts/Drugs	Zone of inhibition (mm)				
	E. coli (ATCC 25922)	*K. pneumoniae* (ATCC 700603)	*S. typhimurium* (ATCC 14028)	*Ps. aeruginosa* (ATCC 27853)	*S. aureus* (ATCC 25923)
Boerhavia	-	-	-	-	-
Calotropis	-	-	-	-	-
Hedychium	-	-	-	8	7
Phyllanthus	-	-	-	8	8
Woodfordia	7	-	-	10	8
Miltefosine	-	-	-	8	9
Gentamycin (10mcg)	20	16	20	30	26
DMSO	-	-	-	-	-

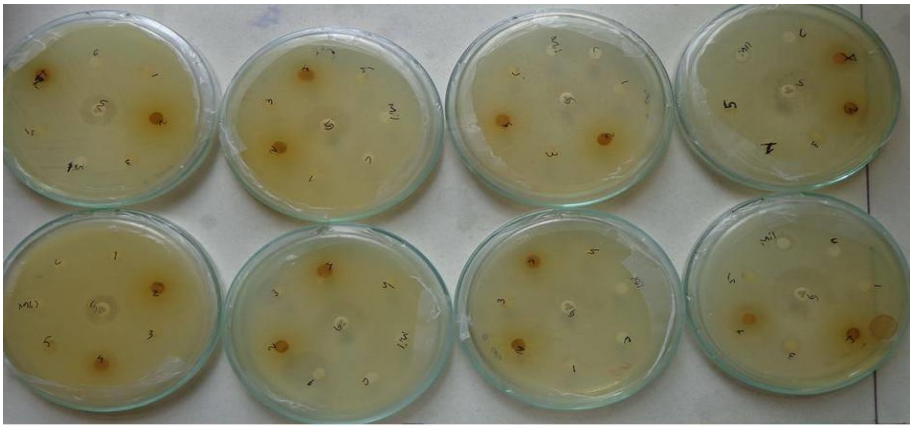


Fig. 4.9a: Antibacterial screening of plant extracts

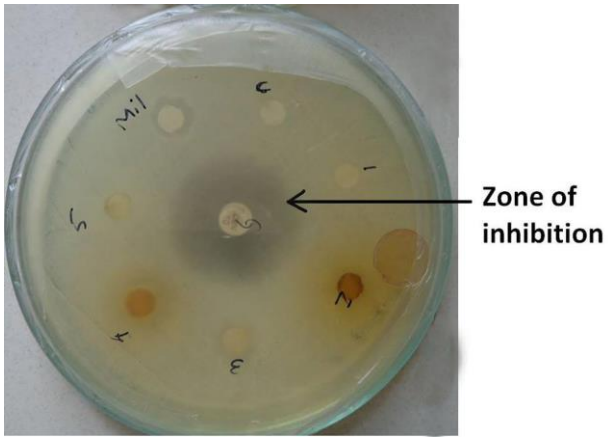


Fig. 4.9b: A test plate showing zone of inhibition

Chapter V

Discussion

From ancient times in the world, plant products are clinically used for curing various ailments. Nepal being a home to numerous valuable medicinal plants is no different in this regard. But unfortunately, this treasure has not been fully explored for fight against leishmaniasis. Leishmaniasis, the second parasitic killer after malaria, afflicts many lives in the southern region in Nepal. And with new cases evolving in the non endemic regions, this disease threatens even more lives in the country. With several limitations associated with chemotherapy, herbal medicine and natural product research is currently a hot topic of interest all over the world, in the field of drug discovery against leishmaniasis (Rocha et al., 2005; Tiuman et al., 2011). This research work is an attempt to study some of the medicinal plants of Nepal for their anti leishmanial potential. In this work, a total of five different medicinally important plants were selected and studied for their anti leishmanial properties. It is important to point out that all five investigated plant species have never been evaluated before for antileishmanial potential. The results obtained show that different plant extracts displayed different levels of activity.

