IN VITRO ANTILEISHMANIAL ACTIVITY OF *BOMBAX CEIBA* LINN. FLOWERS

M. Sc. Thesis
(2011)

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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I believe he would prove his worth for the organization where he would be selected. To best of my knowledge he bears a good moral character. I wish him all success in his future endeavors.

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Dedicated

to

our beloved Parents

Narayandevi *and* **Krishna**, *who encouraged us*

and

our respected

TEACHERS, *who enabled us*

Acknowledgement

Most respectfully, I would like to acknowledge and extend my heartfelt gratitude to my Supervisors **Dr. Krishna Das Manandhar** and **Dr. Rakesh Kumar Singh**, who not only nurtured me under their expertise and excellence but also give me an opportunity to get known to and be a part of such an esteemed University and Department, respected Faculty, Research Team and Fellows, the research ambience and a place so divinely pleasing as heaven of Gods - Varanasi. I have no words to describe my pleasure to get such love, care and appreciation during the entire stay. Dear Sir, this work would not have been possible without you and I will always remain indebted to you.

I am much thankful to respected **Dr. Vinod Kumar Tiwari**, Dept. of Chemistry, Centre of Advanced Study, BHU, for allowing me to work in his revered carbohydrate chemistry research laboratory. Thank you very much Sir, your encouraging and inspiring words will always be remembered. I am grateful to **Dr. Bhuwan Bhushan Mishra**, Dept. of Chemistry, Centre of Advanced Study, BHU, and **Mr. Jalaj Kumar Gour**, Research Scholar, Molecular Immunology Laboratory, Dept. of Biochemistry, BHU, who have guided me on every aspect of this research work. Thanking you is not just sufficient to express my gratitude for your endless efforts, guidance and support. I would like to take this opportunity to acknowledge **Dr. Manish Mishra** and **Dr. Hemant Katiyar,** Dept. of Medicine, BHU, and **Mrs. Surabhi Bajpai**, **Ms. Ankita Srivastava**, **Mr. Vinod Kumar**, **Mr. Jalaj Kumar Gour**, **Ms. Nisha Singh**, Research scholars, Molecular Immunology Laboratory, Dept. of Biochemistry, BHU, for their continued support, guidance, presence and appreciation. I would also like to be thankful to **Dr. Saroj Sir**, Dept. of Chemistry, Orisha, and **Mr. Virendra Prasad, Mrs. Pratibha Mishra, Mr. Navneet Kishore, Mr. Raju R. Kale, Ms. Divya Kushwaha, Mr. Dananjay Kumar**, Research Scholars, Dept. of Chemistry, Centre of Advanced Study, BHU, who were always there to guide me. I am thankful to **Mr. Dananjay Kumar**, for his guidance, presence and support at Dept. of Chemistry, Centre of Advanced Study, BHU.

I would also like to sincerely appreciate and remember kind help and support of **Dr. Kamlesh Gidewani** and **Dr. Vijay Prajapati**, Dept. of Medicine, BHU; thank you very much sir. I am also thankful to **Dr. Ananta Panthi**, Dept. of Geology, BHU, **Dr. Pawan Raj Shakya,** and **Dr. Pramod Kumar Yadav**, Dept. of Chemistry, BHU, for continued support and guidance during our stay. I would also like to be thankful to **Dr. Rameshwor Adhikari**, Central Dept. Of Chmistry, TU, Kirtipur and **Dr. Surendra Kumar Gautam**, Dept. of Chemistry, Trichandra Campus, TU, for valuable suggestions on the work and NMR studies. Dai', **Niranjan Bhandari**, Research scholar, Dept. of Music, BHU, many many thanks for making our visit and stay wonderful and making us known to places at Varanasi; you have been a guardian and I have no words to describe my appreciation to your love and care.

I will always remain obliged to **my home institution/university - Central Department of Biotechonology, Tribhuvan University** and would like to be express my sincere gratitue to honourable Head of Department, **Dr. Tribikram Bhattrai**, honourable **Dr. Lakshmaiah Sreerama**, visiting professor, Dept. of Chemistry, St. Cloud Univerity, USA and respected faculty members, **Dr. Mohan Kharel, Dr. Ganga Kharel, Dr. Sampoornananda Jha, Dr. Rajani Malla, Dr. Krishna Das Manandhar, Dr. Giri Raj Tripathi, Dr. Deepak Raj Pant, Late. Mr. Janardan Pandey, Mr. Bal Hari Poudel, Mrs. Jarina Joshi** and **Mrs. Smeeta Shrestha**.

Also **CHEERS! to our Kala-azar Research Team** at our department. I am thankful to all my friends, collegues, all members of my department and my family members, thank you for being there always; actually you all have made this possible.

Once again, I would like to be thankful to my supervisors, **Dr. KD Manandhar** and **Dr. RK Singh**, I will always remain obliged to you sir, Thank you. Finally thank you God for making this happen, **may God bless all of us. Thank you**!

Om Basukala

Glossary Acronyms

ABC	ATP-binding cassette
ABCD	AmB colloidal dispersion
ABL	AmB lipid complex
ACL	Anthroponotic cutaneous leishmaniasis
AIDS	Acquired immune deficiency syndrome
AmB	Amphotericin B
AMPs	Antimicrobial peptides
APC	Antigen presenting cell
AQ	aqueous
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
AVL	*Aloe vera* leaf exudate
BMA	Bone marrow aspiration
CC_{50}	Cytotoxic Concentration of drug for killing 50% cells
CD	Cluster of differentiation
CEE	Crude ethanolic extract
CI	Confidence interval
CIC	Circulation immune complex
CL	Cutaneous leishmaniasis
CMI	Cell-mediated immune response
CP	cysteine proteases
cRPMI	Complete RPMI
CS	conjunctival swab
CSA	crude soluble antigen
DAT	Direct agglutination test
DC	Dendritic cells
DCL	Diffused cutaneous leishmaniasis
DDT	Dichloro-dephenyl-trichlorethane

DHFR-TS	dihydrofolate reductase thymidylate synthase
DMEM	Dulbecco minimum essential medium
DMSO	Diemethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EDCD	Epidemiology and Disease Control Division
ELISA	Enzyme linked immunosorbent assay
FACS	Florescence activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FD	Freeze-dried
FDR	Fluorescent detection reagent
FML	fructose mannose ligand
gp	glycoprotein
GPl	glycoprotein leishmaniolysin
HAART	Highly active antiretroviral therapy
HASPB1	Hydrophilic acylated surface protein
HIV	Human immunodeficiency virus
IC	Inhibitory concentration
IC_{50}	Concentration inhibition 50% of parasites
IC_{90}	Concentration inhibition 90% of parasites
ICT	immunocromatograchic test
IFAT	indirect fluorescent antibody test
IFN-ϒ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
iNOS	inducible Nitric oxide synthase
ITN	insecticide-impregnated bed nets
ITS1	internal transcribed spacer 1
KAtex	Latex agglutination test

KMP-11	Kinetoplastid membrane protein-11
LACK	*Leishmania* homologue for receptors of activated C kinase
L-AmB	liposomal Amphotericin B
LCA	Lens culinaris agglutinins
LD body	Leishman-Donovan body
LdMT	*Leishmania donovani* miltefosine trasporter
LeIF	*L. braziliensis* elongation and initiation factor
LmsTI1	*L. major* stress-inducible protein-1
LPG	Lipophosphoglycan
LST	Leishmanin skin test
MCL	Mucocutaneous leishmaniasis
MHC	Major Histocompatibility Class
MRP1	multidrug resistance-associated protein 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nano-AB	Nano-amphotericin B
NK	Natural killer
PCR	polymerase chain reaction
PG	Phosphoglycan
P-gp	Permeability glycoprotein
PKDL	Post Kala-azar Dermal Leishmaniasis
PMM	phosphomannomutase
PMN	Polymorphonuclear neutrophils
PMNC	Polymorphonuclear neutrophil cells
PNA	peanut agglutinins
PPG	Proteophosphoglycan
PRP-2	paraflagellar rod protein 2
PSA-2	Parasite surface antigen
rGBP	recombinant gene B protein
rK39 ICT	Recombinant Kinesin 39 amino acid immunochromatographic test
RNA	Ribose-nucleic acid

RPMI	Rosewell Park Memorial Institute
SAG	Sodium anitimony Gluconate
Sb^v	Pentavalent antimony
TDR	Tropical Disease Research, WHO
TGF	Transforming growth factor
Th1/Th2	T helper 1 / T helper 2
Treg	T regulatory cells
TSA	thiol-specific antioxidant
VL	Visceral leishmaniasis
WB	Western Blot
WHO	World Health Organization

CONTENTS

Acknowledgements i

Glossary Acronyms ii

Abstract 1

CHAPTER I: INTRODUCTION 2-22

1.1 Background 2
1.2 A brief history 3
1.3 Taxonomic position 5
1.4 Morphology of the parasite 5
1.4.1 Promastigote form 5
1.4.2 Amastigote form 6
1.4.3 Transformation of forms 7
1.4.4 Lifecycle of *Leishmania* 7
1.5 Clinical menifestions and Geographical distribution 9
1.5.1 Visceral leishmnaiasis 9
1.5.2 Cutaneous leishmaniasis 11
1.5.3 Mucocutaneous leishmaniasis 12
1.6 Vector of leishmnaiasis 13
1.7 Parasite reservoir 14
1.8 Transmission 15
1.9 Immunology and vaccine prospect 15
1.10 Diagnostic options 16
1.11 Treatment options 17
1.12 Prevention and control 18
1.13 Research Plan and Design 19
1.13.1 Research hypothesis 19
1.13.2 Research objectives 20
1.13.3 Research plan 21
1.13.4 Rationale 22

CHAPTER II: LITERATURE REVIEW 23-52

2.1 Chemotherapy in Leishmaniasis 23
2.1.1 Pentavalent Antimonials (Sb^{v}) 24
2.1.2 Amphotericin B (Polyene antibiotics) 25
2.1.3 Pentamidine (Aromatic diamidines) 29
2.1.4 Paramomycin (Aminoside) 29
2.1.5 Miltefosine (Alkyllysophospholipid) 30

2.1.6 Sitamaquine (Primaquine analogue) 32
2.1.7 Other agents 32
2.1.8 Cytokine Therapy/Immunomodulators 33
2.2 Vaccines 33
2.2.1 Killed parasite vaccines 34
2.2.2 Subunit vaccines 34
2.2.3 Polyprotein vaccines 35
2.2.4 Vaccines of *Leishmania* fractions 35
2.2.5 Live-attenuated vaccines 35
2.3 Natural products and Phytotherapy 36
2.3.1 Antileishmanial screening of Nepalese medicinal plants 42
2.3.2 Bombacaceae family and *Bombax ceiba* 45
2.3.3 Phytochemistry of *Bombax ceiba* 47
2.4 Assessment of antileishmanial activity 49
2.4.1 Parasite strain 49
2.4.2 In vitro systems 49
2.4.2.1 Using promastigotes 49
2.4.2.2 Using amastigotes 50
2.5 Assessment of Cytotoxicity 51
2.5.1 RAW 264.7 cell line 52

CHAPTER III: MATERIALS AND METHODS 53-61

3.1 Selection of Medicinal plant (*B. ceiba*) 53
3.2 Plant Material 53
3.3 Phytochemical extraction and fractionation 53
3.4 Parasite culture 55
3.4.1 Determination of parasite count and viability 56
3.5 RAW 264.7 macrophage cell culture 56
3.6 Reconstitution of antileishmanial reference drug, *Bombax ceiba* extract and fractions 57
3.7 *In vitro* antileishmanial assay on *L. donvani* promastigotes and axenic amastigotes 58
3.8 Determination of percentage inhibition, IC_{50}, IC_{90} and absolute inhibition estimation 59
3.9 Assessment of cytotoxicity 59
3.10 Determination of survival index 60
3.11 Thin layer chromatography (TLC) of the crude extract 61
3.11.1 Preparation of TLC plates, sample preparation and application 61
3.11.2 Development of the chromatogram and calculation of R_f value 61
3.12 Statistical Analysis 61

CHAPTER IV: RESULTS **62-78**

4.1 Phytochemical extraction and fractionation 62
4.2 *In vitro* anntileishmanial assay on *L. donovani* promastigotes and amastigotes 63
4.2.1 Determination of required drug concentration for IC_{50} 63
4.2.2 Determination of required drug concentration for IC90 and absolute inhibition 65
4.2.3 Dose response curves: Percentage of inhibition (PI) versus (vs.) Concentration of extract, fractions, control and drug on promastigotes 66
4.2.4 Dose response curves: PI vs. concentration of extract, fractions, control and drug on amastigotes 67
4.2.5 Time dependent efficacy response of fractions over 96 hours 68
4.3 Cytotoxicity of miltefosine, crude extract and fractions 73
4.4 Efficacy comparison on the basis of selectivity indices 74
4.5 Thin layer chromatography and R_f value 74
4.6 Statistical comparison on antileishmanial effects of CEE, fractions and reference drug 76

CHAPTER V: DISCUSSION **79-85**

CHAPTER VI: SUMMARY **86-87**

CHAPTER VII: CONCLUSION **88-89**

References 90-107

Appendices 108-110

List of Tables

Table 1.1: Geographical distributions of Leishmaniasis worldwide and their pathogenic species, vector and reservoir.

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

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

Table 2.3: Some Nepalese medicinal plants with theirs scientific name, distribution and uses.

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

Table 4.1: Physical characterization (Color and consistency) and yield percentage of the crude ethanolic extract and its fractions.

Table 4.2: *In vitro* antileishmanial activity (IC_{50}) *Bombax ceiba* Linn. flowers ethanolic extract and its fractions against *L. donovani* promastigotes and axenic amastigotes.

Table 4.3: Concentration of *Bombax ceiba* fractions required for IC_{90} and absolute inhibition values in 96 hours.

Table 4.4 a: Percentage growth inhibition of *L. donovani* promastigotes by various concentrations of *Bombax ceiba* fractions over 96 hours.

Table 4.4b: Percentage growth inhibition of *L. donovani* amastigotes by various concentrations of *Bombax ceiba* fractions over 96 hours.

Table 4.5: *In vitro* antileishmanial activity (IC_{50}) and cytotoxicity (CC_{50}) of *Bombax ceiba* Linn. flowers CEE and its fractions against *L. donovani* promastigotes and axenic amastigotes.

Table 4.6: Retardation factor (R_f) values of spots visualized on the TLC plates 1-4.

Table 4.7: Comparative statistical analysis on antileishmanial activity of miltefosine vs. *Bombax ceiba* components at their 50% cytotoxic concentration.

List of figures

Fig. 1.1: District map showing geographical distribution of VL in Nepal, April 1999 to March 2009.

Fig. 1.2: Evolution of VL reported yearly incidences in Nepal from 1980 to 2009.

Fig. 1.3: Giemsa stained promastigotes (a) and intracellualar amastigotes (b).

Fig. 1.4: Development of *Leishmania* species in the sandfly vector.

Fig. 1.5: Diagrammatic representation of transmission and life cycle of *Leishmania* parasite

Fig. 1.6: Life cycle of *Leishmania donovani.*

Fig. 1.7: Clinical manifestations and spectrum of Leishmaniasis.

Fig. 1.8 a-d: Geographical distribution of visceral leishmaniasis (VL) worldwide.

Fig. 1.9: Vectors of Leishmaniasis: (a). *Phelebotomus argentipes* in Old world and (b). *Lutzomyia longipalpis* in New world.

Fig. 1.10 Flow chart: Preparation of plant extract and fractionation and Evaluation of the antileishmanial activity of the fractions extracts and accessing their cytotoxicity.

Fig. 2.1: Chemical structure of sodium sitbogluconate.

Fig. 2.2: Chemical structure of Amphotericin B.

Fig. 2.3: Chemical structure of pentamidine.

Fig. 2.4: Chemical structure of Paramomycin.

Fig. 2.5: Chemical structure of Miltefosine.

Fig. 2.6: Chemical structure of Sitamaquine.

Fig. 2.7: *Bombax ceiba* tree (left) dried flower, fresh flower and leaves (right from top).

Fig. 2.8: Chemical structures of the flavonoids from *Bombax ceiba* flowers namely; vicenin 2 (1), linarin (2), sponarin (3), cosmetin (4), isovitexin (5), xanthomicrol (6) and apigenin (7).

Fig. 2.9: Principle of MTT assay.

Fig. 3.1: Flowchart for phyotochemical extraction and fractionation.

Fig. 4.1: Yield of *Bombax ceiba flower* fractions (%) and composition (%) of crude ethanolic extract.

Fig. 4.2 Comparative inhibition parameter exhibited by four fractions of the crude ethanolic extract of *Bombax ceiba*.

Fig. 4.3 a: Dose response curve showing percentage of inhibition of promastigotes at different concentrations starting from control to 1mg/mL of crude ethanolic extract, fractions and reference drug-miltefosine in 48 hrs.

Fig. 4.3 b: Dose response curve showing percentage of inhibition of amastigotes at different concentrations starting from control to 1mg/mL of crude ethanolic extract, fractions and reference drug – miltefosine in 48 hrs.

Fig 4.4 a-d: Effect of different fractions on promastigotes over 96 hours: (a). hexane fraction (b). chloroform fraction (c). acetone fraction and (d). methanol fraction

Fig. 4.5 a-d: Effect of different fractions on amastigotes over 96 hours: (a). hexane fraction (b). chloroform fraction (c). acetone fraction and (d). methanol fraction

Fig. 4.6: CC_{50} value of crude extract and fractions on RAW 264.7 macrophage cells after 72 hrs.

Fig. 4.7: Comparative selectivity indices of CEE, fraction and miltefosine on RAW 264.7 cells after 72 hours.

Fig. 4.8: Thin layer chromatography of crude ethanolic extract (CEE) for demonstration of presence of number of compounds. Solvent systems: (1). 100% ethyal acetate; (2). Hexane:Ethylacetate (2:8); (3). Methanol:Ethylacetate (2:8); (4). Methanol:chloroform (1:9).

Fig. 4.9 a: Comparative antipromastigote activity between miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration.

Fig. 4.9 b: Comparative antiamastigote activity between miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration.

Abstract

In vitro antileishmanial activity of *Bombax ceiba* Linn. flowers

Visceral leishmaniasis (VL) is one of the neglected tropical diseases, as many as 8 million people in the 13 endemic districts of Nepal. Incidence of VL is associated with poverty and is further pushing the poor to the poorest. Available treatment options are limited and unsatisfactory due to several limitations like parenteral administration, long course of treatment, toxic side effects and high treatment cost. Malnutrition and co-infection with diseases such as malaria, pneumonia, HIV and development of drug-resistance by parasites are further worsening the situation. In absence of a vaccine there is an urgent need of alternative treatments. One of the main sources for new antileishmanial agents are secondary metabolites isolated from plants. Nepal is rich in natural products biodiversity and habitats more than 900 types of valuable medicinal plants among 7000 medicinal plants found all over the world. However, these natural resource has largely been unexplored for its medicinal and therapeutic potential against leishmaniasis. Flowers of *Bombax ceiba* has been known for its several enthnomedicinal uses in various afflictions including splenomegaly, a hallmark of VL. To evaluate its potential use in VL an *in vitro* antileishmanial activity of the flowers of the plant was carried out. The crude ethanolic extract of the flower inhibited promastigote growth at IC_{50} of 131.24 ± 12.54 µg/mL. The methanol fraction showed a greater inhibitory effect against promastigotes (IC_{50} of 89.62 ± 0.55 µg/mL) and amastogotes (IC_{50} of 58.73 ± 1.89 µg/mL) than the other fractions. Fraction n-hexane, also had appreciable inhibitory activity against promastigotes (105.12 ± 7.99 µg/mL) and amastigotes (61.39 ± 1.34 µg/mL), indicating the active compounds might have been attributed to these fractions. The reference drug miltefosine had lower 50% inhibitory concentration values for both forms of the parasite (IC_{50} for promastigote: 11.27 ± 0.52 µg/mL and IC_{50} for amastiogote: 4.12 ± 0.13 µg/mL) than any of the fractions or the crude extract. From the dose response curves and time dependent efficacy response graphs it can be conferred that n-hexane and methanol fractions are leishmanicidal and acetone and chloroform fractions are leishmanistatic. Also, hexane fraction was effective at low dose against promastigotes (IC_{100} at 250 µg/mL) and so was methanol fraction against amastigotes (IC_{100} at 125 µg/mL). Cytotoxicity test revealed the components were safe, with selectivity indices values greater than 1. Moreover, methanol fraction of the crude flower extract was found similar in activity of miltefosine when compared to at its CC_{50} concentration (P=0.0638). Identification of the effective compounds from methanol and n-hexane fractions could lead to discovery of lead compounds effective against kala-azar. To the best of our knowledge this is the first report to demonstrate antileishmanial activity of *B. ceiba* Linn. flowers. This work also focuses on the need of screening of medicinal plants native to Nepal for anti-leishmanial activity.

Key words: Visceral leishmaniasis, *Leishmania donovani*, *Bombax ceiba*, Semal, IC_{50}, *In vitro*, antileishmanial acitivity.

Chapter I

INTRODUCTION

1.1 Background

Leishmaniasis is one of the neglected tropical diseases. It is a vector-borne disease, transmitted by the bite of infected female phlebotomine sandfly (*Phlebotamus* and *Lutzomyia*), inoculating *Leishmania* promastigotes. *Leishmania*, the etiological agent of the disease - is a dimorphic, obligate, intracellular protozoan parasite. The disease is known to be caused by about 21 species of *Leishmania* and transmitted by 30 species of sand fly (Herwaldt, 1999; Sharma and Singh, 2008). After infection, the *Leishmania* promastigotes make silent entry into macrophages where it transforms themselves into amastigotes and multiply within them in the mononuclear phagocyte system. Clinically, Leishamaniasis has been grouped as cutaneous leishmaniasis (CL), diffused cutaneous leishmaniasis (DCL), mucocutaneous leishmaniasis (MCL) and Visceral leishmaniasis (VL) with clinical interventions leading from serious disability and disfigurement to fatality, if left untreated (Herwaldt, 1999; Desjeux, 2004; Mishra et al., 2009).

As many as 12 million people are believed to be currently infected, with about 1–2 million estimated new cases occurring every year and threatening about 350 million people in 88 countries. There are 500,000 new cases of visceral leishmniasis (VL) and more than 75,000 deaths are estimated each year of which, over more than 90% of cases occur in just six countries: India, Bangladesh, Nepal, Sudan, Ethiopia and Brazil (Ashford et al., 1992; Desjeux, 2004; Chappuis et al., 2007). However, serious under reporting of the cases is hiding the actual severity of the disease in these endemic areas (Singh et al., 2006) and there has been progressive increase in incidence and is being reported from the newer areas as well (Desjeux, 2001; Joshi et al., 2006). On accounts of available control measures, the disease is one of the most neglected tropical diseases and indeed is not only affecting the poorest of the poor strata, but also preventing the economic development of the affected areas (Desjeux, 1996; Sharma et al., 2004). Further, migration, lack of control measures and HIV co-infection are raising the incidence of VL high making leishmaniasis still a major global health problem (Chappuis et al., 2007).