The basic parameters influencing the quality of an extract and the experiment are the plant parts used as starting material, the solvent used for extraction and the extraction technology (Ncube et al., 2008). In this study, the extraction process was completed by means of soxhlation. During soxhlet extraction, the sample is continually exposed to fresh solvent, which improves the efficiency of the method eluting out maximum of the phytochemicals (Ncube et al., 2008). The crude ethanolic extract obtained were variously coloured indicative of the presence of coloured phytochemicals such as Quinones (Cowan, 1999). Besides the extraction method, time and temperature of extraction; the solvent concentration and polarity also affects the quantity and secondary metabolite composition of an extract. Methanol and ethanol are the two most commonly used solvents that are capable of extracting nearly all the active constituents in plant material. However owing to the higher cytotoxic nature of methanol, the bioassays could lead to erroneous results if traces of the solvent remains in the extract (Tiwari et al., 2011). Hence, ethanol was chosen over methanol and ethanolic extract of the plants were used for the study.

The ethanolic extract of all the plants in this study exhibited a dose-dependent anti leishmanial activity against both the promastigote and axenic amastigote forms of *L. donovani*. Of the five plants used, *Woodfordia fruticosa* exhibited the highest inhibitory

effect against promastigotes (IC_{50} = 35.30 ± 2.43 µg/mL) and axenic amastigotes (IC_{50} = 27.25 ± 1.88 µg/mL). Other three plants namely *Phyllanthus niruri*, *Hedychium spicatum* and *Calotropis procera* also showed appreciable inhibitory activity with IC_{50} values below 100 µg/mL. *P. niruri* had IC_{50} values of 67.46 ± 3.03 µg/mL and 40.23 ± 2.23 µg/mL against the promastigotes and axenic amastigotes respectively. For *H. spicatum,* IC_{50} for promastigote was 73.63 ± 4.34 µg/mL and for amastigote was 46.96 ± 2.11 µg/mL. Similarly, IC_{50} values of *C. procera* were 96.32 ± 8.79 µg/mL against promastigotes and 57.96 ± 0.13 µg/mL against axenic amastigotes. A total inhibition (100%) of the promastigotes was obtained over 96 hours with *W. fruticosa, P. niruri* and *C. procera* extracts indicating their leishmanicidal action. *Boerhavia diffusa* showed the minimum efficacy amongst all with IC_{50} values greater than 100 µg/mL. But there were no significant differences in the activities of all the extracts at a concentration of 1mg/mL as indicated by the p-value (0.5061). A constant observation was that the extracts were more effective against the axenic amastigote form of the parasite with lower IC_{50} values than those required for the promastigotes. This result is encouraging as the amastigotes are the clinically relevant stage of *Leishmania*. Similar results was observed by Singh et al., whereby the IC_{50} values for amastigotes was lower than for promastigotes for the ethanolic extract of *Tinospora sinensis Linn* (Singh et al., 2008). The same pattern was observed by Lakshmi et al. with ethanolic extracts of *Dysoxylum binectariferum* against *L. donovani* promastigotes and amastigotes (Lakshmi et al., 2007).

The reference drugs- Miltefosine and Amphotericin B had much lower 50 % inhibitory concentrations than the extracts. The comparatively higher IC_{50} values of the extracts might be due to the lower quantities of active compounds in them requiring further fractionation and concentration. But a similar trend of higher inhibitory action against the amastigotes was present between the reference drugs and the extracts. This fact is suggestive of some similar mechanism of action between the reference drugs and the extracts under study.