There is only scanty information available on the epidemiology of VL or *Leishmania* infection in Nepal. After being first recorded in 1980 from Dhanusha district (Bista, 1998; Rijal et al., 2010), there has been a steady increase in the reported cases (>1000 cases/year) with approximately 1341 cases reported annually (Pun et al., 2011). Presently 13 districts of eastern Terai in Nepal (Fig. 1.1) bordering North Bihar are endemic with an estimated 8

million people at risk (Rijal et al., 2010; Pun et al., 2011). However, recently VL has been increasingly reported form non-endemic regions as well (Pun et al., 2011). Since 1993, a national kala-azar control programme under the Epidemiology and Disease Control Division (EDCD) has been operational for early diagnosis, treatment and vector control strategies (Fig. 1.2). However, no any detectable impact of the control efforts has been seen between 1993 and 2005 (Rijal et al., 2010).

Substantiating the havoc disease burden in the endemic regions; Tropical Disease Research, Word Health Organization (TDR, WHO) has already initiated the programme for elimination of Kala-azar in the South-East Asia Region, agreed by the Ministers of Health of Bangladesh, India and Nepal in 2005. The target of this campaign is to reduce the incidence rate of VL below 1 in 10,000 per year by 2015 (Joshi et al., 2006; Bhattarai et al., 2010; WHO, 2011).

1.2 A brief History

The descriptions of Oriental sore, now-a-days commonly know as Old World cutaneous leishmaniasis dates back to 7th century BC. On the tablets in the library of King Ashurbanipal, this ancient disease has been described as conspicuous lesions; some of which are thought to have been described from even earlier texts from 1500 to 2500 BC. Arab physicians including Avicenna (10th century) have detailed the condition terming it as Balkh sore from northern Afghanistan (Manson-Bahr, 1986; Cox, 2002; Stanford, 2012).

Old World Visceral leishmaniasis or Kala azar was first noticed in Jessore (now in Bangladesh) in India in 1824, when patients suffering form fevers that were though to be due to malaria, failed to respond to quinine, which by 1862 spread to Burdwan in epidemic proportions (Elliott, 1863). The cause remained unknown and several clinicians including Sir, Ronald Ross were convinced of the disease being a virulent form of malaria (Ross, 1899), until *L. donovani* was discovered in 1900 by a Scottish army doctor, William Leishman, and the Professor of Physiology at Madras University, Charles Donovan independently in the spleens of patients with visceral leishmaniasis (Hoare, 1938; Cox, 2002; Stanford, 2012).

In Nepalese context VL was postulated to be endemic in southern terai of Nepal by an Indian scientist Raghavan in 1953. During 1960's and 1970's VL ceased mainly due to countrywide malaria eradication activities with DDT spraying but with improvement of malaria eradication program insecticide spraying was also reduced. Due to a decade cutting of the spraying activities, particularly in the southern terrain VL cases started reappearing and were first recorded in 1980 with the incidence rate of 1.5 per 100,000 population and case fatality rate of 5.88 percent (EDCD, 2010).

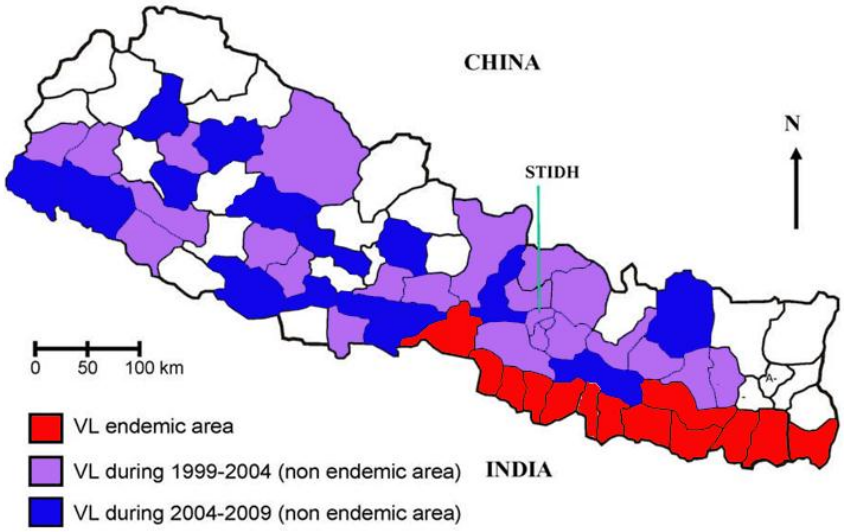

Fig 1.1: District map showing geographical distribution of VL in Nepal, April 1999 to March 2009. VL endemic area: 13 Districts of eastern Terai namely - Jhapa, Morang, Sunsari, Saptari, Siraha, Udayapur, Dhanusha, Mohattari, Sarlahi, Rautahat, Bara, Parsa and Chitwan. [Source: (Pun et al., 2011)]

STIDH: Sukraraj Tropical and Infectious Disease Hospital

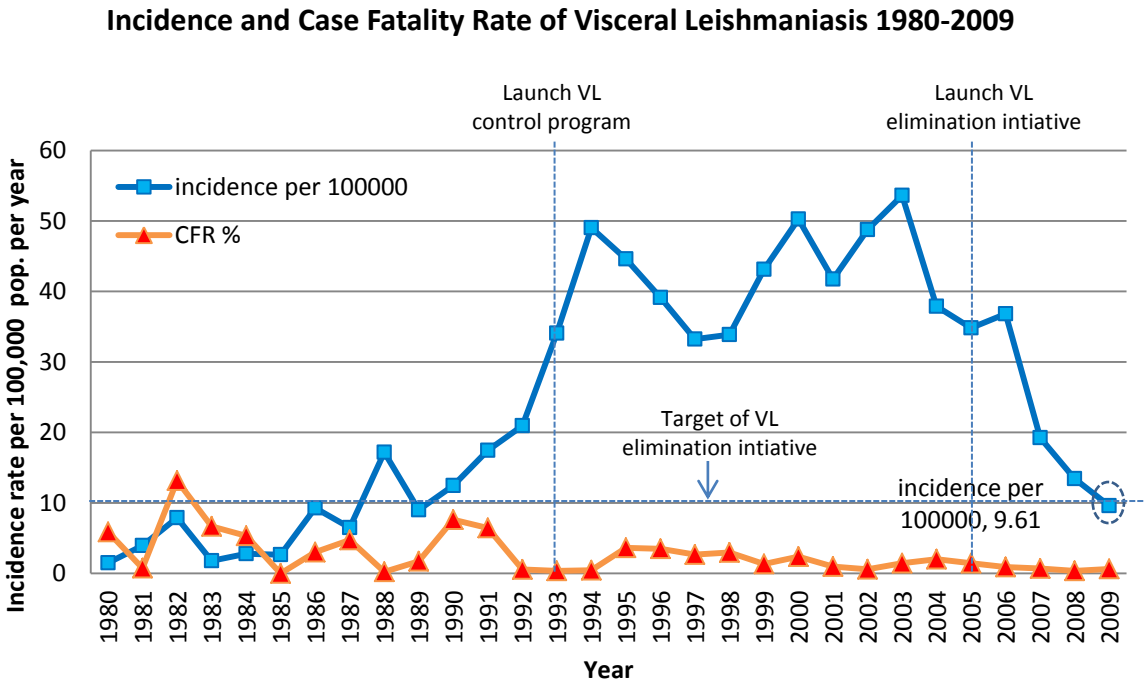

Fig. 1.2: Evolution of VL reported yearly incidences in Nepal from 1980 to 2009.

[Source: Annual Reports, Ministry of Health and Population, Nepal (EDCD, 2010)]

1.3 Taxonomic position

The *Leishmania* belong to the kingdom – Protista and subkingdom Protozoa. With monomorphic nuclei and presence of locomotory organ as flagella, it has been placed to phylum – Sarcomastigophora. Similarly, to subphylum – mastigophora for being flagellated protozoan and to class – zoomastigophora for absence of chlorophyll or chromatophores. *Leishmania* are one among the several genera of Trypanosomatidae family, characterized by the possession of a kinetoplast, which is a unique form of mitochondrial DNA (Sharma and Singh, 2008). The *Leishmania* are further grouped into 2 sub genera, the *Leishmania* and *Viannia* subspecies. The *Leishmania* subspecies undergo development in the anterior portion of the alimentary tract of the vectors, while the *Viannia* subspecies undergo development in the hindgut and midgut of the vectors (Mishra et al., 2009).

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)

1.4 Morphology of the parasite

The *Leishmania* exits in two different morphological stages; i.e. dimorphic parasite: the extracellular promastigote in the invertebrate host (sandfly) and intracellular amastigote in the vertebrate host (Mishra et al., 2009). In these two hosts, the parasite adapts in a varied and heterogeneous environments, eg. : (i) temperature – from 37^{o}C in mammalian host to ambient temperature in sandfly; (ii) pH – from neutral in sandfly stomach to highly acidic in the macrophage phagolysosome; (iii) nutrients and oxygen contents; and (iv) to immune attack – complements, antibodies and T-lymphocytes (Sharma and Singh, 2008). This rapid adaptation to the environment is attributed to the ability of *Leishmania* to modulate the gene expression, which probably occurs by the specific gene amplification or by having several tandem repeats (Singh et al., 2005; Sharma and Singh, 2008).

1.4.1 Promastigote form

Promastigotes are spindle-shaped and flagellated form inhabiting in the intermediate host measuring about 15-20 µm in length and 1.5-3.5 µm in breadth (Herwaldt, 1999). The

forms of promastigote, based on their location in the sandfly body, can be distinguished on morphological ground (viz. Procyclic, nectomonad, haptomonad, and metacyclic promastigotes). After being ingested, amastigotes transform to procyclic promastigotes in posterior midgut of sandfly within hours of ingestion (Bates, 1994; Manandhar, 2008). This is followed by transformations into ncetomonad (elongated, 15-20 µm), haptomonad (attached to stomodael valve), and metacyclic (highly motile, long flagella, 5-8 µm) promastigote form sequentially (Bates, 1994; Herwaldt, 1999; Manandhar, 2008).

In the promastigote form, the nucleus is situated at the center and kinetoplast traversely towards the anterior end. The single and delicate flagellum measures 15-28 µm in length and consists of paraxial rod which has tubular and latticed structure. Presence of a DNA-containing granule located within the single mitochondrion and associated with the flagellar bases; the kinetoplast is the characteristic features of these protozoan parasites. They have absorptive mode of nutrition and are morphologically similar to those grown in culture.

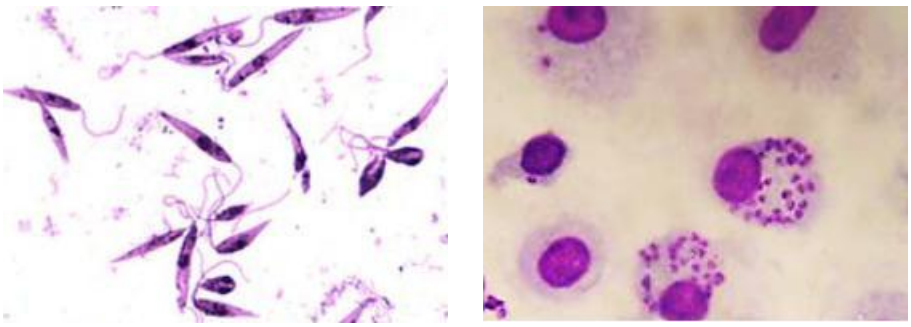

Fig. 1.3: Giemsa stained promastigotes (a) and intracellular amastigotes (b).
[Source:(b). (Manandhar, 2011)]

1.4.2 Amastigote form

The amastigote form inhabits in the parasitophorous vacuoles in the macrophases of the reticuloendothelial system of the vertebrate host. These measuring 3-5 µm in length, are ovoid and nonflagellated form of *Leishmania*. The centrally located round/oval nucleus and adjacent but smaller round/rod shaped kinetoplast is distinguished structural feature of it. Kinetoplast is a dense mass of mitochondrial DNA and composed of several thousand circular DNA molecules linked together in a catenated network. These DNA networks are of two types: kinetoplast containing 25-250 maxicircles of approximately 30 kb, and 5000-10,000 minicircles of about 2 kb (Chen et al., 1995; Chen et al., 1995). Together these constitute the mitochondrial genome. There is a 'flagellar pocket' which is an in-folding of the surface membrane forming an internal space. The flagellum is not functional in amastigotes and does not extend beyond the cell body as well. The function of the pocket is to serve as a site of endocytosis and exocytosis (Webster and Russell, 1993). The cytoplasm

contains both rough and smooth endoplasmic reticulum. The Golgi complex is typically found in the vicinity of the flagellar pocket, which probably reflects the role of this organelle in the endocytic and exocytic pathways. Lysosomes are also found in the cytoplasm together with an organelle unique to kinetoplastids, the glysosome (Opperdoes, 1990).

1.4.3 Transformation of forms

Sandfly during the blood meal introduce the metacyclic promatigotes into the skin where their developmental cycle is initiated by interaction of metacyclic promastigotes with skin macrophages. Metacyclic promastigotes are then uptaken and internalized in a phagosome. Fusion with lysosomes proceeds as normal and the prarasite inhabits in secondary lysosome or phagolysosome. During this process, the metacyclic promastigote transforms into an amastigote with 24-48 hours and continues to grow and divide within the phagolysosomal compartment. Within the transition from the sandfly to the mammalian host, the promastigotes face two major environmental changes, a temperature shift to 35-37^{o}C and a pH shift to around pH 5. The organism sense this new environment and transform into the obligatory intracellular amastigote with loss of flagellum, closing the flagelar pocket, drastic reduction in size and major changes in gene expression (Hommel, 1999).

1.4.4 Life cycle of *Leishmania*

Leishmania spp have complex life cycle with two hosts where they adopt different morphologies. When an invertebrate host feed on blood of *leishmania* infected host, it pools the amastigotes along with. The non flagellated amastigotes convert to flagellated promastigotes which keep on dividing by binary fission and transform into procyclic promastigotes in posterior mid-gut of the sandfly. Procyclic promastigotes continue to divide by binary fission and transform to nectomonad forms. Approximately 3 days after blood feeding, the peritrophic membrane of the gut contacting these parasites begins to breakdown and promastigotes are set free. They then move forward to the anterior midgut of the host. After 5 days in the anterior mid-gut, the nectomonads transform to heptomonad and attach to the stomodeal valve. From 5th day onwards, highly motile metacyclic stage parasites emerge out. They are found in the lumen of the anterior midgut or foregut or both. During next blood feeding, metacyclic forms of these leishmanial parasites enter the human host via proboscis. Within the human host, the amastigotes continue to grow and divide by binary fission within the phagolysosomal compartment. The heavily populated phagolysosomes burst, releasing amastigotes in blood circulation. From the blood they reach to liver, spleen and bone marrow. These parasites again will be taken by sandfly to be transmitted to new host and cycle thus repeats.

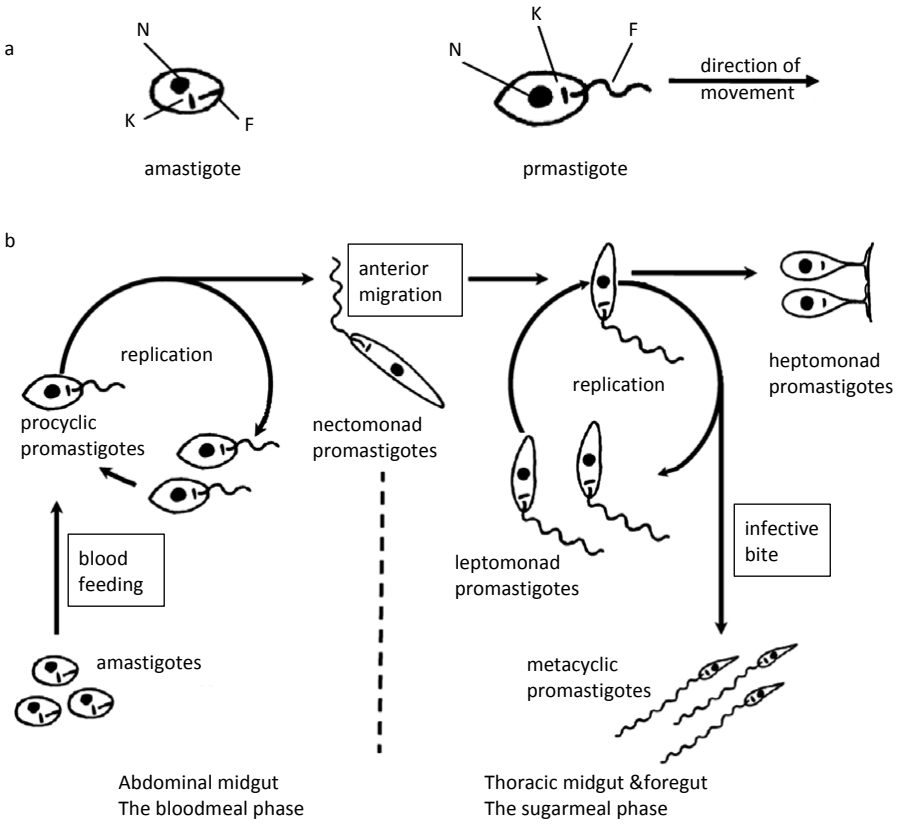

Fig. 1.4: Development of *Leishmania* species in the sand fly vector. [Source: (Bates, 2007)]

(a). The morphology of amastigotes and promastigotes. Each form has a nucleus (N), kinetoplast (K) and flagellum (F). The kinetoplast is the mitochondrial genome. The flagellum in amastigotes is internal and non-functional; in promastigotes the flagellum extends form the cell body, beats and pulls the organism in the direction shown, emerging from the anterior end of the cell. (b). The development sequence of the five major promastigote forms: procyclic, nectomonad, leptomonad, heptomonad and metacyclic prmastigotes.

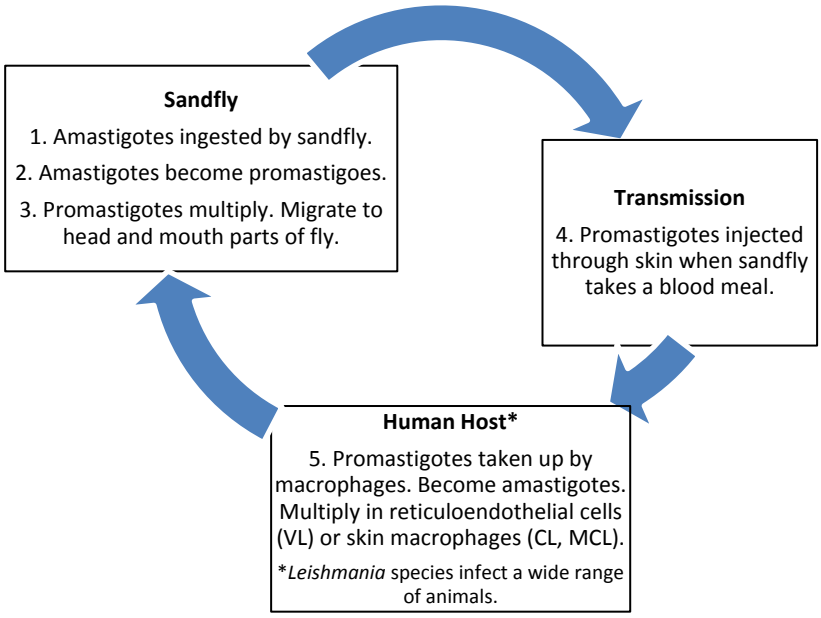

Fig. 1.5: Diagrammatic representation of transmission and life cycle of *Leishmania* parasite .

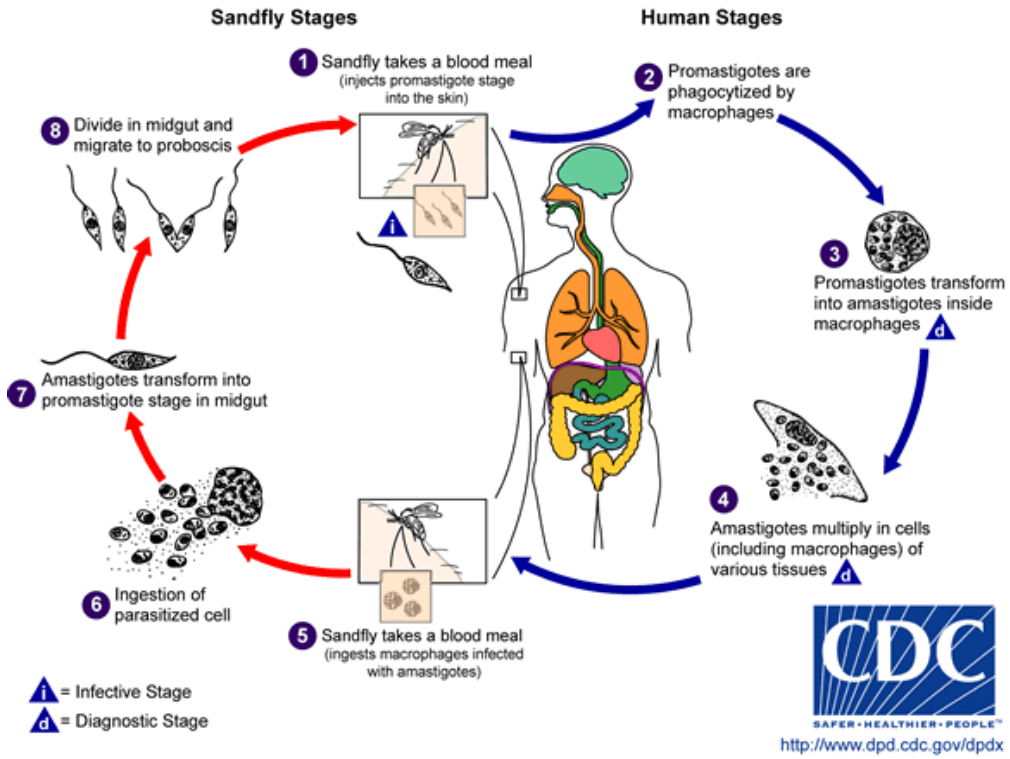

Fig. 1.6: Life cycle of *Leishmania donovani* (Source: CDC website, laboratory identification of parasite)

1.5 Clinical manifestations and Geographical distribution

1.5.1 Visceral leishmaniasis (VL)

Well know as kala-azar (refers to hyperpigmentation with fever which may occur during the disease, visceral leishmaniasis; 'kal' – meaning to death or signifiying a fatal illness; Hindi for black sickness or fever), Visceral leishmaniasis is the most severe form of leishmaniasis. The parasites infect the reticulo-endothelial cells of the visceral organs such as liver, spleen and bone marrow. It is caused by *L. donovani* species complex (i.e. *L. donovani* and *L. infantum* in the old world and *L. chagasi* in new world (Herwaldt, 1999; Chappuis et al., 2007; Mishra et al., 2009).

Clinical features include signs and symptoms of persistent infection characterized by fever, fatigue, weakness, loss of appetite, severe cachexia, splenomegaly, hypergammaglobolinemia, hypoalbulinemia, lymphadenopathy and pancytopenia. The incubation period generally lasting between 2 and 6 months (Chappuis et al., 2007). Since the clinical presentation is fragile to immune system, the patients are prone to develop secondary infections like malaria, pneumonia, tuberculosis, amebic or bacillary dysentery.