Bioactive phytocompounds such as quinones, alkaloids, quinolines, terpenes, saponins, phenolic derivatives, flavonoids and other metabolites present in the crude extracts and essential oils of medicinal plants are found to be responsible for leishmanicidal action (Chan-Bacab and Pena-Rodriguez, 2001; Fournet and Munoz, 2002; Alviano et al., 2012). Phytochemical studies of these selected plants previously have reported the presence of many of such compounds. Phenolics, tannis and flavonoids have been named the main components of *W. fruticosa* flowers (Chauhan et al., 1979; Das et al., 2007). Similarly, a variety of tannins, lignans, terpenoids, flavonoids etc has been isolated from *P. niruri* (Shakil et al., 2008; Thakur et al., 2011). The rhizome of *H. spicatum* consists of alkaloids, flavonoids, terpenoids, and essential oils among many (Ghildiyal et al., 2012). Different types of saponins and flavonoids are reported for *C. procera* that have molluscicidal and

antihelminthic action (Moustafa et al., 2010; Al-Sarar et al., 2012) while *B. diffusa* is reported to contain different alkaloids, flavonoids, terpenoids, glycosides etc (Chaudhary and Dantu, 2011; Apu et al., 2012). The appreciable anti leishmanial potential of these plants in our study could be attributed to the presence of such diverse group of secondary metabolites.

A huge amount of literature also reports the antimicrobial properties of these plants. Among the significant ones are the antifungal properties that have been reported. Antifungal drugs could be a successful treatment option for leishmaniasis given that currently used first line drug- Amphotericin B is also originally antifungal. To add to this trend are the azoles that are in a process of being developed as antileishmanial drugs. The ergosterol component common in both the fungi and the *Leishmania* parasites might explain for this selective action. Amongst the five plants selected for this study, antifungal property has been reported for *B. diffusa* (Agrawal et al., 2003), *H. spicatum* (Bisht et al., 2006) and *C. procera* (de Freitas et al., 2011) which explains the antileishmanial activity of these plants to some extent.

In view of the wide range of biological activities that have been described for these plants, an antimicrobial screening was performed in parallel that included a number of bacteria namely, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The results demonstrated that all the extracts are devoid of any relevant inhibitory activity. This suggests a highly specific pharmacological action of the extracts towards the parasite. This result also indicates a mode of action different than that of the regular antimicrobial compounds. Extracts exhibiting non-selective action in the panel of *in vitro* screens are supposed to give large false positives and hence have to be properly evaluated before asserting their anti infective potential (Maes et al., 2004; Cos et al., 2006). This high selectivity obtained in our results indicates the presence of probable leads for the drug discovery process against leishmaniasis.

Yet another important aspect in course of drug discovery is the cytotoxicity tests. Not necessarily the medicinal plants are safe always. Though the medicinal plants have been used traditionally, one cannot foresee and guarantee their safety. Hence, any drug or drug formulation has to go through the toxicity tests in order to render them safe and benefit from them. The preliminary safety of the extracts under this study was assessed by their toxicity against primary mice peritoneal macrophages via MTT assay. MTT is tetrazolium salt, yellow in colour, and are reduced only by metabolically active cells to a blue/purple colored formazan reflecting their mitochondrial activity. The principle of this assay is that for most viable cells, mitochondrial activity is constant and thereby an

increase or decrease in the number of viable cells is linearly related to mitochondrial activity (Meerloo et al., 2011).

B. diffusa exhibited the least cytotoxicity among all the extracts studied with CC_{50} value of 344.63 ± 5.45 µg/mL against the macrophages. A previous study on this plant has reported the inhibition percentage with regard to cytotoxicity to be 89 % at maximum concentration of 1000 µg/mL (Darsini et al., 2009). A similar result was obtained in this study with an inhibition of 86 % at the same concentration. *P. niruri* had a CC_{50} value of 98.90 ± 8.60 µg/mL in this study which is comparable with 119 µg/mL obtained in a study by Mustofa et al. (Mustofa et al., 2007). A slightly higher safety was reported for *W. fruticosa* flowers with CC_{50} value of >100 µg/mL against Vero cell lines (Choi et al., 2010) as compared to 90.38 ± 6.09 µg/mL in this study. Similarly, for *C. procera*, the CC_{50} value of 192.83 ± 15.82 µg/mL was obtained. However in a previous study, the CC_{50} value of ethanolic extract of this plant against the FL-cells, a human amniotic epithelial cell line, was 31 µg/mL (Ali et al., 2001). *C. procera* was seen to be comparatively safer in our study. These differences in the cytotoxicity in various studies could be due to the differences in cell lines used. Also the extraction procedure and the solvents used for the extraction processes determine the quantity and quality of the phytochemicals responsible for the cytotoxic effects. Besides, the phytochemical constituents in plants also vary with the altitude of their occurrence, species, time of harvest and stages of the plants during harvesting.