WHO has established a definition of Visceral leishmainasis as – a case of Visceral leishmaiasis (VL) is a person showing clinical signs (prolonged irregular fever, splenomegaly and weight loss) with serological (at peripheral geographical level) and/or (when feasible at central level) parasitological confirmation of the diagnosis. In endemic malarious areas, VL must be suspected when fever lasts for more than 2 weeks and no response has been achieved with anti-malarial drugs (assuming drug-resistant malaria has also been considered). The cured VL cases may emerge in new symptomatic form well known by Post Kala-azar Dermal Leishmaniasis (PKDL).

Fig. 1.7: Clinical manifestations and spectrum of Leishmaniasis.

(a). Visceral leishmaniasis; (b). Post kala-azar dermal leishmaniasis (PKDL); (c). Cutaneous leishmaniasis and (d). Mucocutaneous leishmaniasis. [Source: (Chappuis et al., 2007; Manandhar, 2008)]

PKDL, a variation of visceral leishmaiasis

Post Kala-azar Dermal Leishmaniasis (PKDL) is a syndrome that develops at variable times after resolution of VL. It is associated with relapse of visceral disease and manifested by skin lesions that can be of various types. As a complication of visceral leishmaniasis (VL); it is characterized by a macular, maculopapular, and nodular rash in a patient who has recovered from VL and who is otherwise well. The rash usually starts around the mouth from where it spreads to other parts of the body depending on severity. It is mainly seen in Sudan and India where it follows treated VL in 50% and 5-10% of cases, respectively. Thus, it is largely restricted to areas where *Leishmania donovani* is the causative parasite. The interval at which PKDL follows VL is 0-6 months in Sudan and 2-3 years in India. PKDL probably has an important role in interepidemic periods of VL, acting as a reservoir for parasites (Ramesh and Mukherjee, 1995; Zijlstra et al., 2000; Zijlstra et al., 2003); while a recent retrospective cohort study (2000-2010) showed 5.4% (37/680) presented active skin lesions suspect of PKDL with 2.4% confirmed PKDL in south-eastern region of Nepal (Uranw et al., 2011).

1.5.2 Cutaneous leishmaniasis (CL)

Cutaneous leishmaniasis, also known as 'Oriental Sore', is the most common form of leishmainsis and represents 50-75% of all new cases worldwide. The disease is caused by *Lieshmania tropica*, *L. major* and *L. aethiopica* in old world and *Lieshmania mexicana* species complex specially *L. mexicana*, *L. amazonesis* and *L. venzuelensis* and some *Viannia* subgenus of *Leishmania* in new world (Herwaldt, 1999) [Table 1.1]. In this disease, papulae appear in skin initially which are followed by ulceration with raised borders, usually at the site of the vector bite. After few weeks to months, these papulae break with bloody pus. The CL can produce large numbers of skin ulcers, as many as 200 in some cases, on exposed parts of body. They are often self healing wounds but take long time from 3-4 weeks to 12 months. One and half million new cases are arising every year around the world. Approximately 90% of all these cases of CL, now occur in Iran, Syria, Saudi Arabia, Afganistan, Peru and Brazil. However, it is not a major health problem in Nepal and India and it has only been reported from the dry, north-western states of India. The clinically distinguished important forms of CL are categorized into two major forms: (i) Localized cutaneous leishmaniasis: it causes skin ulcers that heal very slowly. The nodular leisons are limited in extent and number. (ii) Diffuse cutaneous leishmaniasis: in this leishmaniasis, cutaneous nodules and plaques do not ulcerate but can sometimes spread over the entire body.

1.5.3 Mucocutaneous Leishmaniasis (MCL)

In muco-cutaneous leishmaniasis, the parasites affect the mucosae, typically starting at the junction of the nose and upper lip. The infection is confined to mucus secreting organs such as nose, throat, mouth, anus and larynx. After initial skin lesions which heal slowly but spontaneously, chronic ulcers appear after months or years with destruction of underlying tissues (e.g. Nasal-cartilage). Tissue destruction with disfigurement can be very severe. Parasites are usually rare in lesions. Death might result from severe respiratory tract infections due to massive destruction of the pharynx (ElHassan et al., 1995). The etiological agent of the disease is *Viannia* subgenus of *Leishmania*, typically of *L. (V). braziliensis* and also of *L. (V.) panamensis, L. (V.) guyuanesnsis and L. (V.) amazonesis* (Herwaldt, 1999). It is mostly related to *Leishmania* species of the New World. At present 90% of all MCL occurs in Bolivia, Peru and Brazil. This form of disease has not been reported in India (Manandhar, 2008).

Table 1.1: Geographical distributions of Leishmaniasis worldwide and their pathogenic species, vector and reservoir.

Clinical manifestations	Geographical distribution	Pathogenic species	Vector	Reservoir
VL	Northest India, Nepal, Bangladesh, Burma	*L. donovani* (Asia)	*Phlebotomus argentipes*	Human
	Mditerrnean basin, Middle East, China, Central Asia	*L. infantum*	*P. perniciosus, P. ariasi*	Dogs, Foxes, Jackals
	Sudan, Kenya, Horn of Africa	*L. donovani* (Africa)	*P. orientalis, P. martini*	Rodents, Cannines, Humans
	Central America, Northern South America, esp Brazil, Venzuela	*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 Savanna, Sudan	*L. major*	*P. duboscqi*	Rodents
	Towns in Middle East, Mediterranean basin, central Asia	*L. torpica*	*P. sergenti*	Humans
	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*	Hyraxes
	Yuctan, 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. guyanensis*	*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

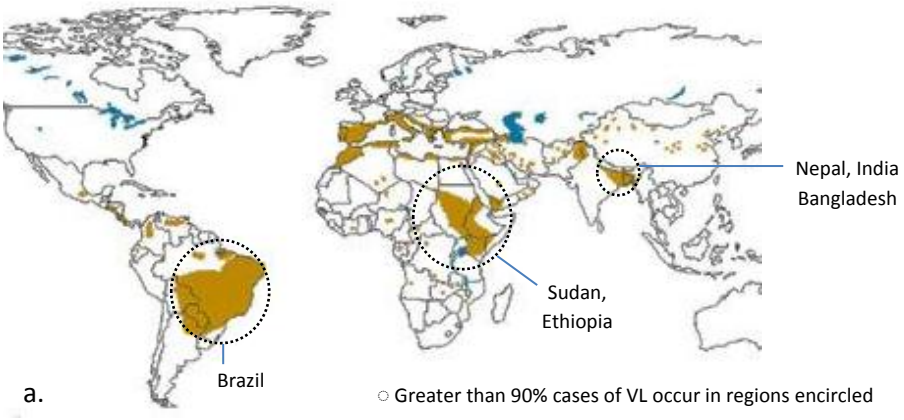

Fig. 1.8a: Geographical distribution of visceral leishmaniasis (VL) worldwide. The majority (>90%) of VL cases occur in just six countries – India, Nepal, Bangladesh, Sudan, Brazil and Ethiopia.

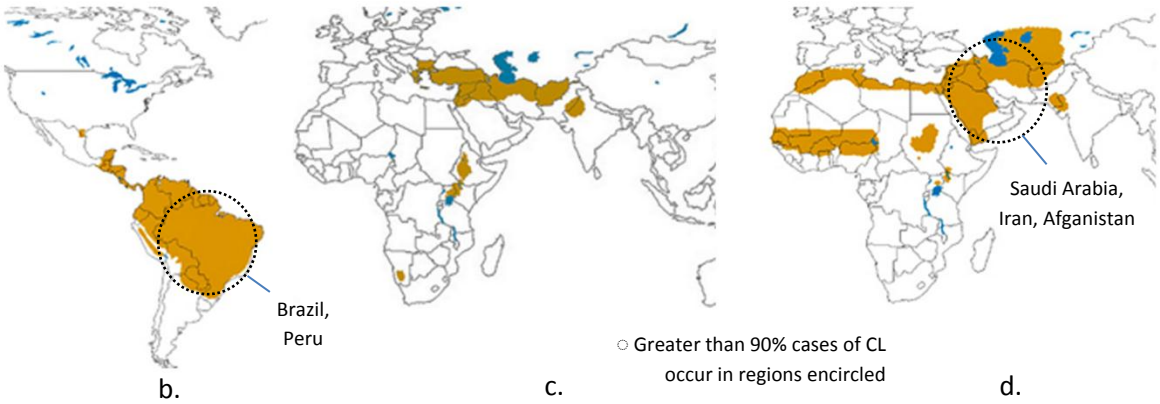

Fig. 1.8b-d: Geographical distribution of: (b). cutaneous and mucocutaneous leishmaniasis in the new world and (c). old world cutaneous leishmaniasis due to *L. tropica* and related speies and *L. aethiopica* & (d). due to *L. major.* [Source: World Health Organisation, (WHO, 2012)]

1.6 Vector of Leishmaniasis

Sandfly of Phlebotominae family (Class Insecta) is the vector of the disease. Out of 600 species of sandfly, only 30 species have been implicated in the transmission of leishmaniasis (Shaw, 1994; Desjeux, 1996; Ashford, 1997). They are tiny insects approximately four times smaller than mosquitoes. There are two genera of sandflies responsible for transmission of leishmnia: *Lutzomyia* in the New World and *Phlebotomus* in the Old World (Killick-Kendrick et al., 1985; Sharma and Singh, 2008). In Indian subcontinent, *Phlebotomus argentipes* is responsible for kala-azar transmission (Swaminath et al., 1942; Sharma and Singh, 2008). It was 1921 when Sergent brothers, Edouard and Etienne experimentally proved that sandflies of genus *Phlebotomus* was responsible for the transmission of leishmaiasis (Sergent et al., 1921). In 1922 it was discovered that the genus

involved was actually *Lutzomyia* in New world. The actual mode of infection, through the bite of the sandfly, was not demonstrated until 1941 (Kean et al., 1978). Over the last two decades, the complex pattern of parasite species, vector, reservoir host and disease have been elaborated by Laison [Table 1.1] (Lainson, 1996).

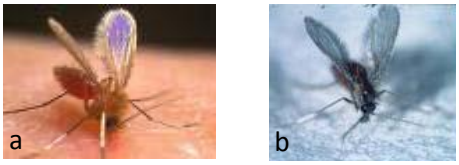

Fig. 1.9: Vectors of Leishamaniasis: a. *Phlebotomus argentipes* in Old World and b. *Lutzomyia longipalpis* in New World. [Source: (Sharma and Singh, 2008)]

The sandfly body and wings are very hairy and fly soundlessly. When at rest the insects hold their wings upright in a V-shape above them. They are poor flyers with flight range of few kilometers and are unable to fly in the presence of any wind produced by fan or ventilator. They are usually active at dawn or dusk (Sharma and Singh, 2008). Sandflies lay their eggs in crevices of rocks, crumble-buildings, rubbish or deep soil cracks where moderate temperature, humid conditions and darkness prevail. *P. argentipes* has been found to pick up the infection form all possible reservoirs of infection. The parasite may develop and propagate at fairly wide range of temperature (16-34^{o}C) in the body of vector. The infection of sandfly is prevalent in considerable number throughout the year in kala-azar endemic area. Its longevity, evening lean density period, has been found to be high enough to transmit infection. The vector is highly endophilic and sufficiently anthropophilic ensuring adequate reservoir/host contact throughout the year in all possible biotypes (mud plastered huts, cattle sheds/outdoor etc.). The vector character of limited dispersal/flight range and multiple behaviors during one gonotropic cycle may transmit the disease within a house or a group of house where it co-inhabits susceptible hosts.

1.7 Parasite Reservoir:

Most forms of leishmaniasis are zoonotic, humans being infected only secondarily, but in anthroponotic forms, humans are the unique reservoir (Desjeux, 1996). Of all potential animal hosts (Dogs, Foxes, Jackals, Rodents, Opossums, Gerbils, Rodents, Sloths), domestic dogs by far play the most important role in harboring and transmitting the disease to humans due to the close association between them. Human is the principle reservoir for anthroponotic VL that is common in Indian subcontinent and Sudan (Sharma and Singh, 2008).

1.8 Transmission:

Sandfly is only the vector known to transmit infection. It may obtain parasite either directly from the infected skin or by ingesting the circulation blood of the infected host. Transmission of infection depends on the presence of a suitable reservoir, vector and a susceptible human population (Swaminath et al., 1942) Although small number of leishmaniasis is anthroponotic, majority of them are zoonotic which involve either wild or domestic animals as reservoir [Table 1.1]. The incubation period for transmission is generally 2-6 months but can also vary form 10 days to 2 years. For this reason sometimes it is impossible to determine the actual transmission period for infection (Rai and Sundar, 1996). The Leishmaniasis in various part of the world is associated with the increased sandfly population and discontinuation of other vector control measures, especially for malaria. Occassionally, sandflies are not involved in transmission, instead, VL can be transmitted by contact of amastigotes to the blood through shared needles, transfusion, transplacental spread or organ transplantation and CL by inadvertent needle stick containing infected materials (Cruz et al., 2002; Morillas-Marquez et al., 2002; Murray et al., 2005; Pagliano et al., 2005).

1.10 Immunology and vaccine prospect

Antileishmanial immunity is mediated *via* both innate (macrophages, neutrophils) and adaptive (B cells, T cells and Dendritic cells) immunity. The pathology of leishmaniasis is determined not only by the parasite species, but also by host genetics and immune factors. In order to develop an effective vaccine a thorough understanding of immune responses during infection is essential (Kedzierski et al., 2009). The fundamental principle of immune regulation of leishmainasis is that the parasite, which replicates in quiescent macrophages, is killed by activated macrophages. Murine models of *L. major* disease exemplify the Th1/Th2 paradigm, in which the outcome of disease is determined by the nature and magnitude of the T-cell and cytokine responses early in infection. In infected inbred mice, production of IFN_{Υ} by Th1 and natural killer cells mediates resistance, whereas expansion of IL-4 pruducing Th2 cells confers susceptibility (Reed and Scott, 1993; Murray et al., 2005; Mougneau et al., 2011).

While, in human cutaneous leishmaniasis, a clear dichotomy in T cell responses has not been reported, instead the patients revealed mixed Th1 and Th2 immunity (Ajdary et al., 2000). Similarly, in human visceral leishmaniasis, there is no strong association between Th1 responses and resistance to disease, instead patients showed co-existing Th1 and Th2 type responses (Khalil et al., 2005). It appears that in humans, the outcome of disease is influenced by the balance between the two T cell populations and is further affected by the

host genetic factors, inoculum size and parasite strain. Despite these shortcomings, the mouse model of leishmaniasis has been extremely beneficial in testing vaccine and drug candidates (Ajdary et al., 2000; Khalil et al., 2005; Kedzierski et al., 2009).

Leishmaniasis in general, but particularly cutaneous leishmaniasis, is probably one of a few parasitic disease that is most likely to be controlled by a successful vaccination program. The relatively uncomplicated leishmanial life cycle and the fact that recovery from a primary infection renders the host resistant to subsequent infections indicate that a successful vaccine is feasible. Extensive evidence from studies in animal models, mainly mice, indicates that a solid protection can be achieved upon immunization with defined protein or DNA vaccine, however, to date such vaccines have been disappointing when tested in field studies and no vaccine either prophylactic or therapeutic is available for human use (Kedzierski et al., 2006; Kedzierski et al., 2009).

1.10 Diagnostic options

Kala-azar diagnosis and treatment follow up is difficult because leishmniasis share its clinical features with many other commonly occurring disease such as malaria, typhoid, tuberculosis, tropical splenomegaly, malnutrition, lymphoma and leukaemia and many of these diseases out break as co-infection with VL (Singh and Sivakumar, 2003; Srivastava et al., 2011). Thus, sensitive and specific laboratory tests to confirm the diagnosis of leishmaniasis as well as monitoring the effectiveness of therapy becomes the first step to achieve the goal of VL elimination (Srividya et al., 2011).

The mean period from the onset of symptoms to diagnosis (mean diagnostic lag period) is 7.7 ± 5.96 months (Sundar et al., 1991) during which the patient not only suffers but also continues to spread the disease. Ideally, all cases of leishmaniasis are confirmed by demonstration of the parasite. The demonstration of LD bodies in the splenic aspirate smear ranges 93.1-98.7% and is regarded as the gold standard for the diagnosis of kala-azar (Sundar, 2003; Srivastava et al., 2011). However, bone marrow and the lymph node smears have lower sensitivity ranging from 52–85% (Zijlstra et al., 1992; Srivastava et al., 2011) and 52–58% (Siddig et al., 1988; Zijlstra et al., 1992), respectively. Splenic aspiration procedure is invasive and carries risk of complication including fetal hemorrhage. Culture and animal inoculation (such as hamsters, mice or guinea pigs) are other methods of parasitological diagnosis but are time taking and costly.

Non-invasive and sensitive, serological techniques are the best and most suitable in the endemic regions. These tests include Direct Agglutination Test (DAT), Enzyme Linked Immunosorbent Assay (ELISA), Recombinant K39 Immunochromatorgraphic Test (rK39 ICT), Western Blot (WB), indirect fluorescence antibody test (IFAT), Latex Bead Agglutination

Test (Katex) etc. These methods are either based on the antigens or antibodies. Serological tests on IFA, ELISA or WB have shown high diagnostic accuracy in most studies but are poorly adapted to field settings (Sinha and Sehgal, 1994; Iqbal et al., 2002; Sreenivas et al., 2002; Srivastava et al., 2011). DAT based on whole promastigotes of *L. donovani* or *L. infantum* and the rK39-ICT (39 amino acid recombinant leishmanial antigen from *L. chagasi*) are the two serological tests used widely for the diagnosis of VL (Chappuis et al., 2007; Srivastava et al., 2011). Molecular assays for detecting parasite DNA have been developed, but none have become popular in field diagnosis (Srivastava et al., 2011). Despite rK39-ICT and DAT widely used in screening is quick with high sensitivity and specificity; however a major drawback is, it fails to differentiate symptomatic cases (patients) form asymptomatic cases and treated patients (Srivastava et al., 2011; Srividya et al., 2011).

In Nepalese context, the recommended diagnostic test is bone marrow microscopy at the level of district hospitals and rK39 in primary health care center (EDCD, 2010). DAT, IFAT and ELISA have also been used for the immune-epidemiological studies (Rijal et al., 2010; Pun et al., 2011).

Accurate diagnosis of VL still remains a major problem of kala-azar control programs, thus there is an urgent need of a diagnostic marker. In regards to the drugs currently available, with toxic and associated with several complications, accurate diagnosis needs to be ascertained which could make a clear distinction between acute disease and asymptomatic infection.

1.11 Treatment options

Despite tremendous progress made in understanding of the parasite pathogenesis and immune response and leishmaniasis being one of the few parasitic diseases likely to be controlled by vaccination; till date one effective – either prophylactic or therapeutic is not available, limiting the treatment options for the disease to chemotherapy (Kedzierski et al., 2009; Kedzierski, 2010). But available and recommended chemotherapeutic drugs are tagged with severe toxic side effects, several reports of emerging resistance and limitations like parenteral administration, long treatment course and high treatment cost (Maltezou, 2008; Kedzierski et al., 2009; Maltezou, 2010).

Sodium stibogluconate (Sb^V), used as first line therapy has developed resistance and treatment failures rate up to 65% in Bihar (Sundar, 2001). Second line therapy as amphotericin-B and its liposomal formulations, despite are considered by many experts as best existing drug against VL, its use is limited in developing countries due to high market price, in addition to long-parenteral course of therapy and hospitalization (Chappuis et al., 2007; Matlashewski et al., 2011). Use of pentamidine as second line therapy has been

discouraged due to decreased cure rates and serious toxic effects associated with its use (Kedzierski et al., 2009; Mishra et al., 2009).

First oral drug miltefosine (an analogue of phosphatidylcholine), initially developed as an anticancer agent, however is effective and promising but is not attractive for the risks associated with non adherence to the recommended regimen – possibly leading to widespread parasite resistance. Further the drug has long half life (parasite resistance is easily induced *in vitro*), potentially teratogenic and abortifacient (Perez-Victoria et al., 2006; Chappuis et al., 2007; Matlashewski et al., 2011). Paramomycin, sitamaquine and various antimycotic azoles are other drugs with considerable usefulness in the therapy and could potentially supplement current available drug regimen for combination therapies but their progress and development is slow and unsatisfactory (Kedzierski et al., 2009; Mishra et al., 2009; Mishra et al., 2009). This urges the search of novel antileishmanial chemotherapeutics against the disease for its global effective control.

1.12 Prevention and Control

For no drugs are currently available for chemoprophylaxis and no vaccine either prophylactic or therapeutic is available in routine use for human, the preventive measures to control leishmaniasis depend chiefly on the treatment of infected individuals and reduction in the transmission of the disease. Active case detection and treatment of cases is important to control spread to the disease particularly in Indian subcontinent type of kala-azar, in which infected man himself acts as the reservoir of the infection. The measures to reduce transmission of the infection to man are achieved by (i) reducing sandfly population, (ii) preventing exposure of the people to infected sandflies and (iii) reducing reservoir of the infection (Murray et al., 2005; Chappuis et al., 2007; Sharma and Singh, 2008).

Sanflies are almost invariably susceptible to spraying insecticides notably DDT, HC, dieldrin and malathion. House spraying with residual insecticides is recommended by the WHO against endophilic sandfly species and it has been the most often used vector control measure so far. The eradication of sandfly breeding places or resulting site is the environmental method to control the sandfly population.

Adequate clothing, bed netting and window screens have proved to be effective in reducing exposure of the people to the bite of the infected sandfly. Repellants either applied directly to the skin or impregnated into the clothing, window mesh or screens, etc. have proved to successful in prevention of exposure to the sandflies. Insecticide-impregnated bed nets (ITN) were shown over the past two decades as one of the most effective methods of

reducing man-vector contact and intra-and peridomicilliary transmission of vector-borne diseases. In most studies the insecticides used were synthetic pyrethroids (permethrin, deltamethrin, lambda-cyhalorthrin). Currently large scale implementation of ITNs is being under experiment in Bihar, India and Nepal under Kala-net project funded by European Union to explore the efficacy against Indian VL due to *Phlebotomus argentipes*.

Since man is the only reservoir in the kala-azar in Indian subcontinent, active and passive case detection and treatment of those found to be infected (including PKDL), is an important attempt to abolish the human reservoir and control the disease. Health education in addition is the most important for the effective control of VL (Murray et al., 2005; Sharma and Singh, 2008).

1.13. Research Plan and Design

1.13.1 Research Hypothesis

1. Flowers of *Bombax ceiba* have been used in the treatment of splenomegaly and other hepatic ailments, which has similarity with symptoms of visceral leishmaniasis therefore; its constituents should have antileishmanial activity.
2. The medicinal plants have least side effects in treatment so cytotoxicity test will show significant result.

1.13.2 Research objectives

General objectives:

Assessment and evaluation of *in vitro* antileishmanial activity of *Bombax ceiba* flowers.

Specific objectives:

1. Extraction and fractionation of phytochemical components from the *B. ceiba* flowers.
2. To analyse the efficacy of the extract and fractions in the *in vitro* promastigote culture of *L. donovani* (MHOM/IN/80/Dd8).
3. To analyse the efficacy of the extract and fractions in the *in vitro* axenic amastigote culture of *L. donovani* (MHOM/IN/80/Dd8).
4. Evaluation of the cytotoxicity of the extract and fractions against RAW 264.7 cell line.
5. Comparative analysis of efficacy among extract, fraction and the reference drug.