Moreover, selectivity indices were calculated as a measure of the relative safety of the extracts. The resulting selectivity indices of all the extracts were greater than 1 indicating their preferential selectivity towards the parasites. The selectivity was even better in case of the amastigotes. *W. fruticosa* showed the best activity against the parasites with selectivity indices of 2.56 and 3.31 against promastigotes and axenic amastigotes respectively. The selectivity index against amastigotes was highest (3.38) for *B. diffusa*. The cytotoxicity tests thus confirmed the safety of the extracts.

The reference drugs used showed very high levels of toxicities at lower doses as compared to the extracts. CC_{50} value of Miltefosine was 49.69 ± 1.42 µg/mL and that of Amphotericin B was the least with 14.00 ± 0.89 µg/mL. But their selectivity indices are even higher than that of the extracts exhibiting better activity than the extracts. Moreover, when the antileishmanial activity of miltefosine at its cytotoxic concentration was compared to the antileishmanial activity of the extracts at their respective cytotoxic concentrations, there were significant differences in their activities. However, the toxicity such as nephrotoxicity and neurotoxicity associated with prolonged use of the reference drugs cannot be neglected that limits their use (Sundar et al., 2012).

Though ethanol extracts most of the phytochemicals in any plant and many of them are attributed for their antileishmanial activity, the complete mechanism is difficult to interpret. Not a single phytochemical is solely responsible for this inhibition. There are high chances that these phytochemicals act synergistically. There could also be some antagonistic behaviour. Hence, a complete profiling of the phytochemicals extracted and their possible mechanism needs to be studied before giving away the final results.

One of the properties most commonly associated with the herbal medicines is their immunomodulatory properties. They are known to enhance the immunity of the hosts adding to the anti infective potential of the drugs. In case of a disease like leishmaniasis where the parasites establish an infection modulating the macrophage functions and which disturbs the host immune response hugely, an immunomodulatory drug could be used rationally for the treatment. An immunomodulator used either as an adjunct or in combination with other drugs is thus a novel approach for the treatment of leishmaniasis (Bhattacharya and Ali, 2013). The plants under this study have been reported to enhance the immune responses in the host encouraging their use as potent antileishmanials. Similarly, reports of the plants being antihelminthic, larvicidal, hepatoprotective etc speak of their wide therapeutic use. The findings of this study support the antileishmanial potential of these selected plants. Further studies on immunomodulation and *in vivo* tests are required to validate these findings even more. This research work also attempts to document the importance of such medicinal plants and natural products and urges for their conservation.

Chapter VI

Summary

Kala-azar is one of the neglected tropical afflictions of the poor caused by protozoan parasite, *Leishmania.* It is a fatal disease if not treated in time. However, unfortunately, the treatment options are limited to the highly toxic regimens of chemotherapy. It is even more worrisome as the current drugs are rapidly gaining resistance. The statistical figures show that 6 million people residing in 13 districts of eastern and central regions in southern plains of Nepal are at risk of this global health problem.

The search for novel drugs has led the researchers all over the world to team up with nature and find their answers in phytotherapy. Medicinal plants have been used in this world for various ailments since ages. These traditionally used plants are considered to be very effective and safe overall. Nepal is no different in this regard but only a few of this enormous treasure has been studied and utilised and even fewer has been screened against this parasitic threat- kala-azar.

A literature search for medicinal plants native to Nepal was carried out based on their traditional uses in ailments similar to kala-azar or any parasitic or protozoal or helminthic diseases. Five such plants were selected that have never been documented for their antileishmanial properties namely, *Boerhavia diffusa*, *Calotropis procera*, *Phyllanthus niruri*, *Hedychium spicatum* and *Woodfordia fruticosa*. Air dried and finely powdered plant parts were subjected to soxhlation with ethanol for 36 hours. The ethanolic extracts of all these plants were then assessed *in vitro* for their antileishmanial potential against both the stages of *Leishmania donovani.*

Promastigotes were cultured in cRPMI media (pH 7.2) at 26°C which were later allowed to convert into amastigotes in an acidic cRPMI media (pH 5.5) at 37°C. The conversion was confirmed with the morphological changes like oval/rounded structure and loss of flagella under the microscope. All the extracts were found to inhibit the parasites effectively and selectively. The extract of *W. fruticosa* flowers was found to be most active with IC_{50} value 35.30 ± 2.43 µg/mL against the promastigotes and 27.25 ± 1.88 µg/mL against the axenic amastigotes. *P. niruri* extract also had appreciable inhibitory activity against promastigotes (67.46 ± 3.03 µg/mL) and axenic amastigotes (40.23 ± 2.23 µg/mL). For *H. spicatum,* IC_{50} for promastigote was 73.63 ± 4.34 µg/mL and for amastigote was 46.96 ± 2.11 µg/mL. Similarly, IC_{50} values of *C. procera* were calculated to be 96.32 ± 8.79 µg/mL against promastigotes and 57.96 ± 0.13 µg/mL against axenic

amastigotes. *B. diffusa* had the highest values of IC_{50} (294.7 ± 18.09 µg/mL for promastgote and 101.75 ± 0.73 µg/mL for amastigote).

The diferences in IC_{50} values signify the differences in doses required for the extracts to be effective against the parasites. As per the values, *W. fruticosa* was the most effective at lower doses. An ANOVA test carried out to see if there are any significant differences in the inhibition of parasites by different extracts at the concentration of 1 mg/mL in 48 hours resulted in no significant differences between them. This suggested that all the extracts were equally effective at higher concentrations (1mg/mL). Similarly the efficacies of the treatments against the two forms of the parasites were also compared in terms of significant differences. The results showed that the extracts were more effective against the amastigotes forms than the promastigotes forms. This good antileishmanial activity of the plants against the clinically relevant (amastigote) stage is encouraging.

Cytotoxicity tests are mandatory in course of new drug formulation as they enable to remove potentially toxic compounds early in the drug discovery process. The efficacy of the extracts can only be considered significant when they are safe to use and do not pose any threat to the host cells. All the extracts were tested against the macrophages and cytotoxicity assessed via MTT assay. The results were exciting and encouraging with selectivity indices greater than 1. This high selective index indicates their safety towards macrophages. Similarly an antibacterial screening was conducted in parallel to determine the selectivity of the extracts. The results showed a selective action towards the parasites. From both the antibacterial and cytotoxicity tests, it can be suggested that the leishmanicidal efficacy of the extracts was not due to *in vitro* cytotoxicity.

This work demonstrates the antileishmanial activity of the selected plants for the first time. Further studies on the active components in the extracts and their isolation followed by immunomodulatory studies and their efficacy in the animal models should also be looked for, that might ultimately lead to the discovery of a novel drug.

Chapter VII

Conclusion

Plants are a source of diverse group of phytocompounds. The potentialities of these phytocompounds have been studied time and again and provided modern medicine with very effective drugs. Nepal has been a land of diverse forms of life. More than 900 valuable medicinal plants among 7000 all over the world are present here (Manandhar, 2000). However, these immensely precious natural resources has largely been unexplored for their medicinal potential against leishmaniasis. Neglected diseases such as leishmaniasis have been affecting many lives but receiving less attention. With increasing failures and severe drawbacks of current medications, there is an urgent need to develop effective, safe, non toxic and cheaper drugs.