1.13.3 Research Plan

In view of contemporary research problem and need, and objectives chosen, the present work was planned as in following flow chart (Fig. 1.6).

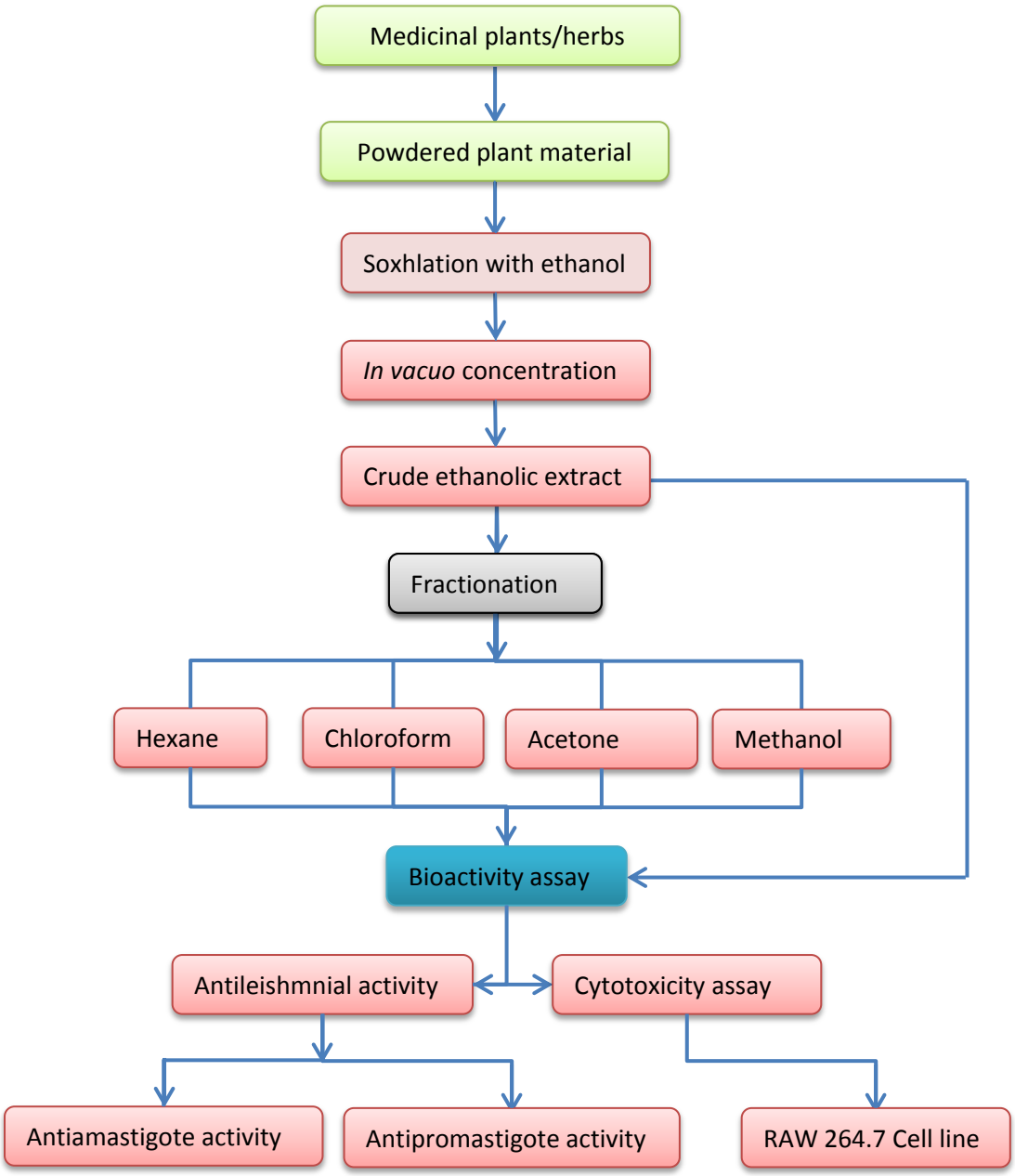

Fig. 1.10: Flow chart: Preparation of plant extract and fractionation and Evaluation of the antileishmanial activity of the fractional extracts and accessing their cytotoxicity.

1.13.4 Rationale

The chemotherapeutic options against visceral leishmaniasis are limited and facing serious concerns of toxicity, high cost and emerging drug resistance. In addition, increase in incidence of co-infection with HIV/AIDS and absence of a vaccine candidate has created a more critical situation. The plant kingdom is definitely a valuable as a source of new medicinal agents. There is a greater interest in novel drug developments from traditionally used medicinal plants which offers unprecedented diversity in structures, bioactivity and immune-modulatory activities in addition to fighting against the etiological agent. Study of effective herbal extracts of medicinal plants of Nepalese origin is thus of immense importance which would help in herbal therapy of the disease, singly or in combination with other chemotherapeutic drugs. The current research work was undertaken in an attempt to validate the traditional uses of the medicinal plants in laboratory demonstration. Nepal is enormously rich with potential natural resource of medicinal plants, available to us as a boon; an assessment of antileishmanial activity would surely be a rational approach that would make defacto small but a hopeful contribution in the field of research on neglected tropical disease of the poor.

Chapter II

Literature Review

2.1 Chemotherapy in Leishmaniasis

Leishmaniasis has recently earned more public attention as one of the "most-neglected diseases" owing to least prioritized in the field of drug research and development (R&D) albeit, it is the utmost need to improve the drug pipeline for VL (Manandhar, 2008).

Chemotherapy is the only effective measure to control leishmaniasis worldwide. Soon after realization that *Leishmania* causes this disease, pentavalent antimonials, the generic sodium stibogluconate (pentosam) and branded meglumine antimoniate, have been the main stay of leishmanial chemotherapy; and still they are the first line drugs of choice where resistance is not reported (Singh et al., 2006). The other second line drugs like amphotericin (polyene antifungal drug) and its liposomal formulations, liposomal AmB (L-AmB: Ambiosome), AmB colloidal dispersion (ABCD: Amphocil) and AmB lipid complex (ABL: Abelcit) are being used in the treatment with more efficacy but their high cost, and longer administrative period which necessitates prolonged hospitalization are the major drawbacks. The first oral drug, miltefosine, originally developed as anticancerous agent, which is an alkylphosphocholine (hexadecylphosphocholine) moiety was considered a major breakthrough in anti-leishmanial chemotherapy but its teratogenic and abortifacient nature limits its use (Jha et al., 1999; Croft and Coombs, 2003; Sundar et al., 2006). Other drugs like paromomycin, sitamaquine and pentamidine also have shown some usefulness against leishmaniasis and could potentially supplement the drugs regimen currently available for the treatment of leishmanial infection but their progress and development is far from satisfactory (Davis and Kedzierski, 2005; Mishra et al., 2009).

These currently used drugs are unsatisfactory for they are either toxic or necessitates hospitalization and close monitoring of the patients; leading to over burdening of already compromised healthcare delivery system. The number of infected people is steadily rising in several parts of the world, in part due to the lack of effective drugs which pose no serious toxic side effects (Croft and Coombs, 2003; Davis and Kedzierski, 2005). Unfortunately, no vaccine candidate either prophylactic or preventive is available for human use, and in addition emergence of resistance to several drugs has further worsened leishmaniasis therapy. The drugs those have been used and currently in use so far have been reviewed and discussed in the following subheadings.

2.1.1 Pentavalent Antimonials (Sb^V)

Since the early 1940s, this drug has been the anchor of treatment for VL and has been used as the first line drug (Berman, 1997). In most of the world these compounds are still used to treat all forms of leishmaniasis. This is primarily because it is affordable, effective and is certainly a time tested therapy. These are available as branded product (Meglumine antimoniate [Glucantime®; Aventis, France] and sodium sitbogluconate [Pentostam®, David Ltd, Calcutta, India]). These drugs can be administered either intravenously or intramuscularly, which is distributed in high concentration in the plasma, liver and spleen. Mean total apparent volume of distribution is 0.22 ± 0.057 L/Kg of body weight and the half-life is 2 hours (Monzote, 2009). In liver, it transforms to its trivalent state (Sb^{III}) and about 50% of antimony is excreted from 24 hours to 76 hours through urine (Franco et al., 1995). The recommended dose is 15-20 mg Sb^V/kg of body weight per day for 21-28 days by intravenous route. Intralesional administration of the drug has shown promising results by injection of 0.2-1mL of Sb^V (Gasser et al., 1994). The long course treatment allows antileishmanial levels of the drug to accumulate in tissues, particularly in liver and spleen. Side effects of the treatment with antimonials include: nausea, abdominal pain, myalgia, pancreatic inflammation, cardiac arrhythmia and hepatitis, leading to the reduction or cessation of treatment (Thakur et al., 2004; Monzote, 2009).

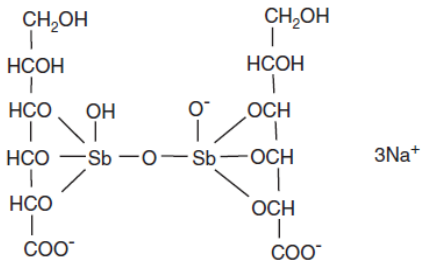

Fig. 2.1: Chemical structure of sodium sitbogluconate.

Based on widely accepted mechanism of action of antimonials, their antileishmanial action depend on the *in vivo* reduction of Sb^V form to a more toxic Sb^{III} form, due to that only amastigotes are susceptible to the Sb^V (Berman et al., 1989). A general consensus is that Sb^V acts upon several targets that include influencing the bioenergetics of *Leishmania* parasite by inhibiting parasite glycolysis, fatty acid beta-oxidation and inhibition of ADP phosphorylation (Chakraborty and Majumder, 1988). It has also been reported to cause non specific blocking of SH groups of amastigote proteins and cause inhibition of DNA topoisomerase I (Wyllie et al., 2004). More recently, it was demonstrated that antimony can alter the thiol-redox potential in both forms of parasite by actively promoting efflux of

thiols, glutathione and trypanothione, thus rendering the parasite more susceptible to oxidative stress (Ameen, 2007).

Currently, several limitations have decreased the use of antimonials: the variable efficacy against CL and VL, as well as the emergence of significant resistance has been increased (Croft and Coombs, 2003). Thiol metabolism is supposed to play a key role in both clinical resistance and laboratory-generated resistance mechanisms. It has been found that elevated intracellular thiol levels and overexpression of tryparedoxin peroxidase are associated with high levels of Sb^{III} resistance (Cortes-Selva et al., 2005; Mukherjee et al., 2007; Wyllie et al., 2008). However, resistance appears to be multifactorial - in natural antimonial resistance the impaired thiol metabolism results in inhibition of Sb^{V} activation and decreased uptake of the active form Sb^{III} by amastigotes; these processes being accomplished by lower expression of the genes ϒ-glutamylcysteine synthase, ornithine decarboxylase, and aquaglyceroporin I, which are involved in the metabolism of glutathione and trypathione, and uptake of Sb^{III} respectively (Decuypere et al., 2005; Carter et al., 2006; Kothari et al., 2007). Overexpression of the membrane-bound ATP-binding cassette (ABC) transporters on the surfaces of *Leishmania* is another mechanism of antimonial resistance. It has been found that, in contrast to infection with Sb-sensitive *L. donovani* isolates, infection with Sb-resistant *L. donovani* isolates upregulates the multidrug resistance-associated protein 1 (MRP1) and the permeability glycoprotein (P-gp) in host cells, thus inhibiting intracellular drug accumulation by decreasing antimony influx (Mukherjee et al., 2007; Mookerjee Basu et al., 2008; Mandal et al., 2009). In animal models, inhibition of the proteins MRP1 and P-gp by lovastatin reverses their action on drug accumulation, and allows them to escape a fatal outcome (Mookerjee Basu et al., 2008). Flavonoid dimers are also known to reverse antimonial resistance in leishmanias *in vitro* by inhibiting ABC transporters and increasing the intracellular accumulation of the drug (Wong et al., 2007).

2.1.2 Amphotericin B (Polyene antibiotics)

Amophotericin B is a polyketide (polyene) antifungal agent produced by the gram-positive filamentous soil bacterium *Streptomyces nodosus*. It is an amphoteric compound composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other. Amphotericin B is poorly soluble in water. It was found to have antileishmanial acitivity in the early 1960s (Ramos et al., 1996; Singh and Sivakumar, 2004). The drug is poorly absorbed by gastrointestinal tract. Amphotericin B exhibits multicompartmental distribution and is found to be present in low concentrations in aqueous humour, pleural, pericardial, peritoneal and synovial fluids. The elimination in adult is approximately 24 hours and can be found in blood for upto 4 weeks and in urine for 4-8 weeks in case of discontinuation of therapy (Monzote, 2009).

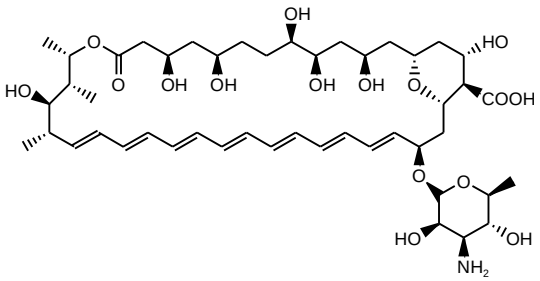

Fig. 2.2: Chemical structure of Amphotericin B

The antileishmanial activity of amphotericin B is attributable to its selectivity for 24-substituted sterols, namely ergosterol vis-a-vis cholesterol, the primary sterol counterpart in mammalian cells eventually helping to increase drug selectivity towards the microorganism. However, higher concentrations (>0.1 µM), it triggers cationic and anionic influx via formation of aqueous pores resulting in cell lyses (Ramos et al., 1996).

Amphotericin B has excellent leishmanicidal activity and constitutes an option in patients that showed resistance to treatment with antimonials. In VL, amphotericin B deoxycholate used in doses of 0.75 to 1mg/kg body weight for 15-20 infusions either daily or on alternate days had consistently produced cure rate of 97% (Sundar and Rai, 2002). With increasing unresponsiveness to Sb^{V}, now it is being used as a first line drug in the regions with high level of unresponsiveness; (Mishra et al., 1994; Thakur, 1998; Sundar and Rai, 2002). Response to the treatment is excellent with long term cure. While major limitations of this treatment is the need for prolonged hospital stay, IV infusion, serious toxicities and high cost (Thakur et al., 1991). The infusions can lead to fatal conditions like renal dysfunction, hypokalemia (low potassium level in blood), hepatic dysfunction, bone marrow suppression and myocarditis, and first dose anaphylaxis are not uncommon (Sundar and Chatterjee, 2006; Chappuis et al., 2007). Fever with chills, shock, ache and pains all over the body, nausea and vomiting are common and can occur acutely during each infusion.

Currently amphotericin B, despite being toxic and expensive, remains the most valuable drug for the treatment of kala-azar. There is an excellent response to this treatment, with quick return of the temperature to normal, splenic regression and return of the laboratory parameters towards normalcy. Long term cure rate is greater than 97% (Giri and Singh, 1994). Many workers feel that in geographical regions where antimony failure rate is more than 10% amphotericin B should be considered as the first-line drug (Sundar et al., 2002).

Liposomal Drug Delivery System

The major limiting factor about the use of amphotericin is toxicity. Currently, toxic effects of amphotericin B have been largely ameliorated with the advent of lipid formulations.

Based on Magic bullet concept, these drugs are delivered to the selective tissues where they exert their pharmacological effects; not only enhancing the desired therapeutic result but also minimizing the occurrence of unrelated responses and toxic side effects. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and facilitating its preferential uptake by reticuloendothelial cells, especially in liver and spleen where they end up in the lysosomal apparatus. In lysosomes, they are disrupted and the drug is released acting either locally or after diffusion outside the organelle in other cell compartments (Giri and Singh, 1994). Thus, this drug delivery result in increasing efficacy and reduced toxicity. Three lipid-associated formulations of amphotericin are commercially available: liposomal amphotericin B (AmBisome), amphotericin B lipid complex (Abelcet) and amphotericin B colloidal dispersion (Amphocil). These compounds have been considered between the most striking advances in leishmaniasis therapy (Yardley and Croft, 1997; Balana-Fouce et al., 1998).

Liposomal Amphotericin B

Liposomal formulation of amphotericin B was first developed by Lopez-Berenstein who found it to be very effective and safe in patients with systemic fungal infections. Liposomal Amphotericin B (AmBisome) is approved in several European countries for primary treatment of VL. Food and Drug Administration of USA has also approved it and recommended a total dose of 21 mg/kg (Meyerhoff, 1999). However, regional variation is seen in the response to AmBisome. Unfortunately due to the 25-30 fold higher cost of AmBisome compared to conventional amphotericin B, even single dose regimen remains beyond the reach of most of the patients.

Amphotericin B Lipid Complex (ABLC)

Blood level of amphotericin B is much lower with ABLC than with same dose of deoxycholate. In four clinical trials with ABLC, it was found very safe with minimum infusion reactions and other toxicities associated with amphotericin B, and no organ specific toxicity was seen. It was possible to infuse a cumulative dose of 10 mg/kg within 24 hours. A total dose of 10-15 mg/kg could cure 90-100% patients and duration of therapy could be compressed to 2-5 days (Sundar and Murray, 1996; Sundar et al., 1997; Sundar et al., 1998; Sundar et al., 1999). In HIV/VL co-infected patients in Europe, its efficiency was low (33-42%) and similar to Sb^{v} (37%) (Laguna, 2003).

Amphotericin B Colloidal Dispersion (Amphocil)

Amphocil is a novel formulation of amphotericin B based on its unique affinity for sterols and is a stable complex of amphotericin B and sodium cholesteryl sulphate, a naturally occurring cholesterol metabolite. It is not a liposomal formulation but a colloidal dispersion of amphotericin B and sodium cholesteryl sulphate. It is administered by intravenous infusion at a rate of 1 to 2 mg/kg/hour. It is reported to be 4 to 15 times more effective than conventional amphotericin B in hamster with visceral leishmaniasis as it gets concentrated in reticuloendothelial system in particular macrophage of liver and spleen (Berman and Gallalee, 1985). Brazil stands first to use it in a total dose of 10 and 14 mg/kg administered as 2 mg/kg daily for 5 and 7 days in 10 patients each, and a cure rate of 90% and 100% respectively (Dietze et al., 1993). Recently evaluation was carried out in a large number of VL patients from North Bihar.

Although, lipid formulations of amphotericin B is a quantum leap forward in the chemotherapy of VL their high cost offsets the advantages associated with it. The use of nanoparticles and microspheres for the delivery of conventional amphotericin B also increased its efficacy against experimental VL (Manandhar et al., 2008; Prajapati et al., 2011). The cost of drug has to be brought down considerably, if these formulations are to be applied meaningfully in developing countries like Nepal, India and Bangladesh.

Amphotericin B fat Emulsion

As a cheap alternative to the costly lipid formlations of amphotericin B, the drug was mixed in 100 ml of commercially available 20% fat emulsion. In a WHO sponsored trial it was found that alternate day infusion of the amphotericin B (2 mg/kg) mixed fat emulsion for a total of 5 infusions, 93% of patients (study group of 70 VL patients) were complete responders at six month follow up. Another major advantage pronounced was a considerably shortened duration of treatment i.e. 10 versus 20-40 days (Sundar et al., 2000). However, a recent study on safety and efficacy of high-dose infusions single bolus dose (15 mg/kg) of amphotericin B fat emulsion could achieve definitive cure rate of 100% with excellent safety and efficacy (Sundar et al., 2009).

2.1.3 Pentamidine (Aromatic diamidines)

Aromatic diamidines were first synthesized as hypoglycemis drugs and their chemotherapeutic profile against antiprotozoal therapy was early discovered. Chemically, pentamidine is 4-[5-(4-carbamimidoylphenoxy) pentoxyl benzenecarboximidamide, synthesized in the late 1930s. It can be administered parenterally, by intramuscular or intravenous route. Drug distribution shows their concentration to be considerably higher in the liver, kidneys, adrenal glands and spleen, while only a small amount is found in lungs (Monzote, 2009).

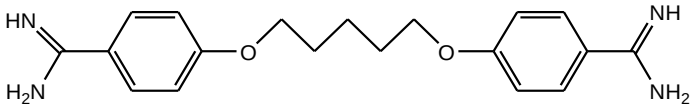

Fig. 2.3: Chemical structure of pentamidine.

Pentamidine acts on the genome of parasite by hampering replication and transcription at the mitochondrial level. Polyamines are substituted at nuclei acid binding sites, which preferentially bind to kinetoplast DNA (Mishra et al., 2007). The regimen consists of 4 mg/Kg three times a week for 3-4 weeks (10–12 injections). Commonly, the treatment with pentamidine causes myalgias, pain at the injection site, nausea, headache and less frequently result in a metallic taste, a burning sensation, numbness and hypotension. Reversible hypoglycemia occurs in about 2% of cases. It causes irreversible insulin dependent diabetes mellitus and death (Sundar and Chatterjee, 2006). Despite suggestions that pentamidine in lower doses combined with allopurinol is safer and effective, these adverse results led to its use totally abandoned in India (Das et al., 2001).

2.1.4 Paramomycin (Aminosidine)

Paromomycin (aminosidine) is an aminoglycosidic aminocyclitol produced by *Streptomyces riomosus* var. Paromomycinus, which was isolated in 1956. It is effective against a wide range of bacteria and protozoa (Monzote, 2009). Antileishmanial activity of paromomycin was demonstrated by Neal et al. in the 1960s (Neal, 1968). The drug is poorly absorbed into systemic circulation after oral administration, but rapidly absorbed from intramuscular sites of injection. Peak concentration in plasma occurs in 30-90 min and half-life varies between 2 and 3 hours in patients with normal renal function. Their clearance is almost entirely by glomerular filtration (Monzote, 2009).

Paromomycin is well known to inhibit protozoan protein synthesis mechanism. It binds to the 30S ribosomal subunit, interfering with initiation of protein synthesis by fixing the 30S-50S ribosomal complex at the start codon of mRNA, leading of accumulation of abnormal

initiation complex (Sundar and Chakravarty, 2008). In parallel, experimental evidences have shown that paromomycin promoted ribosomal subunit association of both, cytoplasmatic and mitochondrial forms, following low Mg^{2+} concentration, induce dissociation and also cause dysfunction in respiratory systems (Maarouf et al., 1997).

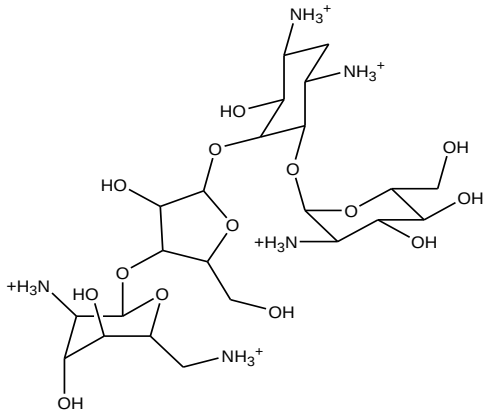

Fig. 2.4: Chemical structure of Paramomycin.

Three preparations of paromomycin ointments have been used for CL: paromomycin 15% plus methylbenzethonium chloride 12%, paromomycin 15% with urea 10% and paromomycin plus gentamicin 0.5%. These formulations have shown variable results according to the species of *Leishmania* involved and the epidemiologic situation (Sundar and Chakravarty, 2008). The most common side effect associated with the paromomycin is the ototoxicity, and in liver function (Sundar et al., 2007). In patients treated with the ointment formulation skin rashes, local pruritus and burs have been the side effects encountered (Balana-Fouce et al., 1998).

2.1.5 Miltefosine (Alkyllysophospholipid)

Lysophosphatidylcholine was found to have immunomodulatory activity in 1960s. More stable derivatives including ether phospholipids and structurally related alkylphosphocholines were made in the 1970s and 1980 (Croft and Coombs, 2003). One of them was the miltefosine and their *in vitro* activity on amastigotes of *L. donovani* was reported in 1987 (Croft et al., 1987). Depending on the individual weight, the recommended therapeutic regimen for patients weighing less than 25 Kg is a single oral dose of 50 mg for 28 days by oral route, whereas individuals weighing more than 25 Kg require a twice daily dose of 50 mg for 28 days (Sundar and Chatterjee, 2006). Adverse effects of miltefosine include gastrointestinal disturbances and renal toxicity. Fortunately, these symptoms are reversible and they are not a major cause for concern. As miltefosine is teratogenic, it is contraindicated in pregnancy and women of child bearing age group (Sundar and Chatterjee, 2006).

The antileishmanial mechanism of action of this compound can be extrapolated from its effect on mammalian cells, where it causes modulation of cell surface receptors, inositol metabolism, phospholipase activation, protein kinase C and other mitogenic pathways, eventually climaxing in apoptosis (Verma and Dey, 2004). The intracellular accumulation of miltefosine appears to be the critical step for its action. It includes: binding to plasma membrane, internalization in the parasite cell (two proteins, the miltefosine trasporter LdMT and its beta subunit LdRos3, are the most significant), and intracellular targeting and metabolism (Perez-Victoria et al., 2006). It has been found that miltefosine induces an apoptosis like cell death in *L. donovani* by producing numerous defects (Perez-Victoria et al., 2006). It is also known to induce several immunologic and inflammatory effects on macrophages. It has been indicated for possibility in being used in T-cell-deficient patients for it does not require T-cell-dependent immune mechanisms for its actions as revealed in animal models (Perez-Victoria et al., 2006; Marques et al., 2008). More recently, it enhanced IFN-ϒ receptors and thus IFN-ϒ responsiveness in *L. donovani* infected macrophages and induced an IL-12-dependent Th1 response and reversed the Th2 response to Th1 (Wadhone et al., 2009).

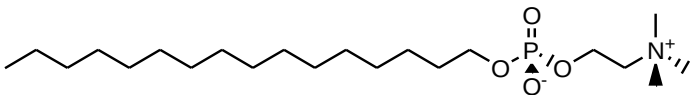

Fig. 2.5: Chemical structure of Miltefosine.

One major fear to using of miltefosine is the probable attainment of field resistance for miltefosine resistance strains can be easily generated in laboratory. Resistance to miltefosine may emerge easily during treatment due to single point mutations (Seifert et al., 2003; Seifert et al., 2007). Decrease in drug accumulation being the common denominator in all miltefosine resistant *Leishmania* lines studied to date, and this could be achieved through decreased uptake, increased efflux, faster metabolism, or altered plasma membrane permeability. Decreased uptake being attributed to mutated LdMT and LdRos3, rendering parasites remarkably less sensitive to miltefosine and overexpression of ABC transporters, reducing the intracellular drug accumulation/increased efflux (Perez-Victoria et al., 2006; Seifert et al., 2007; Maltezou, 2010). More recently, a novel flavonoid derivative was designed and it was shown that the use of suboptimal doses in order to overcome the overexpression of LtrMDRI (a P-glucoprotein-like transporter belonging to the ATP-binding cassette superfamily) was associated with a four-fold increase of intracellular miltefosine accumulation in the resistant *Leishmania* lines (Perez-Victoria et al., 2006).

Miltefosine was registered in India (March 15, 2002) for the treatment of patients with VL including the approval for treatment in children (2-12). It has been registered for treatment of CL and VL in Colombia and VL in Germany with access to patients in entire Europe. In Nepal, it is the first line drug and drug of choice of national program and is provided to all confirmed cases of Kala-azar if there are no contraindications (EDCD, 2010).

2.1.6 Sitamaquine (Primaquine analogue)

Sitamaquine is an 8-aminoquinoline analogue (8-aminoquinoline (8-[6-(diethylamino)hexyl] amino)-6-methoxy-4-methylquinoline) known as WR 6026. This new primaquine was originally developed by Walter Reed Army Institute of Research (United States) for malaria (Dietze et al., 2001).

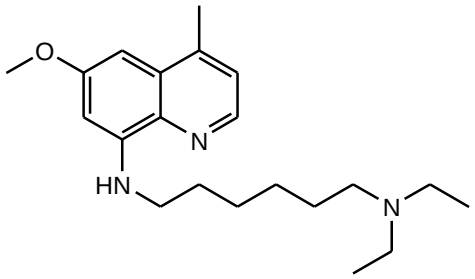

Fig. 2.6: Chemical structure of Sitamaquine.

It was shown to have high antileishmanial activity. The mode of action is not well understood, however, there is evidence that sitamaquine is rapidly metabolized in more polar compounds, which may play an important role in anti-leishmanial activity (Polonio and Efferth, 2008). In recently conducted phase II studies in India, 89% and 100% patients were cured with 28 days of 1.75 and 2.0 mg/kg/day dose of sitamaquine, respectively (Jha et al., 2005). Except nephropathy at higher doses, the durg is well tolerated in VL patients.

2.1.7 Other Agents

Other agents under clinical trial include several antifungal azoles, ketoconazole, itraconalzole and fluconazole for CL and VL (Sundar et al., 1996; Alrajhi et al., 2002). Azoles are potent inhibitors of 14-α-demethylase, an enzyme involved in sterol metabolism responsible for ergosterol biosynthesis, though it has been reported that the parasite is able to survive with an altered sterol composition, induced by the treatment with certain azoles (Roberts et al., 2003).

Allopurinol, a purine analogue, has also been investigated in clinical trials for CL and VL, however, the results were disappointing (Monzote, 2009). The azalide antibiotic has been investigated in cutaneous leishmaniasis with varying success. In *in vitro* models,

azithromycin showed activity against the viscerotropic *Leishmania chagasi*, both at promastigote and amastigote leishmanial forms (de Oliveira-Silva et al., 2008). Azithromycin may constitute an option for combination treatment against leishmaniasis.

2.1.8 Cytokine Therapy/Immunomodulators

Cure of leishmaniasis appears to be dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen metabolites to kill the intracellular amastigotes. This process is suppressed by the infection itself, which down regulates the requisite signaling between macrophage and T cell such as the IL-12, the IFN-ϒ and the presentation of major histocompatibility complex. One alternative in leishmaniasis treatment is the association of antileishmanial drugs with products that stimulate the immune system. The purpose is to enhance the immune response by the activation of macrophages and the increase of the nitric oxide production among other mechanisms to eliminate the infection (Croft and Coombs, 2003; Murray et al., 2003).

The first report about the use of immunomodulators was the superiority of human IFN-ϒ as an adjunct antimony therapy for VL, which was demonstrated in Kenya and India (Badaro et al., 1990). Amphotericin B in conjunction of IL-12 or IL-10 was more efficient than monotherapy and led to a reduction of the amphotericin dose (Murray et al., 2003). Other studies have been reported, using immunomodulators like BCG (Convit et al., 1987) and protein-A (Ghose et al., 1999). Nevertheless, the price of immunomodulators is exorbitantly high for poor population (Sundar and Murray, 1995). Cytokines are produced by recombinant DNA technology and are expensive; being heat labile proteins they also need effective cold chain. In a developing country like Nepal, field applicability of these products remains a distant possibility.

2.2 Vaccines

Most individuals who were once infected with *Leishmania* are resistant to clinical infections, when later exposed. Leishmaniasis in general, but particularly cutaneous leishmaniasis is probably one of a few parasitic diseases that are most likely to be controlled by a successful vaccination program (Kedzierski et al., 2006; Reithinger et al., 2007). An ideal antileishmanial vaccine would need to possess several attributes, such as; i) safety, ii) affordability, iii) induction of robust CD4+ and CD8+ T cell responses and long-term immunological memory, iv) cross-species effectiveness against cutaneous and visceral forms of the disease, v) stability at room temperature eliminating the need for a cold chain

to preserve potency, vi) effectiveness as a prophylactic as well as a therapeutic vaccine (Kedzierski et al., 2009).

Immunisation against leishmaniasis was achieved in the past by inoculating humans with live parasites that induced loacalized self-healing cutaneous lesions (leishmanization). Since then, a first generation vaccines composed of formulations including killed parasites were developed against CL but not VL and were used in large clinical trials on humans populations of endemic areas. Second generation vaccines can be divided into three categories according to their composition: live mutant vaccines, defined subunits and crude fractions. The third generation vaccines are composed of cDNA encoding leishmanial antigens cloned into a eukaryotic expression vector (Manandhar, 2011; Nagill and Kaur, 2011).

2.2.1 Killed parasite vaccines

Killed parasite vaccines have been proposed as both prophylactic and therapeutic vaccines. The therapeutic application may be particularly important in cases of drug resistant refractory disease. However, the whole-cell, killed vaccines have been rather poorly defined and variable in potency (Tabbara, 2006).

2.2.2 Subunit vaccines

Subunit vaccines are the mostly studied vaccines; these were delivered either as recombinant proteins, DNA vaccines, poly-protein vaccines or dendritic cells loaded with peptides derived from leishmanial antigens. Different molecules tested to date include antigens such as surface expressed glycoprotein leishmaniolysin (gp63) (Kedzierski et al., 2006), GPI-anchored membrane protein gp46 or Parasite Surface Antigen 2 (PSA-2), that belongs to a gene family present in all *Leishmania* species except *L. braziliensis* (McMahon-Pratt et al., 1992). LACK (*Leishhmania* homologue for receptors of activated C kinase) antigen is another extensively tested antigen expressed throughout leishmania life cycle, however demonstrated its protective efficacy against *L. major* model, has failed to protect against VL (Melby et al., 2001). Several other antigens from different species have been tested in animal models. These include amastigote cysteine proteases (CP) (Rafati et al., 2005), cysteine proteinase A2 and amastigote membrane proteins P4 and P8 (Soong et al., 1995), kinetoplastid membrane protein-11 (KMP-11) (Basu et al., 2005), amastigote LCR1 (Streit et al., 2000), hydrophilic acylated surface protein B1 (HASPB1) (Stager et al., 2000), leishmanial antigen ORFF (Tewary et al., 2005), acidic ribosomal protein P0 (Iborra et al., 2003), paraflagellar rod protein 2 (PRP-2) (Saravia et al., 2005), and NH36, a main component of the fucose-mannose ligand (Aguilar-Be et al., 2005) and proteophosphoglycan (PPG) (Samant et al., 2009). In addition, molecules such as ATP

synthase alpha chain, beta-tubulin and heat shock 70-related protein 1 precursor have been recently identified as novel vaccine candidates (Bhowmick and Ali, 2009; Kedzierski, 2010).

2.2.3 Polyprotein Vaccines

Leish-111f is the only vaccine to date that could make up the clinical trials. Leish-111f is a single polyprotein composite of three molecules - *L. major* homologue of eukaryotic thiol-specific antioxidant (TSA), the *L. major* stress-inducible protein-1 (LmSTI1) and the *L. braziliensis* elongation and initiation factor (LeIF) (Coler and Reed, 2005). Initial immunisation trials in mice demonstrated that Leish-111f was able to protect mice against *L. major* and *L. amazonensis* infection (Skeiky et al., 2002). There is some evidence that the Leish-111f vaccine can also induce partial protection against visceral leishmaniasis in animal models (Coler et al., 2007), however, Leish-111f failed to protect dogs against infection and did not prevent disease development in a recent Phase III vaccine trial in dogs (Gradoni et al., 2005).

2.2.4 Vaccines of *Leishmania* Fractions

Leishmune®, the first vaccine against canine visceral leishmaniasis, consists of a purified *L. donovani* fraction, named fructose mannose ligand (FML) and a saponin adjuvant. FML has been characterized as a major antigenic complex of *L. donovani* and the main antigen in this complex is NH36, an essential enzyme involved in the construction of the parasite's DNA. Leishmune® is considered a promising tool for the prevention of canine visceral leishmaniasis and further more its potential as a transmission-blocking vaccine is promising for the control of zoonotic visceral leishmaniasis (Dunning, 2009).

2.2.5 Live-attenuated Vaccines

Failure of the subunit vaccines tested so far to develop long-term immunity has led to the requirement of live, persistent parasites for maintaining effector T cell immunity (Uzonna et al., 2001). Avirulent microorganisms can be generated by targeted gene deletion, eliminating the risk of parasite reversion to the virulent phenotype. This type of vaccine would activate the same immunological pathways as virulent infection, since live-attenuated parasites presumably would be taken up and presented the same way as the wild type parasites (Kedzierski et al., 2009). Different attenuated mutant lines such as dihydrofolate reductase thymidylate synthase (DHFR-TS) knockout parasites, cysteine proteinase or lpg1 genes delelted parasites, lpg2 gene deletion has been tested on various animal model but these prepartions couldnot confer protection. Centrin null mutants

(LdCEN-/-) of *L. donovani* parasite and *L. major* phosphomannomutase (PMM) deficient mutants are promising and under development (Kedzierski et al., 2009).

The process of vaccine validation has been hampered by the lack of unambiguous immunological correlates of protection. The mouse models of the disease also lack the demonstration of the complete disease picture, for it mimics only some aspects of the human disease. Further immune responses needed for protection in humans are not clear as in mouse models. Unless understanding of the parasite pathogenesis and the complexity of the immune responses improves; antileishmanial treatment will have to rely on chemotherapy.

2.3 Natural products and Phytotherapy

Due to the limited availability of effective pharmaceuticals, most people in areas where leishmaniasis is endemic depend largely on popular treatments and traditional medicine to alleviate the symptoms. Some of the most popular methods for the treatment of leishmanisis (CL) include cauterization procedures using copper sulphate, battery acid or the application of a hot source such as hot water or red hot metal objects (Chan-Bacab and Pena-Rodriguez, 2001). While treatment of leishmaniasis following the traditional medical practices of different cultures depend heavily on the use of native plants. Such treatments usually consist of oral administration of the crude plant extract for the systemic form of the disease and as topical preparations of the corresponding extract for the treatment of skin infections. With the knowledge of search for new and and better pharmaceuticals of high availability and low toxicity, the Tropical Disease Program of the WHO has considered the investigation of plants used in traditional medicine practices for the treatment of leishmaniasis as essential and of high priority (Chan-Bacab and Pena-Rodriguez, 2001; Monzote, 2009).

Plant-derived products form a large group of compounds containing several products with leishmanicidal activity that include quinones, alkaloids, terpenes, saponins, phenolic derivatives and other metabolites. Various researchers around the globe have worked out in plants, and reported the *in vitro* antileishmanial activity of plant extracts. As an example it is important to mention that the first pharmaceutical products developed for the treatment of malaria and amoebiasis were the alkaloids quinine and emetine, obtained from different species of the genus *Cinchona* and *Cephaelis*, respectively. Recently, the clinical use of artemisinin, a sesquiterpence lactone produced by *Artemisia annua*, for the treatment of malaria has prompted interest to discover new pharmaceuticals of plant origin with antiprotozoal activity (Chan-Bacab and Pena-Rodriguez, 2001).

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). Efforts aimed at screening medicinally important plants to identify novel therapeutic agents have attracted great interest in recent years (Wright and Phillipson, 1990; Iwu et al., 1994; Akendengue et al., 1999; Kayser et al., 2003). Since then a number of studies demonstrating plant extracts with activity against *Leishmania* have been going on to till date (Delorenzi et al., 2001; Rocha et al., 2005; Tiuman et al., 2005; Santos et al., 2008; Singh et al., 2008; Martin-Quintal et al., 2009; Sharma et al., 2009; Sane et al., 2010; Singh et al., 2010).

Several plant extracts were evaluated *in vitro* for leishmanicidal activity by testing on amastigote stages of *Leishmania amazonensis*, and *in vivo* using cutaneous *L. amazonensis* lesions in mice. Among the selected species, *Faramea guianensis* showed activity (Sauvain et al., 1996). Fournet et al. evaluated fourteen plants used topically in folk medicine in Bolivia to treat cutaneous leishmaniasis, which were collected in the tropical regions of colonization and in the rain forest occupied by Chimane Indians. Two plants employed by those in the colonial region showed an *in vitro* antileishmanial activity: *Bocconia integrifolia* and *B. pearcei*. Three other plants, *Ampelocera edentula, Galipea longiflora and Pera benensis*, employed by Chimane Indians, were effective in mice infected with L*. amazonensis* (Fournet et al., 1994).

A preliminary examination of the crude methanol extracts of eight plant species collected from the Sudan revealed that only three plant species had a considerable in vitro antileishmanial activity on *L. major* promastigotes at a concentration of 0.5 mg/ml. The plants *Azadirachta indica, Maytenus senegalensis* and *Eucalyptus globulus* showed IC_{50} values of 11.5, 55 and 78 mg/mL, respectively (Tahir et al., 1998). Singha et al. (1992) evaluated a total of 23 plants from Madras, India, for antileishmanial activity, with *L. donavani* infected hamsters. Extracts derived from five plants (viz., *Alstonia scholaris, Swertia chirata, Tibouchina semidecandra, Tinospora cordifolia* and *Nyctanthes arbortristis*) showed more than 75% inhibition (at 1 g/ kg/day x 5 orally) of multiplication of parasites on day 7 and/or 28 post treatment with an increased survival period (Singha et al., 1992).

The drugs used currently for treatment of Kala-azar, cause severe toxic side-effects and acute immunosuppression in the treated individuals. Picroliv, a standardized mixture of iridoid glycosides, prepared from the alcoholic extract of the root and rhizome of *Picrorhiza kurroa*, has shown strong hepatoprotective activity against several models of hepatotoxicity. Therefore, study was undertaken to study the effects of picroliv (12.5 mg/kg body wt. x 7 days oral) alone and in combination with SSG on parasitemia, lipid peroxidation and hepatic marker enzymes of golden hamsters during *L. donovani* infection.

The results indicated a marked hepatoprotective effect of picroliv in terms of biochemical markers, and a significant antileishmanial activity, implying that it can be utilized as an adjuvant to chemotherapy or in combination therapy of Kalaazar along with SSG, thus enhancing the efficacy of antileishmanials (Mittal et al., 1998).

The ethanolic extract of *Yucca filamentosa*, showed potent activity against *L. amazonensis* at a concentration of 5 mg/mL (Plock et al., 2001). Other plants with marked activity against *L. donovani* were *Khaya senegalensis* and *Anthostema senegalense* with IC_{50} values of 9.8 and 9.1 mg/mL, respectively (Abreu et al., 1999; Rocha et al., 2005). Studies carried out in Colombia with *Annona muricata* against *L. braziliensis* and *L. panamenis* showed that its activity was greater than that of meglumine antimoniate (Glucantime) (Jaramillo et al., 2000).

Dutta et al. described the potential herbal therapy of leishmaniasis by *Aloe vera* leaf exudate (AVL). AVL was found to contain antileishmanial activity against various strains of *Leishmania,* causing the various disease forms – IC_{50} in promastigotes of *L. tropica, L. major, L amazonensis, L. braziliensis, L. infantum* respectively with 176, 102.5, 182, 177.5 and 167.5 µg/mL. In axenic amastigotes of *L. donovani* the IC_{50} was 6.0 µg/mL. In addition AVL also caused activation of host macrophages evident by an increased release of members of reactive oxygen species that was attenuated by preincubation with free radical scavengers. The safety index of AVL was up to 300 µg/mL; remained non-toxic to monocytes and macrophages. Also in a *L. donovani*-BALB/c mouse model, oral or subcutaneous administration of AVL (15 mg/kg body weight x 5 days) reduced parasitemia by >90% in the liver, spleen, and bone marrow without impairment of hepatic and renal functions (Dutta et al., 2007; Dutta et al., 2008).

Leaves of Paan (*Piper betle* Linn.) have also been shown to contain antileishmanial activity which mediates via apoptosis. In the study Sarkar et al., evidenced the ethanolic extract of leaves of *Piper betle* both in promastigotes and amastigotes, with IC_{50} values of 9.8 and 5.45 µg/mL respectively (Sarkar et al., 2008). A Nepalese medicinal plant, Tulsi (*Ocimum sanctum* L.), showed strong activity leading to isolation and structural elucidation of the active constituents from *O. sanctum* L. and from the ethyl acetate soluble fraction of the plant, seven new novel neolignan derivatives were isolated along with 16 known compounds, some of these compounds showed leishmanicidal activity (Suzuki et al., 2009).

Singh et al. recently made an assessment of leishmanicidal effects of herbal extracts obtained from plants in the visceral leishmaniasis endemic area of Bihar, India. In the study, *Azadirachta. indica* and *Piper longum* plant extracts eliminated *L. donovani* promastigotes after 48 h at concentrations of 0.1 and 0.5mg/ml, respectively. *Eclipta. alba* and *Agave.*

americana eliminated the promastigotes at a concentration of 0.5mg/ml and 0.05 mg/ml. respectively within 24h. The axenic amastigote killing response was 1.90-, 2.52- and 1.3-fold higher than the promastigote killing response with *A. indica*, *A. americana* and *E. alba* plant extracts, respectively (Singh et al., 2010). More recently hydroalcoholic extracts *Bidens pilosa* L. (Asteraceae) and *Punica granatum* L. (Punicaceae) inhibited the growth of intracellular amastigotes (*L. amazonensis*), with IC_{50} values of 42.6 and 69.6 µg/mL, respectively. Their antileishmanial activity has been suggested to be the results of flavonoids present in their extracts (Garcia et al., 2010).

Similarly a huge amount of research work and review have been conducted on natural products (plant crude extracts, fractions, isolated compounds and essential oils), signifying efforts of researchers around the world to locate compounds with antileishmanial activity. These efforts are now validating natural products as genuine sources for drug discovery (Tiuman et al., 2011) and these results show the importance of the investigation of plants with therapeutic potential in the treatment of Leishmaniases. A summary of plant crude extracts, fractions, isolated compounds, and essential oils evaluated against the *Leishmania* genus has been illustrated in the table below.

Table 2.1 : Plant crude extracts, fractions, isolated compunds, and essential oils evaluated against the *Leishmania* genus (Tiuman et al., 2011).