This present study illustrates the antileishmanial activity of five selected medicinal plants native to Nepal. Based on the antileishmanial and cytotoxicity assays, these plants were found to be impressively active against *Leishmania donovani, Woodfordia fruticosa* being the best of all. Further investigations are required to isolate and identify the active compounds in the extracts. Similarly, the immunomodulating activity and effects on host macrophagic systems are to be studied so as to understand the proper mechanism of action followed by the *in vivo* tests.

What has been done is only a small effort in recognition of the valuable plants for their antileishmanial activity. Many more of such plants need to be recognised, screened, assessed and documented for their medicinal values and therapeutic use. Researches regarding their phytochemistry and pharmacology could lead to an effective drug originating from the plants. Such novel drugs someday could put an end to this global threat called Kala-azar.

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Appendices

RPMI (Roswell Park Memorial Institute) complete medium

RPMI powder : 10.40 gm

$NaHCO_3$: 2.00 gm

HEPES : 1.40 gm

L-Glutamine : 2 mM

DDW : 1 L

Gentamycin : 20 µg/ml

Streptomycin : 100 µg/ml

Penicillin : 100 U/ml

pH : 7.2

PBS (Phosphate Buffer Saline)

NaCl : 8 gm

$Na_2HPO_4.2H2O$: 1.44 gm

KCl : 0.2 gm

KH_2PO_4 : 0.2 gm

pH : 7.3 to 7.4

DDW : 1 L

Preparation of Giemsa stain

Giemsa stock solution:

- giemsa powder : 1 gm
- glycerol : 60 ml
- methanol : 66 ml

Giemsa buffer:

- Na_2HPO_4 : 9.5 gm/L
- KH_2PO_4 : 9.07 gm/L

Giemsa stain: 10% giemsa stock in giemsa buffer

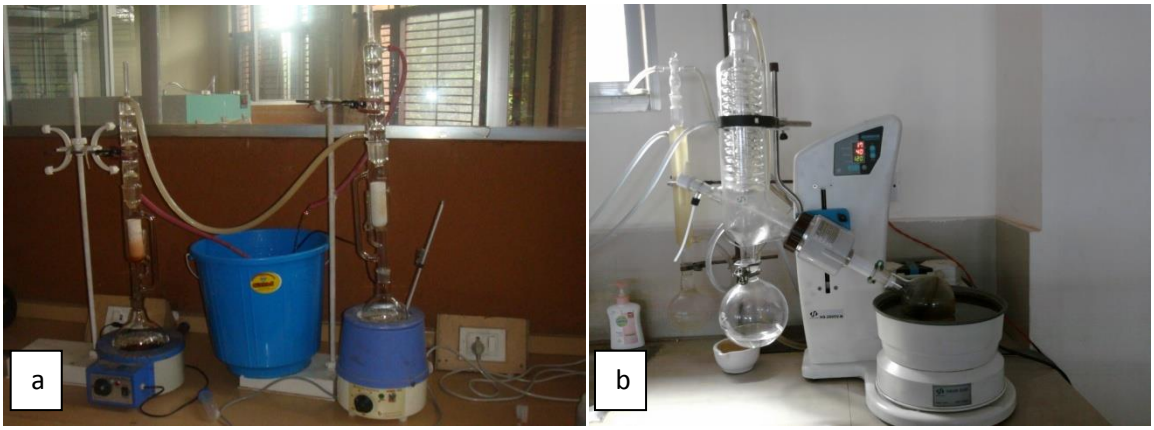

Plate 1. (a) Soxhlation with ethanol, (b) *in vacuo* concentration using Rotatory evaporator