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

Family/plant species	**Extracts or compounds**	***Leishmania* species**	**Leishmania species IC_{50} (µg/ml)**	
			PRO	**AMA**
Aloeaceae				
Aloe nyeriensis	Methanolic extract	*L. major*	68.4	ND
	Aqueous ext ract	*L. major*	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

Family/plant species	Extracts or compounds	*Leishmania* species	Leishmania species IC_{50} (µg/ml)	
			PRO	**AMA**
Pagiantha cerifera	Dichloromethane extract	*L. amazonensis*	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-Hydroxy-ursolic 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

Family/plant species	Extracts or compounds	*Leishmania* species	Leishmania species IC_{50} (µg/ml)	
			PRO	AMA
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*	NT	>100

Family/plant species	Extracts or compounds	*Leishmania* species	Leishmania species IC_{50} (µg/ml)	
			PRO	**AMA**
	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
	Buddlejasaponin 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

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

2.3.1 Antileishmanial screening of Nepalese medicinal plants

In context to our country, it is well known that we are rich in all forms of biodiversity - among flora and fauna; species, genetic and habitat. More than 900 types of valuable medicinal plants among 7000 medicinal plants found all over the world are found in Nepal (Manandhar, 2000). However, these natural resource has largely been unexplored for its medicinal and therapeutic potential against leishmaniasis. Therefore, extensive exploration of the medicinal plants commonly used in the traditional medicine for treating parasitic disease is of utmost importance.

A literature review of medicinal plants of Nepal documented by Adhikari et al. (2007) was carried out for identifying medicinal plants of value, native to Nepal and has been implicated traditionally in various ailments and stating various uses and typically those that are being used in symptoms similar to kala-azar, or ailments with similar pathogenesis or stating to be active against helminthes, fungus, protozoans or parasites. A short review of information on the plants with local name, scientific name and parts used and uses has been summarized in the Table 2.3. Plants that have never been reported to have

antileishmanial activity were considered while choosing the plants to be accessed for antileishmanial activity.

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**
Leguminosae Koiraalo [*Bauhinia variegata* L.*, B. candida* Aiton]	150-1900 m, east to west	Root and bark	The bark is alterative, tonic, and blood purifier. It is also useful in diarrhea, dysentery, piles and liver complaints. The decoction of root is valuable drink for reducing corpulence. Fresh flowers used as laxative.
Lauraceae Kapoor [*Cinnamomum camphora*]	1300-1500 m, east to west	Plant	Plant is anodyne, antispasmodic, diaphoretic, anthelmintic, stimulant, carminative and used in insecticidal preparation. White crystalline substance known as Japan camphor, obtained from leaves and twigs is used as disinfectant.
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 dysmenorrhea. Fruits are used as anthelmintic, especially for tapeworms and are laxative in dropsy and colic.
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. It is also used in blood purifier, diuretic, and insecticide. Leaf stalk is used in toothache. It is given in indigestion and nervousness.
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, jaundice, anemia, constipation, cough and bronchitis. Root is diuretic and promotes urination in dropsy. Leaf juice is given in jaundice and other liver complaints.
Ranunculaceae Atees [*Aconitum heterophyllum* Wall. Ex Royale = *Aconitum* L. *atees* Royle]	3200-3700 m, central	Root	Roots are antiperiodic, aphrodisiac, astringent, tonic, and useful in diarrhea, dyspepsia, and cough.
Asclepiadaceae Aank [*Calotropis gigantea*(L.) Dryand.*, Asclepias gigantean*]	100-1200 m, east to west	Roots and root bark , leaves latex and flowers	Dried root bark is febrifuge, anthelmintic, expectorant and is useful in cutaneous diseases and dysentery. Leaves are useful in dropsy and enlargement of abdominal viscera. Dry leaves are smoked for the cure of asthma and cough. The 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. Antihelminthic, Enlargement of abdominal viscera (Al-Qarawi et al., 2001).
Flacourtiaceae Taalishpatree, 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 is also used in biliousness. Leaves are also used in diarrhea. Fruits are astringent, digestive and liver tonic.
Bombacaceae Simal [*Bombax ceiba, Salmalia malabaricum*]	200-900 m, east to west	Roots, gums,bark, leaves , flowers and seeds	The gum is efficacious remedy for diarrhea, dysentery, hemoptysis of pulmonary tuberculosis, influenza and vomiting of blood and menorrhagia. Root of young trees is useful in dysentery. Bark is mucilaginous and is used in healing wounds. Leaves are good for skin eruptions. Flowers are good in skin troubles, splenomegaly and hemorrhoids. Young fruits are useful in calculus affections and ulcerated of the bladder and kidney. Seeds are useful in treating gonorrhea and chronic cystitis.
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.
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.
Zingiberaceae Pankhaaphool, Paaneesaro [*Hedychium spicatum, Hedychium album*]	1500-2100 m, east to west	Rhizome	Rhizome is astringent, fragrant, stomachic, carminative, tonic, stimulant, emmenagogue, expectorant and good in liver complaints, vomiting, diarrhea, 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. Antimicrobial activity (Bisht et al., 2006; Joshi et al., 2008) Good in liver complaints.

[Source: (Adhikari et al., 2007)]

2.3.2 Bombacaceae family and *Bombax ceiba*

The family Bombacaceae consists of about 22 tropical genera and 150 species. The largest genera includes Bombax (60 species), Ceiba (15 species), Durio (15 species), Salmalia (10 species) and Adansonia (10 species). The family has mostly large trees with buttressed trunks. In most species, it has palmate type of compound leaves. They bear complete flowers which are bisexual, generally large and showy, arranged in crowded fascicles with the calyx mostly five toothed, persistent and petal are often elongated. The seed are generally found embedded in hairs from the wall of the fruit, with little or no albumen.

Taxonomic Position:

Kingdom:	Plantae
Division:	Mangnoliophyta
Class:	Magnoliopsida
Order:	Malvales
Family:	Malvaceae (Bombacaceae)
Genus:	Bombax
Species:	ceiba
Botnical name:	*Bombax ceiba L.*
	Bombax malabaricum D.C.
	Salmalia malabarica (DC.) Schott & Endl.

[*Ref: (Chakraborty and Charkraborty, 2010)*]

Bombax ceiba is a widely as used ethnomedicinal plant (Jain et al., 2011). It is a large, deciduous tree commonly known as Silk Cotton Tree, Semal, Indian Red Kapok tree, roktasimul, Shalmali etc; found in temperate Asia, tropical Asia, Africa and Australia (Chakraborty and Charkraborty, 2010; Anandarajagopal et al., 2011). In Nepal it is distributed east to west at 200-900 m, and flowers and fruits during Feb.-March (Adhikari et al., 2007). In traditional system of medicine the plant has been described for various medicinal purposes. Various parts of the tree; root, bark, flowers, fruits, seeds, gum have been reported to have anti-inflammatory, antibacterial, antiviral, anti-microbial (Faizi and Ali, 1999; Anandarajagopal et al., 2011; El-Hagrassi et al., 2011), analgesic (Yoshimi et al., 2001), antioxidant (Yu et al.; Creczynski-Pasa et al., 2009; Vieira et al., 2009; El-Hagrassi et al., 2011), antitumor, hypotensive (Saleem et al., 1999; Saleem et al., 2003), hypoglycemic (Saleem et al., 1999), antiangiogenic (You et al., 2003) and heptoprotective (Dar et al., 2005) activities.

Fig. 2.7: *Bombax ceiba* tree (a) dried flower (b), fresh flower (c) and leaves (d).

***Bombax ceiba* Linn.**

Synonymous:	*Bombax malabaricum* DC., *Salmalia malabarica* (DC.) Schott & Endl.
Local name:	Simal
Saskrint name:	Salmali
Common name:	Silk cotton tree, red silk cotton tree, Semal
Trade name:	Semal

Habitat and distribution: It is common in dry as well as moist deciduous forests; the tree is strong light demander, fast growing, grows best in deep sandy loams or well-drained soils, particularly in valleys, in regions receiving 50-460 cm annual rainfall.

In ethnomedical aspect, the paste of flowers and leaves are applied externally to relieve swellings, boils and various skin troubles. The traditional healers of Chattisgarh Plains are known to boil the flowers at night and keep it as such whole night and next day it is given with mustard seeds internally as treatment of enlarged spleen (Chakraborty and Charkraborty, 2010). These traditional uses of the *B. ceiba* flowers, typically being used for splenomegaly (a hallmark of VL) and various other documented pharmacological studies lead this study on the antileishmanial activity of the ethanolic extract of the flowers of the tree. To the best of our knowledge this is the first scientific report to describe the antileishmaial activity of *Bombax cebia* extracts.

2.3.3 Phytochemistry of *Bombax ceiba*

Phytochemical studies of different parts of the plant have revealed several groups of compounds. Hemigossypol-6-methyl ether was isolated from the root bark of *Bombax malabaricum* along with isohemigossypol-l-methyl ether, isohemigossypol-1, 2-dimethyl ether, 8-formyl-7-hydroxy-5-isopropyl-2-methoxy-3-methyl-4-naphthaquinone, 7-hydroxycadalene (Chakraborty and Charkraborty, 2010). Shamimin, a new flavonol C-glycoside has been isolated as a pale yellow powder from the ethanolic extract of fresh, undried leaves of *Bombax ceiba* in the year 1999 (Faizi and Ali, 1999). Shamimicin, 1’’’, 1’’’’’’-bis-2-(3, 4- dihydroxyphenyl)-3, 4-dihydro-3, 7- dihydroxy-5-O - xylopyranosyloxy- 2H-1-benzopyran along with lupeol were isolated from *Bombax ceiba* stem bark (Saleem et al., 2003). In the same year, mangiferin, a xanthone was isolated by repeated column chromatography of the n-BuOH fraction of the 70% EtOH of the dried leaves of *B. malabaricum* (Shahat et al., 2003). A new sesquiterpene lactone, 5-isopropyl-3-methyl-2, 4, 7-trimethoxy-8, 1-naphthalene carbolactone together with naphthoquinone, 8-formyl-7-hydroxy-5-isopropyl-2-methoxy3methyl 1, 4-naphthoquinone were isolated from the root bark of *Bombax malabaricum* (Vijaya Bhaskar Reddy et al., 2003). By employing concerted 1 and 2D NMR techniques, exact NMR spectral assignments have been made of the acyl and methyl derivatives of mangiferin which was isolated from the leaves of *Bombax ceiba* by workers in the year 2006 (Faizi et al., 2006). Phytochemical investigation of the chemical constituents of the roots of *Bombax malabaricum* afforded nine cadinane sesquiterpenoids, including five new compounds (bombamalones A-D; bombamaloside), and four known compounds (isohemigossypol-1-methyl ester; 2-O-methylisohemigossylic acid lactone; bombaxquinone B; and lacinilene C) (Zhang et al., 2007). A new naphthoquinone together with 7-hydroxycadalene and 8-formyl-7-hydroxy-5-isopropyl-2-methoxy-3-methyl-1, 4 naphthoquinone were isolated from the heartwood of *Bombax malabaricum.* The new naphthoquinone was characterized as 7-hydroxy-5-isopropyl-2-methoxy-3-methyl-1, 4-naphthoquinone based on spectral and chemical studies

(Sreeramulu et al., 2001). A sesquiterpene lactone isolated from *Salmalia malbaricum* (syn *Bombax malbaricum*) roots was previously identified as hemigossylic acid lactone-7-methyl ether. 2D NMR experiments have shown this is a new compound, isohemigossylic acid lactone-2-methyl ether (Puckhaber and Stipanovic, 2001). In the recent chemical research on the flowers of *Bombax ceiba* it was found that three new compounds, bombasin, a colorless gum with molecular formula $C_{19}H_{20}O_6$, bombasin 4-O-b-glucoside, a white amorphous powder, with molecular formula $C_{25}H_{30}O_{11}$, and bombalin, white amorphous powder with molecular formula $C_{16}H_{18}O_8$ were isolated by 70% (v/v) aq. EtOH at room temperature for 3 days which was then further extracted with BuOH five times and then compounds were separated by column chromatography (Wu et al., 2008).

A recent phytochemical analysis of its flower revealed 14 compounds including cholesterol, sitgmasterol, campesterol and alphamyrin while 10 residual compounds being hydrocarbons in the n-hexane fraction and polar methanol fraction afforded seven flavones: vicenin 2 (1), linarin (2), sponarin (3), cosmetin (4), isovitexin (5), xanthomicrol (6) and apigenin (7) (El-Hagrassi et al., 2011).

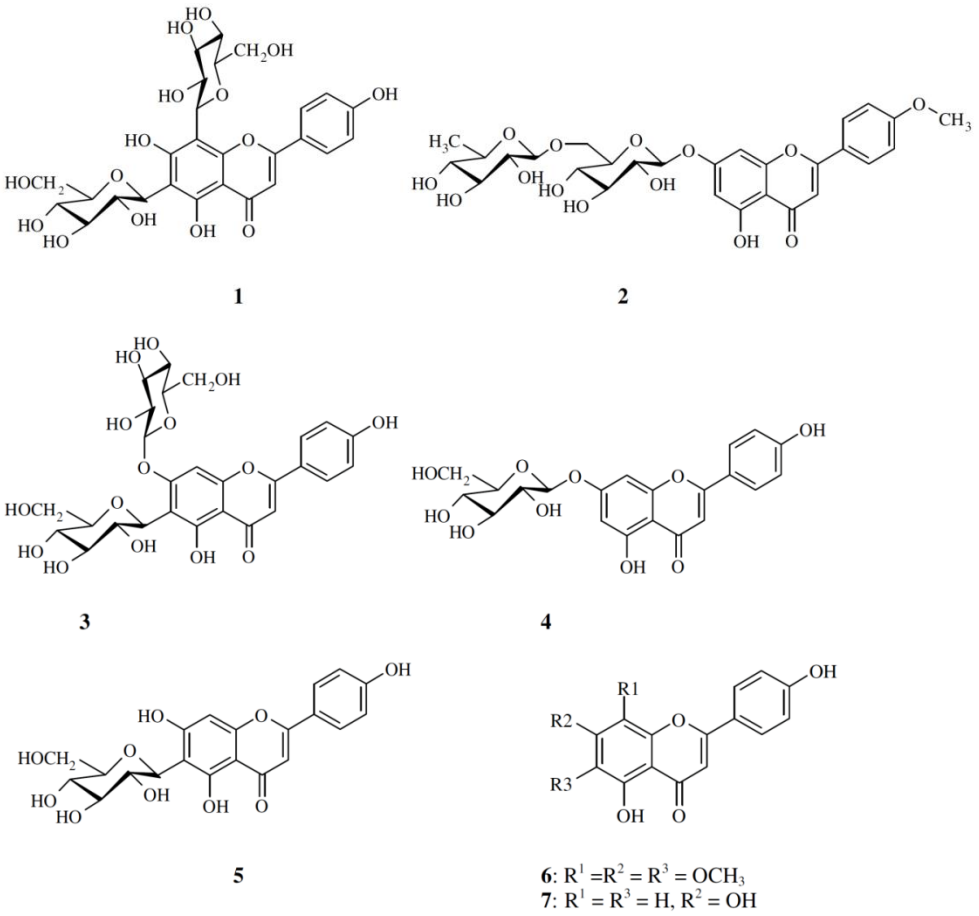

Fig 2.8: Chemical structures of the flavonoids from *Bombax ceiba* flowers namely; vicenin 2 (1), linarin (2), sponarin (3), cosmetin (4), isovitexin (5), xanthomicrol (6) and apigenin (7).

2.4 Assessment of antileishmanial activity

Various screening methods have been developed and used through years to make an assessment of antileishmanial activity of the potentially effective compunds/extracts of plants *in vitro* or *in vivo*. *Leishmania* can be grown *in vitro* as promastigotes and amastigotes in axenic condtions or conversion of promastigotes to amastigotes by raising infection to macrophage cells. Both of these stages have been exploited for the development of primary drug screening procedures. While *in vivo* models include various animal models.

2.4.1 Parasite strain

The parasite *L. donovani* (strain MHOM/IN/80/Dd8), originally isolated from a human patient from Bihar (India) in 1979 was procured in 1981 from the Imperial College, London, through the courtesy of Dr. P.C.C. Garnham.

2.4.2 *In vitro* systems

In vitro system is of potential use, which can be used to access direct lethal action on parasite. But compounds which are effective through their metabolites or those acting through host defense system will not show any action. *In vitro* testing systems, thus at times may not be transferable to *in vivo* situation. However, *in vitro* drug testing has many advantages, e.g. (i) the parasites from few animals are sufficient to test many compounds; (ii) the requirement of test compound is very minute; (iii) the turnover of screening results are quick; and (iv) the results are consistent.

To add one more advantage is, in leishmaniasis, a close correlation exists between *in vitro* and *in vivo* results. The test parasite (disease producing stage: amastigote) can be maintained *in vitro* as axenic amastigotes and in macrophage culture in semi- *in vivo* culture conditions (Bhatnagar et al., 1989; Gupta, 2011). *In vitro* test is the first initial phase for any kind of vaccine development procedure and/or drug discovery process. More recently, assay design has been focused on features that make the system adaptable to high throughput screening (HTS), with additional requirements of (i) small amounts of compound (<1 mg), (ii) quick throughput, and (iii) low cost of tests. However, the test results of *in vitro* system always need to be verified in animals.

2.4.2.1 Using promastigotes

Promastigotes are grown in simple media as test parasite to screen potential antileishmanial agents. Simplicity of this system makes it widely popular. The simplest

model being one that utilizes promastigotes multiplying in cell free media (Neal, 1984). For drug testing, promastigotes are seeded to a concentration of 1.0-2.0 x 10^6 per ml of cultivation medium and the drugs in appropriate concentrations are added to the experimental culture. The inhibition of promastigote multiplication is assessed after approximately 3 days, during which the control organism multiply 3-6 times (Fumarola et al., 2004; Gupta, 2011). The technique is simple and easily applicable. However, the metabolism and ecology of promastigotes differ so widely from those of amastigotes which makes the screening data from *in vitro* test on promastigotes of very little value in animals (Peters et al., 1983; Croft et al., 2006; Gupta, 2011).

2.4.2.2 Using amastigotes

Ideally to be efficient and exhaustive, a drug screening procedure requires conditions that mimic the environment encountered by the target cell. In case of *Leishmania,* intracellular amastigote form might represent the ideal conditions which are the disease producing form of vertebrate hosts; reside exclusively within the macrophages of reticulo-endothelial system.

Axenic amastigotes

Axenic amastigotes are found to demonstrate specific susceptibility to many, if not all drugs tested when compared to the drug susceptibility towards antileishmanial drugs. Screening against axenic amastigotes present several advantages; (i) the test is directed against the relevant stage of parasite, (ii) this stage is as easy to manipulate as the promastigote model and (iii) quantification of drug activity is simple and often inexpensive. For these reasons axenic amastigotes system have been used by many investigators for drug screening (Callahan et al., 1997; Gupta, 2011).

Intracellular amastigotes

The discovery that macrophages infected with amastigotes can be maintained *in vitro* has scored over the usage of promastigotes for screening. The macrophage-amastigote system has received more recognition as it has close resemblance to *in vivo* situation. The system may be called as semi - *in vivo* system.

These widely used models are for testing drug against *Leishmania* species and have involved either murine peritoneal macrophages or human-monocyte transformed macrophages (J774A, THP-1, RAW 264.7, U937, and HL-60) as host cells. These models show species/strain variation in drug sensitivity. In these differentiated macrophages, the rate of amastigote division in host cells and drug activity can be clearly assessed. The

activity of test drug is measured either by microscopically counting of percentage of infected cells or number of amastigotes per macrophage or by colorimetric or fluorometeric methods (Neal and Croft, 1984).

Though, the system (macrophage-amastigotes) is the most preferred *in vitro* screening method, the results of *in vitro* testing many times do not correlate with that obtained in animals because of the problems of bio-availability, bio-transformation and pharmacokinetics of the drugs. The discovery that macrophages infected with amastigotes can be maintained *in vitro* has solved many problems that were being faced with screening against promastigotes. This system (macrophage-amastigote) has received favor since the system provides a more-or-less *in vivo* environment for parasites (Fumarola et al., 2004; Gupta, 2011).

2.5 Assessment of Cytotoxicity

Assessment of cytotoxicity aims to measure the toxicity of the chemicals affecting basic functions of the cells by accessing cellular damage. *In vitro* cytotoxicity assays rapidly evaluates the potential toxicity of large number of compounds, to limit animal experimentation whenever possible, and to carry out test with small quantities of compound. These tests lead to screen compound libraries to remove potentially toxic compounds early in the drug discovery process. Cytotoxicity measurements are based on three basic parameters (i) measurement of cellular metabolic activity, (ii) measurement of membrane integrity (iii) measurement of cell number (Weyermann et al., 2005; BioDiscoveries, 2012).

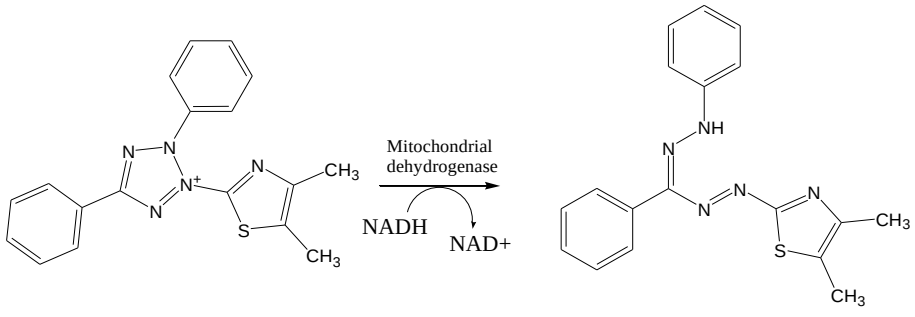

Fig. 2.9: Principle of MTT assay.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, a yellow tetrazole] is a colorimetric assay based on the metabolic activity of viable cells. Tetrazolium salts are reduced only by metabolically active cells, thus MTT is reduced to a blue/purple colored formazan. The insoluble formazan is dissolved using a solubilization solution (usually either

dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) to a colored solution. The absorbance of thus formed colored solution is quantified by measuring at 500 to 600 nm wavelength depending on the solvent used (Mosmann, 1983; Masters, 2000). The reductions take place only when reductase enzymes are active, and therefore conversion is often used as a measure of viable cells. Changes in metabolic activity can give large changes in MTT results while the number of viable cells is constant. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death, or changing metabolism of cells, can be deduced through the production of a dose-response curve (Mosmann, 1983; Ferrari et al., 1990; Masters, 2000).

2.5.1 RAW 264.7 cell line

RAW 264.7 cells are a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice. The cells used by the Alliance for Cellular Signaling (AfCS) were obtained from the American Type Culture Collection (ATCC; cat. no. TIB-71; lot no. 2263775)(ATCC, 2012).

Chapter III

Materials and Methods

3.1 Selection of Medicinal plant (*B. ceiba*)

A review of literature on medicinal plants, native of 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. Several plants used for various purposes having use in similar signs and symptoms as of VL, or ailments with similar pathogenesis were selected (Table 2.3). 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, 2011), ISI Citation Indexes at Web of Science (ISI) using Endnote X2 software (Reuters, 2008), and SciFinder software (CAS, 2011) using the search query "(Plant name)" "and/or" "antileishmanial activity", "leishmanicidal activity". Preliminary screening of the plants *Viscum album* twigs and *B. ceiba* flowers were carried out with antipromastigote activity. *B. ceiba* flowers were found to inhibit promastigotes with higher percentage of inhibition compared to *Viscum album* twigs. Further assessment of antileishmanial activity was carried out with *B. ceiba*.

3.2 Plant Material

Flowers of *B. ceiba* were collected from local herbs market at Nardevi, Kathmandu, Nepal. A voucher specimen (Voucher no. BT 015/068) (Fig. 2.9) has been deposited at Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal, after being authenticated with Annoted Checklist of Flowering Plants in Nepal (Press et al., 2000) and verified by Dr. Deepak Raj Pant, Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal.

3.3 Phytochemical extraction and fractionation

B. ceiba flowers were air dried. The dried flowers (50 g) were powdered in a metallic miller to fine powder and filled in a cellulose thimble and fed to the soxhlet chamber. The soxhletion apparatus was fitted with its RB (round bottom) flask containing 2 L of ethanol and condenser, at the bottom and top respectively. Soxhlation was carried out for 38 hours at 60-70°C using a heating mantle. The extract collected in the RB flask after soxhlation was then concentrated using a rotary evaporator to yield crude extract of 2.05 g. A portion of

crude extract (CEE) was separated for its preliminary bioactivity against promastigotes and the next portion was partitioned using organic solvents.

For partitioning, the crude extract was taken in a 250 ml RB flask and 100 ml of n-hexane was added to it. The mixture was stirred for 24 hours in magnetic stirrer. The solvent was allowed to sediment, decanted and filtered using a Watmann filter paper no. 1 and then collected in a conical flask. The residue was further added with 100 ml of fresh n-hexane and again stirred for 24 hours. The solvent was again collected in the same conical flask after letting to sediment, decanted and filtered and thus obtained 200 ml of solvent was *in vacuo* concentrated using rotary evaporator. The collected solvent free n-hexane fraction was stored at -20°C until use for bioactivity assay.

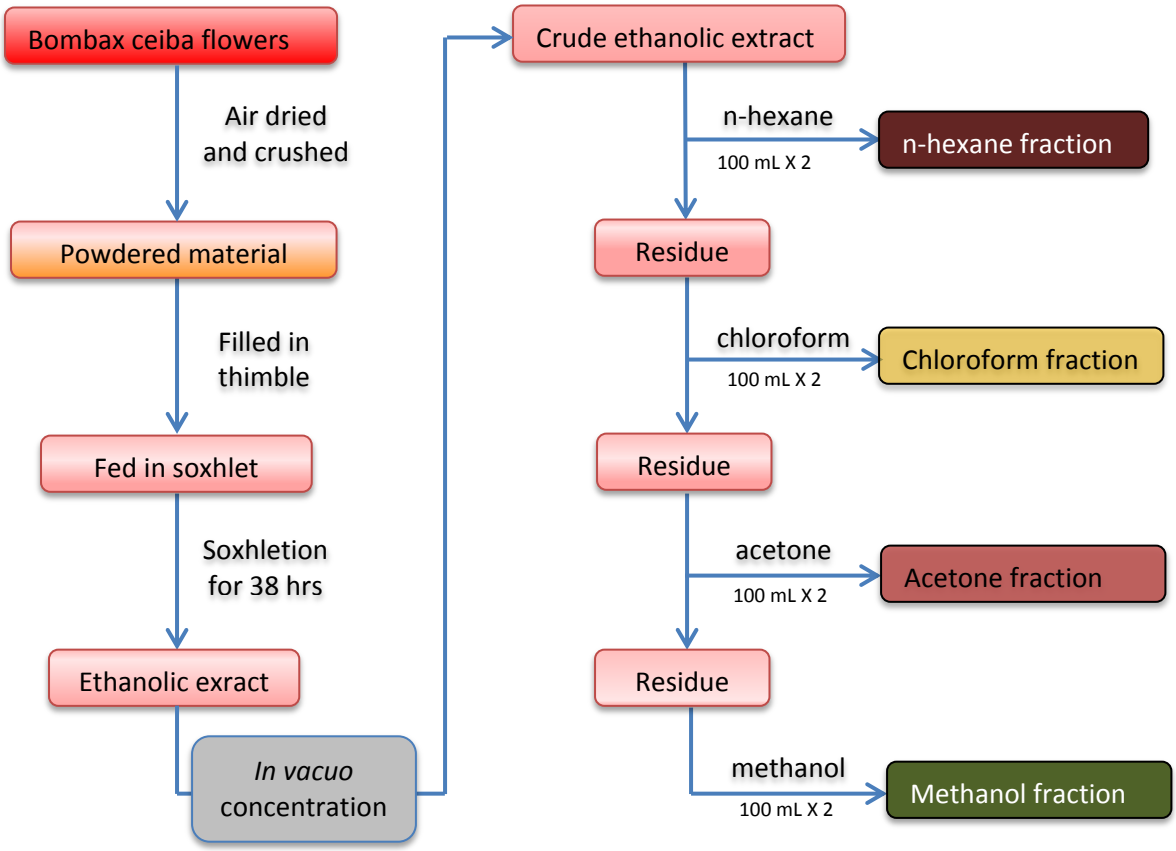

Fig. 3.1 Flowchart for phyotochemical extraction and fractionation:

Dried and powdered flowers of *B. ceiba* (50 g) were extracted with ethanol (Merk, 2 L) for 38 hours using soxhlet apparatus at 60-70°C. The extract was *in vacuo* concentrated using rotary evaporator (Heidolph) to yield crude extract of 2.05 g. The crude extract was further fractionated using magnetic stirrer in increasing order of polarity as n-hexane (100 mL X 2), chloroform (100 mL X 2), acetone (100 mL X 2) and methanol (100 mL X 2). The solvent free extracts were stored at -20°C until use.

To the residue, again 100 ml of chloroform was added and stirred for 24 hours, let to sediment, decanted and solvent was collected by filtration. Again another 100 ml of

chloroform was added and collected likewise; concentrated and stored. Similarly, to the consecutive residues were added with acetone and finally methanol thus yielding acetone and methanol fractions of the crude extracts. They were also concentrated and stored at same temperature. The partitioning solvents sequence was consecutively added on the basis of increasing polarity of the solvents.

3.4 Parasite culture

Promastigotes: A cloned line of *Leishmania donovani* (MHOM/IN/80/Dd8) was maintained *in vitro* in Roswell Park Memorial Institute (RPMI, Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA), and antibiotics (gentamycin 20 µg/ml, streptomycin 100 µg/ml, penicillin 100 U/ml, Sigma, USA) throughout the study. The promastigotes were maintained in a BOD incubator at 26°C.

Amastigotes: Axenic amastigotes were maintained in the same medium acidified to pH 5.5 and cultured at 37^{o}C in a humidified CO_2 incubator with 5% CO_2. Axenic amastigotes were characterized by their round and oval shape without flagella and presence of amastigote specific megasomes under phase contrast microscope. Biochemical characterization was done by lectin agglutination test as described by Balanco et al. (Balanco et al., 1998). Briefly, axenic amastigotes were agglutinated by both peanut (PNA) and Lens culinaris (LCA) agglutinins, respectively at 50 and 12.5 µg/mL. Promastigotes were not agglutinated by PNA and agglutinated in the presence of LCA at concentrations of 100 µg/mL and higher. For biological assays stationary phase parasites were harvested and counted in Neubauer's chamber.

Permanent slides of Giemsa stained promatigotes and amastigotes were prepared for morphological observation.

Passaging: For regular maintenance and availability of the parasite lines, parasite cultures 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^{o}C in BOD incubator for 7 days to harvest stationary phage promastigotes.

Cryopreservation: For cryopreservation, stationary phage promastigotes were spun at 2000 rpm, for 7 minutes at 10^{o}C. The pellet was washed twice with 1X PBS and resuspended in heat inactivated FBS so that the parasite count be 1-2 X 10^7 per mL. Cryovials containing aliquots of 0.5 mL of the cell suspension were replenished with 0.5 mL 3% DMSO (prepared in FBS). The contents of the vials were mixed by inverting and reverting for 3/4 times and

immediately the vial was transferred to Mc. Frosty Can (containing isopropanol) and then kept at -70^{o}C for overnight. The cryovials were stored at liquid nitrogen until use.

Defrosting: The cryopreserved parasites were thawed in lukewarm water (37^{o}C) until a piece of ice was left. The parasite suspension was poured into 15 ml polypropylene tube containing 10 mL cRPMI media. The tube was quickly inverted and reverted for 3/4 times for proper mixing without delay. The tube was then centrifuged at 2000 rpm at 10^{o}C for 10 minutes. The supernatant was discarded and parasites were resuspended in 2 mL complete media. The content was transferred to culture flask containing 5 ml cRPMI media. The culture flask was incubated at 26^{o}C in BOD incubator for 7 days to harvest stationary phage promastigotes.

3.4.1 Determination of parasite count and viability

Neubauer's chamber was used to determine the parasite count throughout the experimental procedures. Neubauer's chamber 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 well homogenized parasite suspension was loaded in the counting chamber and covered with Neubauer's coverslip and observed in light microscope. Parasite count was determined using the formula:

$$\text{Parasite number} = \frac{\text{Total no. of parasite counted in 4 corner squares}}{4} \text{ X dilution factor X } 10^4$$

Prmastigotes were loaded to the Neubauer's chamber and counted excluding non-motile, distorted and dead parasites. While in case of axenic amastigotes, 10 µL of homogenized parasite suspension was loaded after preparing a 1:1 dilution of the parasite with 0.4% Trypan blue. Trypan blue is a vital dye. Its reactivity is based on negatively charged chromophore which do not interact with the cell unless the membrane is damaged (Masters, 2000). Dead parasites were stained blue while live remained colorless.

3.5 RAW 264.7 macrophage cell culture

The RAW 264.7 macrophages were obtained from National Centre for Cell Sciences (NCCS), Pune, India and maintained in RPMI-1640 containing 10% heat-inactivated FBS and antibiotics at 37^{o}C in humidified air containing 5% CO_2. Fresh cRPMI was partially replaced in the flask and was left for proliferation of the macrophages for 3 days. Old media was replaced with approx. 7.5 ml fresh cRPMI-1640 media. With the help of cell-scrapper the adherent cells were scrapped and divided into 3 flasks. The volume was maintained at 5 ml

in each flask and incubated for 3 more days. These cell lines were cultured and subcultured in cRPMI-1640 media and were cryopreserved for further use. For execution of cytotoxicity experiments, cells (2 x 10^4 in 1 ml of complete medium) were seeded in 96-well tissue culture plates (Nunc, Demark).

Passaging: From about 80% confluent growing culture flask, cells were gently scrapped until all cells were dislodged. The cell count was determined using Neubauer's chamber as described in methodology 3.3.1. Then the cells at 1 x 10^6 per mL were seeded in a new flask with 5 mL prewarmed cRPMI medium. The culture flask was incubated at 37^{o}C in humidified air containing 5% CO_2 for 2-3 days (80% confluent cells) before next passaging or harvesting for experimental procedures.

Cryopreservation: Well grown RAW 264.7 cell line as mentioned above transferred to a polypropylence tube and spun at 1200 rpm, for 7 minutes at 10^{o}C. The pellet was washed twice with 1X PBS and resuspended in heat inactivated FBS so that the cell count be 1-2 x 10^7 per mL. Cryovials containing aliquots of 0.5 mL of the cell suspension were replenished with 0.5 mL 20% DMSO (prepared in FBS). The contents of the vials were mixed by inverting and reverting for 3/4 times and immediately the vial was transferred to Mc. Frosty Can (containing isopropanol) and then kept at -70^{o}C for overnight. The cryovials were stored at liquid nitrogen until use.

Defrosting: The cryopreserved cells were thawed in lukewarm water (37^{o}C) until a piece of ice was left. The cell suspension was poured into 15 ml polypropylene tube containing 10 mL cRPMI media. The tube was quickly inverted and reverted for 3/4 times for proper mixing without delay and dipped in ice for 10 minutes. The tube was then centrifuged at 1200 rpm at 10^{o}C for 10 minutes. The supernatant was discarded and cells were resuspended in 2 mL complete media. The content was transferred to culture flask containing 5 ml cRPMI media. The culture flask was incubated at 37^{o}C in humidified CO_2 incubator for 2-3 days (80% confluent cells) before next passaging or harvesting for experimental procedures.

3.6 Reconstitution of antileishmanial reference drug, *B. ceiba* extract and fractions

Miltefosine was used as a reference drug. It was dissolved in Milli-Q water to obtain a stock solution of 2 mg/mL. Crude ethanolic extract of *B. ceiba* was dissolved in 100% of dimethyl sulfoxide (DMSO, Sigma) at 50 mg/mL and syringe filtered (0.22 µm). Stocks of the fractions of the crude extract were also reconstituted at the same concentration as the crude extract. Stock solutions of the reference drug and extract/fractions were stored in dark at

4°C until use in *in vitro* tests. 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

For *in vitro* anti-leishmanial assay, working solutions of reference drug, crude ethanolic extract and fractions were prepared. A 2-fold dilution of the drugs and extracts was setup in 96-well plate (NUNC, Denmark). Briefly, 200 µL aliquots of miltefosine, crude ethanolic extract and fractions were dispensed in triplicate in the row of a 96-well plate as shown in the Table 3.1 below for promastigotes and amstigotes.

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

Drug/extract/fraction	For promastigotes		For amastigotes	
	200 ul dispensing conc. (µg/mL)	Final conc. (µg/mL)	200 ul dispensing conc. (µg/mL)	Final conc. (µg/mL)
Miltefosine	125 to 7.81	62.5 to 3.91	62.5 to 3.91	31.25 to 1.95
CEE	2000 to 62.5	1000 to 31.25	2000 to 62.5	1000 to 31.25
n-hexane	2000 to 31.25	1000 to 15.63	1000 to 15.63	500 to 7.81
Chloroform	2000 to 62.5	1000 to 31.25	2000 to 62.5	1000 to 31.25
Acetone	2000 to 62.5	1000 to 31.25	2000 to 62.5	1000 to 31.25
Methanol	2000 to 62.5	1000 to 31.25	500 to 15.63	250 to 7.81

*CEE: crude ethanolic extract

100 µL of medium was dispensed in the remaining wells. From the drug/extracts well, 100 µL of the drug/extracts was aspirated and transferred to the successive well of the second row and so on and so forth up to the second last row to obtain a two fold drug/extracts dilution. The last row was used as control well. 100 µL of the promastigote suspension at 10^6/mL was dispensed to obtain 5 x 10^4 parasites per well, in all the drug rows, extract and control rows to obtain drug (miltefosine) concentrations from 62.5 to 3.91 µg/mL; crude ethanolic extract and fractions concentrations from 1000 to 15.625 µg /mL (detailed in Table 3.1) respectively in triplicate. For anti-promastigote assay, the plates were incubated at 25°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.

For anti-amastigote assay, a similar 2-fold dilution series of drug/extract/fractions dilution set up was made in 96-well plate as in the Table 3.1. Axenic amastigote suspension in 100 µL at 10^6/mL was dispensed in all the drug rows, extract and control rows to obtain drug

(miltefosine) concentrations from 62.5 to 1.953 µg/mL; crude ethanolic extract and fractions concentrations from 1000 to 7.81 µg /mL (detailed in Table 3.1) respectively in triplicate. The plates were then incubated at 37^{o}C in humidified CO_2 incubator with 5% CO_2. Each time after incubation for 24, 46, 72 and 96 hours, amastigotes were counted by trypan blue dye exclusion method to determine the number of live parasites.

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

Percentage inhibition (PI) by the drug, extract and fractions was determined by equating the mean parasite counts of the control wells as 100% survival and converting all the other counts at different drug/extract/fraction (treatments) dilutions into percentages in reference to the control. The concentration of treatment(s) inhibiting 50% of the parasites (IC_{50}) was obtained by plotting a graph between percentage inhibitions versus different treatment(s) concentration using Microsoft Excel 2007 software. Likewise, IC_{90} 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. Calculation of Percentage of inhibition was done by using the formula,

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

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

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).

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

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

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 concentration of signal >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 RAW 264.7 macrophage cell line, obtained from National Centre for Cell Sciences, Pune, India; was performed for assessment of cytotoxicity by determining the percentage reduction in cell

viability by the reference drug and extract/fractions. Briefly, in a 96 well tissue culture plate, macrophages $2x10^4$cells/well in complete RPMI-1640 medium were seeded and plates were incubated in a humidified CO_2 incubator containing 5% CO_2 at 37^oC for 4 hours. The cells were washed with fresh RPMI-1640 medium (without serum) to remove non-adherent cells and 100µl of fresh complete medium was dispensed. The 100 µl reference drug at different concentration starting from 200µg/mL to 6.25 µg/mL and extract/fractions at different concentration starting from 4mg/mL to 0.03125mg/L were dispensed in each well in a series of two fold dilution and plates were 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 followed by centrifugation at 5000 rpm for 10 min at 4^oC. The supernatant was discarded from each well and 100µl of DMSO was added in each well to dissolve blue formazan formed by reduction of yellow tetrazolium salt within the cells. The absorbance of the plate was read at 540 nm in BioRad Microplate Reader S/N 18551. The experiments were done in triplicate and cells without drug served as control. Results were expressed as percentage reduction in cell proliferation, compared with controls. Wells with cells in media only was used as control and wells with media only served as blank in the experiment.

% cell survival = $\frac{(\mathrm{At} - \mathrm{Ab})}{(\mathrm{Ac} - \mathrm{Ab})}$ X 100; where, At = absorbance of test,

Ab = absorbance of blank,

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 extract and fractions on RAW 264.7 macrophage 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 is considered to be more selective for the parasite.

Selectivity Index (SI) = $\frac{\mathrm{CC_{50}\ (Cytotoxicity\ to\ cell)}}{\mathrm{IC_{50}\ to\ parasites}}$

3.11 Thin layer chromatography (TLC) of the crude extract

A thin layer chromatography of the crude ethanolic extract was carried out to visualize various numbers of compounds present in the extract and retardation factor (R_f) values of the spots on the TLC plates were also determined.

3.11.1 Preparation of TLC plates, sample preparation and application

For preparation of TLC plates 30 grams of silica gel was homogenized with 60 ml of distilled water and this suspension was well distributed over clean and dried TLC glass plates. The plate was then air dried until the transparency of the layer disappeared and then oven dried at 110^{o}C for 30 minutes and then stored in a dry atmosphere until use. Crude ethanolic extract was dissolved in 10 mg/mL in methanol and 5-10 µl of this sample was applied on the TLC plate making a spot 1 cm above the bottom with the help of a capillary tube and the spot was air dried.

3.11.2 Development of the chromatogram and calculation of R_f value

After application of the sample, TLC plates were kept in the solvent in TLC chamber and allowed the mobile phase to move through the adsorbent phase up to 3/4th of the plate. The solvent system was varied – 1) 100% ethyl acetate; 2) Hexane:Ethylacetate (2:8); 3) Methanol:Ethylacetate (2:8); 4) Methanol:chloroform (1:9) for optimized and well resolved visualization of different groups of compounds in the extract. The plate was again air dried and viewed in UV chamber 254nm), iodine chamber and finally charred with 5% of H_2SO_4 to visualize different spots of the compounds spot visible in one or the other.

R_f (Retardation factor) value is the relative distance travelled by the individual spots in reference to the distance travelled by the solvent. R_f were generated using AlphaInnotech software version 1.0.1.14 (AlphaInnotech, 2007). R_f values of the spots were calculated using formula:

$$R_f = \frac{\text{distance travelled by the individual spot}}{\text{distance travelled by the solvent}}$$

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. GraphPad Prism version 5 was used for statistical analysis. An unpaired t-test (two-tailed) was applied to compare the inhibitory concentration between drug, extract or fractions. 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 Phytochemical extraction and fractionation

Preliminary observation of *in vitro* antileishmanial activity against *L. donovani* of the crude ethanolic extract of the plant promoted us to perform a bioassay-guided fractionation to locate the antiliehmanial activity. The crude ethanolic flower extract (1.025 g) of *Bombax ceiba* after partitioning with organic solvents yielded four fractions: n-hexane, chloroform, acetone and methanol fraction. The crude ethanolic extract was transparent yellow in soxhlet collection flask and turned dark brown, solid in color after *in vacuo* concentration, possibly due to crystallization of colored compounds present due to super saturation; yielding 2.05 gm from a starting material of 50 gm of dried powdered flowers. The hexane extract was solid yellow in color with total yield of 0.248 gm (24.20% yield) from 1.025 gm of the crude extract taken. Similarly, the chloroform extract, acetone extract and methanol extract were light brown, pale yellow and dark greenish yellow in color with 0.136 gm (13.27% yield), 0.050 gm (4.88% yield) and 0.052 gm (5.07% yield) yield respectively [Table 4.1]

All the extracts were solid in consistency except the methanol fraction which was semi solid after *in vacuo* concentration in the evaporator. The crude ethanolic extract after *in vacuo* concentration yielded 4% of the starting powdered plant material (flower). After fractionation, hexane fraction had the highest percent composition (51%) in the crude followed by chloroform fraction (28%), methanol fraction (11%) and acetone fraction (10%) [Fig. 4.1].The color and consistency of the extracts and their respective yield percentage are shown in Table 4.1 and Fig. 4.1. All the fractions were subjected to anti-promastigote and anti-amastigote assay.

Table 4.1: Physical characterization (Color and consistency) and yield percentage of the crude ethanolic extract and its fractions.

Fractions	**Color Of Extract**	**Consistency**	**Yield (g)**	**yield %**	**% composition of fraction**
Crude	Dark Brown	Solid	2.050	4.10[1]	-
Hexane	Yellow	Solid	0.248	24.20[2]	51
Chloroform	Light Brown	Solid	0.136	13.27[2]	28
Acetone	Pale Yellow	Solid	0.050	4.88[2]	11
Methanol	Dark Greenish Yellow	Semi Solid	0.052	5.07[2]	10

[1] Extract yield from total plant material taken for extraction

[2] Extract yield out of crude extract (1.025 g)

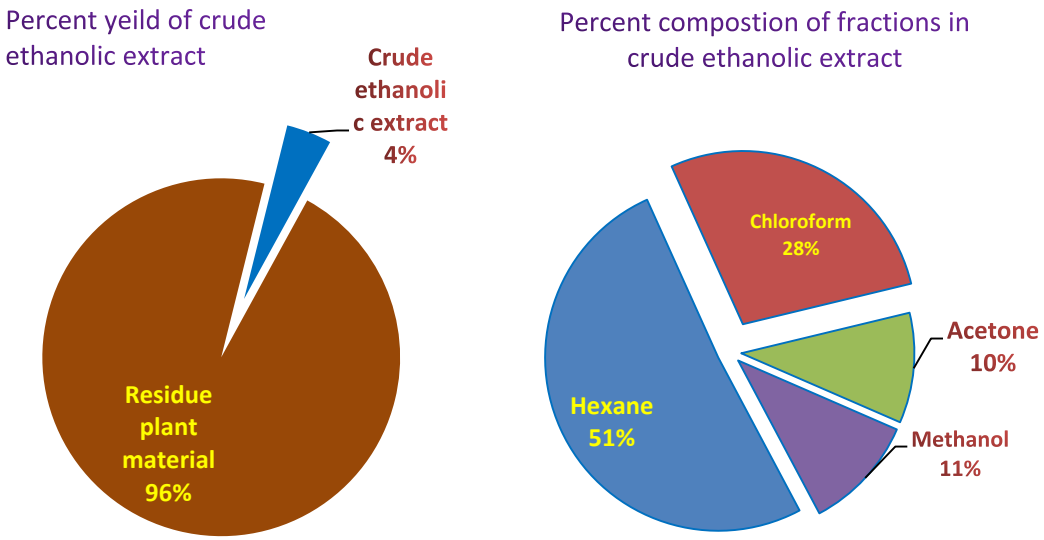

Fig. 4.1: Yield of *Bombax ceiba* flower fractions (%) and composition (%) of crude ethanolic extract.