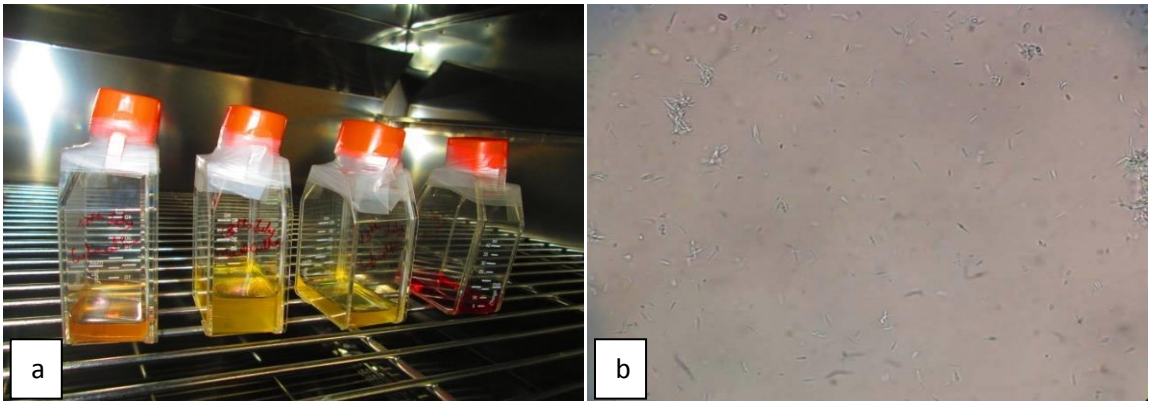

Plate 2. (a) *L. donovani* culture, (b) *L. donovani* parasites under an inverted microscope (40X)

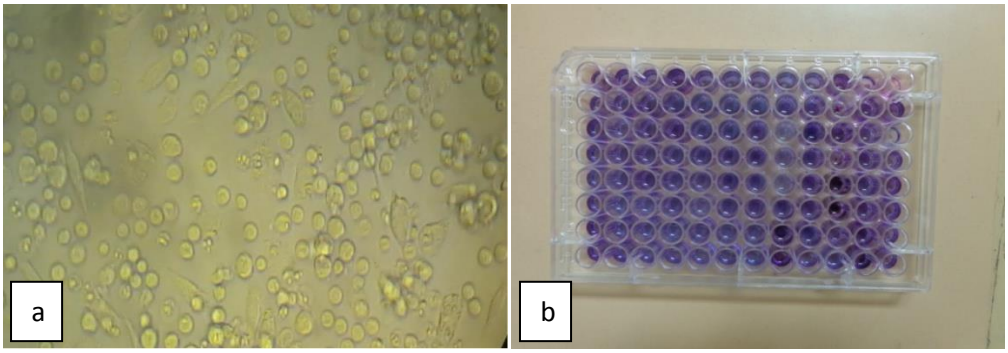

Plate 3. (a) Primary mice peritoneal macrophages, (b) MTT assay

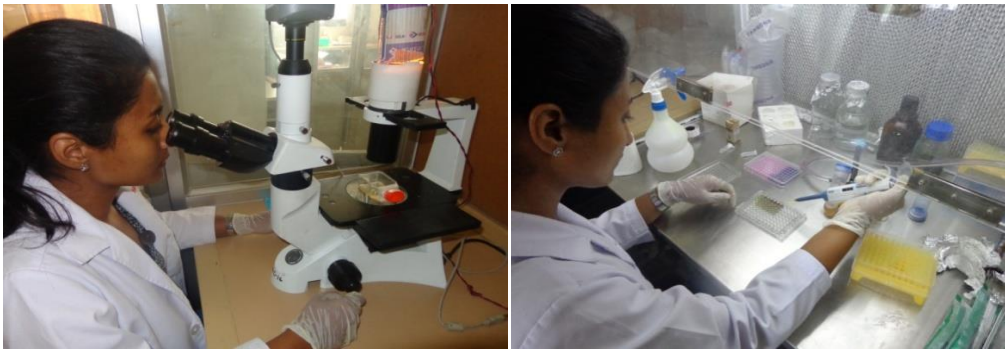

Plate 4. Working in the laboratory