4.2 *In vitro* antileishmanial assay on *L. donovani* promastigotes and amastigotes

Addition of the crude extract on the promastigotes after overnight incubation induced various morphological abnormalities like loss of flagella, immobile, and distorted shape etc. as induced by reference drug-miltefosine, such effect was not seen in the control where the promastioges were intact and alive. Similarly, most of the axenic amastigotes in the drug and extract wells were stained blue with trypan blue stain, while in the control well they remained stainless. That is, the crude ethanolic extract of the flowers were effective against both the promastigotes and amastigotes. Further they were incubated for next 24 hours and so forth and so on up to 96 hours to determine the time dependent inhibition by the extract and fractions.

4.2.1 Determination of required drug concentration for IC_{50}

The IC_{50} value of the crude ethanolic extract against promastigotes was found to be 131.24 ± 12.54 µg/mL. Promastigotes were largely inhibited by the methanol fraction among the various fractions with IC_{50} value 89.62 ± 0.55 µg/mL, while the IC_{50} values of acetone, chloroform and n-hexane fractions were higher than 100 µg/mL; as of crude ethanolic extract, precisely with 191.64 ± 11.94, 144.25 ± 2.64, 105.12 ± 7.99 µg/mL respectively. The reference drug miltefosine showed IC_{50} concentration as low as at 11.27 ± 0.52 µg/mL.

Table 4.2: *In vitro* antileishmanial activity (IC_{50}) *Bombax ceiba* Linn. flowers ethanolic extract and its fractions against *L. donovani* promastigotes and axenic amastigotes.

Extract fraction/drug	Promastigotes IC_{50} (µg/mL)[a]	Axenic amastigotes IC_{50} (µg/mL)[b]
Methanol fraction	89.62 ± 0.55	58.73 ± 1.89
Acetone fraction	191.64 ± 11.94	283.32 ± 6.82
Chloroform fraction	144.25 ± 2.64	180.37 ± 2.73
n-hexane fraction	105.12 ± 7.99	61.39 ± 1.34
Crude ethanolic extract	131.24 ± 12.54	125.96 ± 3.62
Miltefosine	11.27 ± 0.52	4.12 ± 0.13

Results are expressed as mean ± SD of three independent experiments after 48 hrs of treatment.
IC_{50} [a] – Concentration of test sample that inhibited 50% growth of promastigotes.
IC_{50} [b] – Concentration of test sample that inhibited 50% parasite growth of axenic amastigotes.

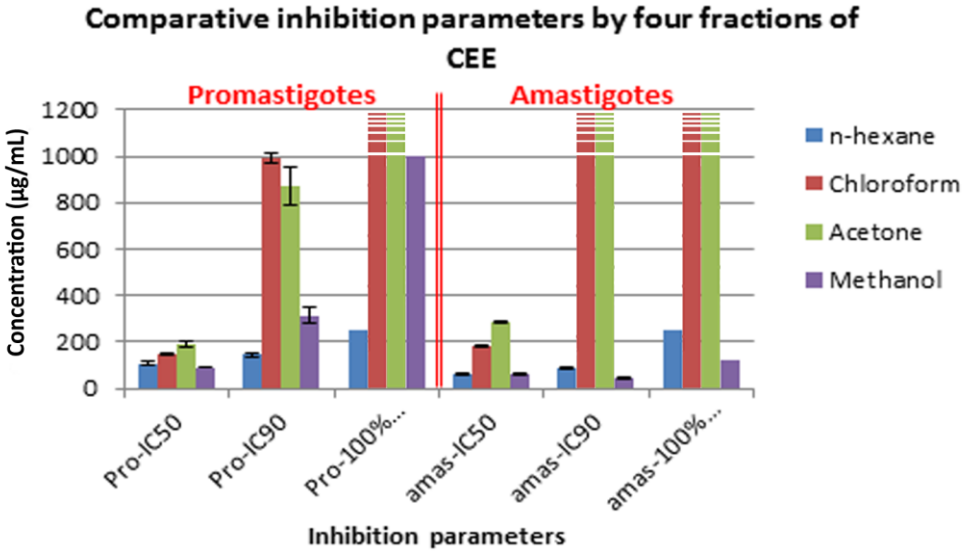

Fig. 4.2: Comparative inhibition parameter exhibited by four fractions of the crude ethanolic extract of *Bombax ceiba*.

IC_{50} values shown are at 48 hrs and IC_{90} and absolute (100%) inhibition values are at 96 hrs. The waning out bars indicates the values are greater then 1000 µg/mL.

IC_{50} values of the crude ethanolic extract against axenic amastigotes was found to be 125.96 ± 3.62 µg/mL which is lower than the anti-promastigote IC_{50}. Miltefosine had also similar effect on the axenic amastigotes with lower IC_{50} than for the promastigotes. IC_{50} of miltefosine for axenic amastigotes was found to be 4.12 ± 0.13 µg/mL, which is about 3 folds lower than the IC_{50} value for the promastigotes. To add to this pattern, methanol and n-hexane also showed lower IC_{50} values for axenic amastigotes, with IC_{50} values 58.73 ± 1.89 (1.52 fold lower than promastigote IC_{50}), 61.39 ± 1.34 µg/mL (1.18 fold lower than promastigote IC_{50}). While acetone and chloroform fractions had higher IC_{50} values for axenic amastigotes than the promastigotes with IC_{50} values being 283.32 ± 6.82 (1.47 fold

higher), 180.37 ± 2.73 µg/mL (1.25 fold higher) respectively. The complete table showing *in vitro* aniti-leishmanial acivity of crude ethanolic extract of *Bombax ceiba* and its fractions against *L. donovani* (MHOM/IN/80/Dd8) with their respective IC_{50} values for promastigotes and amastigotes are shown in Table 4.2 with miltefosine as reference drug [fig. 4.2].

4.2.2 Determination of required drug concentration for IC_{90} and absolute inhibition

Further IC_{90} (concentration that inhibits 90% of parasites) of the fractions were determined. IC_{90} at 96 hours was also found to be lower for amastigotes as compared to the promastigotes in case of methanol and hexane fraction, while for acetone and chloroform fraction IC_{90} was determined to be lower for promastigotes than amastigotes. Precisely, IC_{90} of methanol was determined to be 314.41 µg/mL for promastigotes and 43.81 µg/mL for amastigotes. Likewise IC_{90} of hexane fraction was determined to be 143.60 µg/mL and 88.67 µg/mL respectively for promastigotes and amastigotes. In case of acetone and chloroform fraction, IC_{90} values were determined to be 873.41 and 991.73 µg/ml respectively; and that for amastigotes were higher than 1 mg/mL. Comparative IC_{90} and absolute inhibition of the fractions against promastigotes and amastigotes at 96 hours is shown in Table 4.3 and Fig. 4.2.

The inhibition pattern was studied over 96 hours, in order to check the complete inhibition of the parasites to find whether the fraction achieve the target. Chloroform and acetone fraction did not completely inhibit the both forms parasites at the highest dose (1 mg/mL) used even after 96 hours. Hexane and methanol fraction achieved the target inhibiting 100% parasites within 96 hours period at concentration lower than 1 mg/mL. Hexane absolutely inhibited both forms of the parasite at 250 µg/mL and methanol achieved absolute inhibition of promastigotes at 1 mg/mL and amastigotes at 125 µg/mL.

Table 4.3: Concentration of *Bombax ceiba* fractions required for IC_{90} and absolute inhibition values in 96 hours.

Fractions	Promastigotes		Amastigotes	
	IC_{90} (µg/mL)	Absolute inhibition (µg/mL)	IC_{90} (µg/mL)	Absolute inhibition (µg/mL)
Methanol	314.41	1000	43.81	125
Acetone	873.41	>1000	>1000	>1000
Chloroform	991.73	>1000	>1000	>1000
Hexane	143.60	250	88.67	250

4.2.3 Dose response curves: Percentage of Inhibition (PI) versus (vs.) Concentration of extract, fractions, control and drug on promastigotes

The antileishmanial activities of the fractions were traced in reference to the concentration and time up to 96 hours. An integrated dose response curve has been shown in the Fig. 4.3a with dose dependent inhibition of the promastigotes of the various fractions and the crude extract along with the miltefosine at 48 hrs. of treatment.

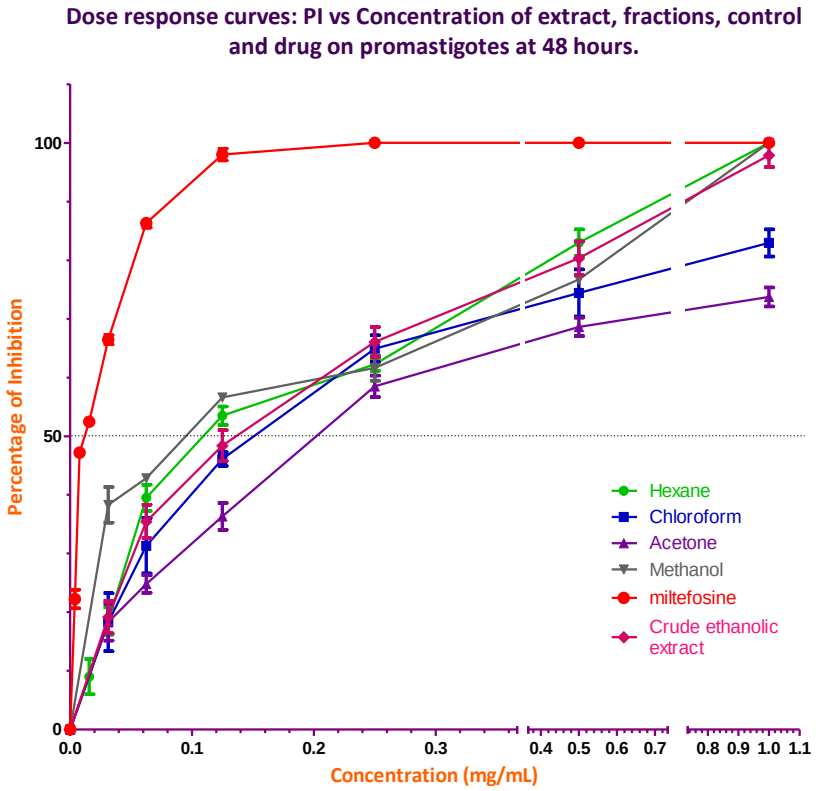

Fig. 4.3a: Dose response curve showing percentage of inhibition of promastigotes at different concentrations starting from control to 1 mg/mL of crude ethanolic extract, fractions and reference durg – miltefosine in 48 hrs.

From the dose response curves against promastigotes (Fig. 4.3a), it can be inferred that reference drug, crude and fractions inhibited promastigotes in a dose dependent manner with higher percentage of inhibition with increase in concentration of the treatments. Miltefosine inhibited the total parasite at lower dose than the extract or any fractions. Chloroform and acetone fraction didn't inhibit 100% parasite even at 1 mg/mL in 48 hrs of incubation, while the crude extract killed all parasites at same concentration, and also hexane and methanol fraction inhibited all the promastigotes at 1 mg/mL. It can be stated

from this observation that the effective compound(s) might have been concentrated in the hexane and/or methanol fraction.

4.2.4 Dose response curves: PI vs. Concentration of extract, fractions, control and drug on amastigotes

Anti amastiogte activity of miltefosine, crude extract and fractions were accessed on the various doses of the treatments and an integrated dose response curve was generated at 48 hrs of treatment (Fig. 4.3b).

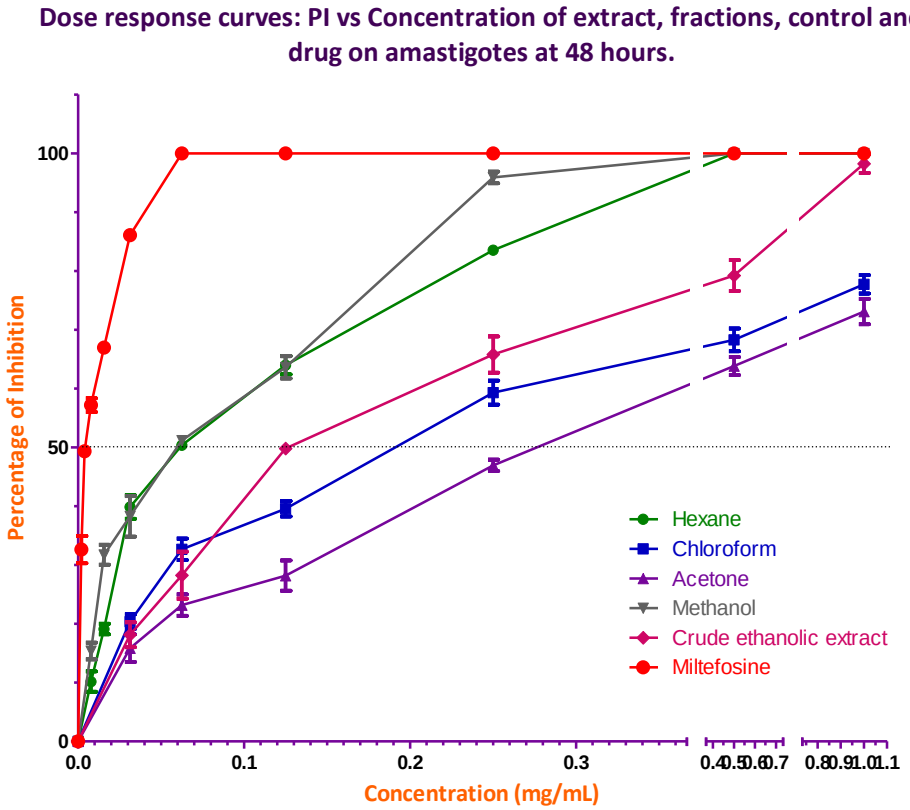

Fig. 4.3b: Dose response curve showing percentage of inhibition of amastigotes at different concentrations starting from control to 1 mg/mL of crude ethanolic extract, fractions and reference durg – miltefosine in 48 hours.

The dose response curve deciphered that axenic amastigotes have been increasingly inhibited by the increasing doses of treatments. Miltefosine inhibited all amastigotes at lower dose than fractions or crude extract. Hexane and methanol fraction inhibited all amastigotes at 0.5 mg/mL and crude extract inhibited nearly all amstigotes at 1 mg/mL. While chloroform and acetone fraction failed to inhibit 100% amastigotes even at 1 mg/mL, however could inhibit >50% amastigotes at that dose. From this observation, it can be inferred that methanol and hexane fraction are more active against amastigotes than

chloroform and acetone fraction, probably the antileishmanial constituents are present in methanol and/or hexane fraction.

4.2.5 Time dependent efficacy response of fractions over 96 hours

Time dependent inhibition of promastioges indicate hexane fraction could totally inhibit the promastigotes at 0.25 mg/mL at 96 hours and at 1 mg/mL within 48 hours (Fig. 4.4a). Methanol fraction also could completely inhibit the parasite at 1 mg/mL in first 48 hours (Fig. 4.4d) while such absolute inhibition was not seen in case of chloroform (Fig. 4.4b) and acetone fraction (Fig. 4.4c).

It seems, from the dose response curves and time dependent efficacy response graphs that hexane and methanol are leishmanicidal while acetone and chloroform are leishmanistatic. Also comparing the graphs of the promastigotes and amastigotes, the later seems be inhibited at lower doses than the promastigotes in the methanol and hexane fractions, extract and reference drug. May be similar active principles are present in the extract as the reference drug or possibly the extract have similar mode of action as miltefosine.

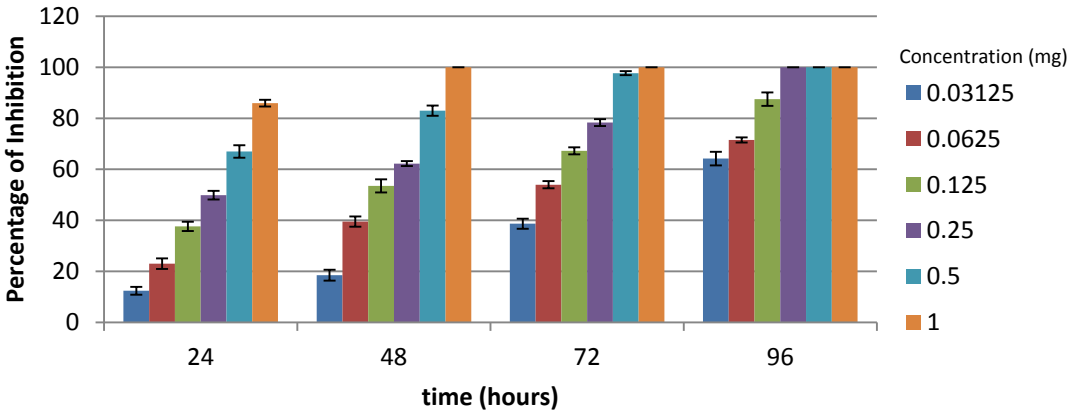

Fig. 4.4a: Effect of hexane fraction on *L. donovani* promastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

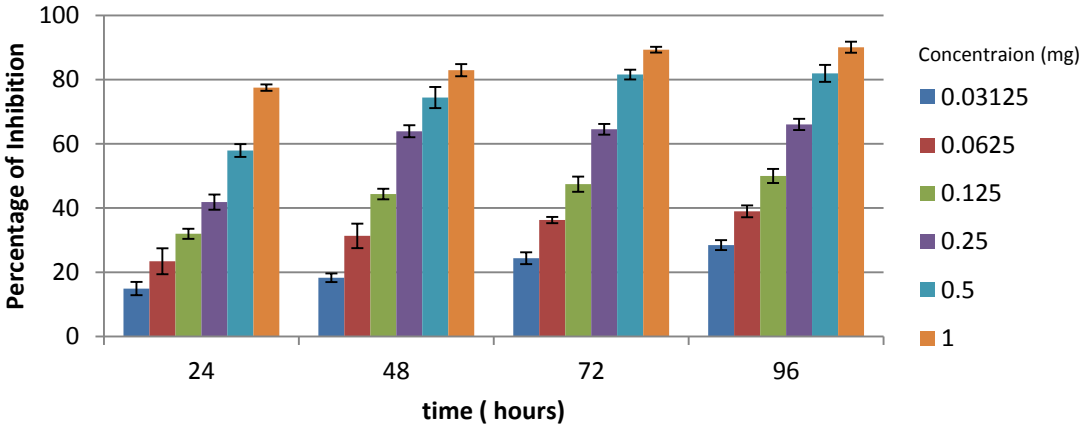

Fig. 4.4b: Effect of chloroform fraction on *L. donovani* promastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

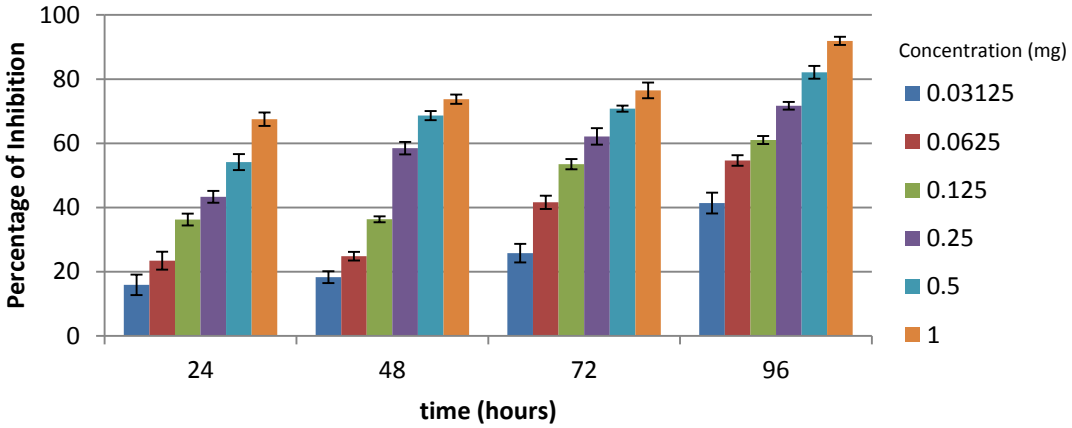

Fig. 4.4c: Effect of acetone fraction on *L.donovani* promastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

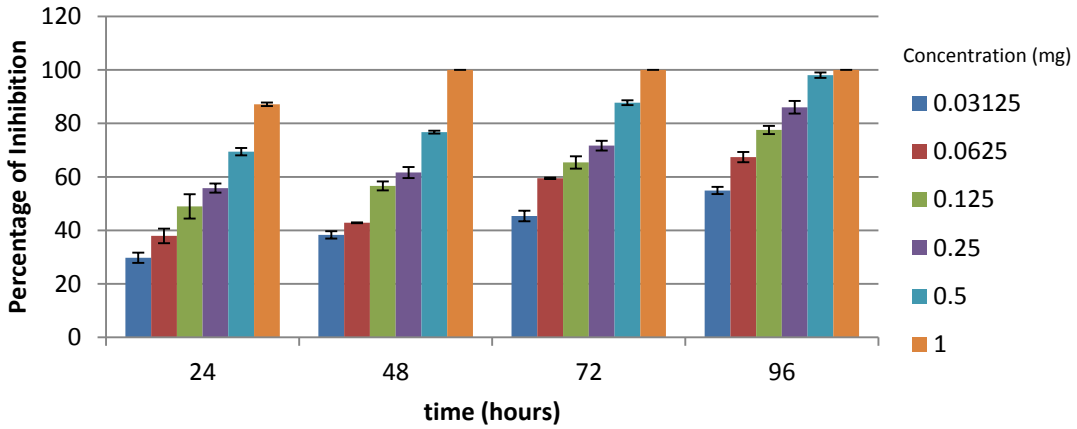

Fig. 4.4d: Effect of methanol fraction on *L. donovani* promastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

Fig 4.4 a-d: Effect of different fractions on promastigotes over 96 hours: (a). hexane fraction (b). chloroform fraction (c). acetone fraction and (d). methanol fraction

While in case of amastigotes, absolute inhibition of hexane fraction was obtained at 0.25mg/mL at 96 hours, while a two fold higher dose – 0.5 mg/mL could inhibit all amastigotes at just 48 hours (Fig. 4.5a). As in case of promastigotes, chloroform and acetone fraction (Fig. 4.5 b and c) could not absolutely inhibit all the parasites even at 96 hours. In case of methanol fraction, 0.125 mg/mL inhibited entire parasites at 96 hours of incubation while a two fold higher dose – 0.25 mg/mL could take over all the parasites within 72 hours (Fig. 4.5d). From these observations (Table 4.3 and Fig. 4.4a-d and Fig. 4.5 a-d) it can be inferred that hexane fraction is best among the fractions at low dose against promastigotes and so is methanol fraction in case of amastigotes at low dose. A summary of percentage of growth inhibition of *L. donovani* by various concentrations of *B. ceiba* fractions over 96 hours has been shown in Table 4.4.

Table 4.4a: Percentage growth inhibition of *L. donovani* promastigotes by various concentrations of *Bombax ceiba* fractions over 96 hours.

			Concentration (mg/mL)					
L. donovani	**Fractions**	**Time (hrs)**	**0.03125**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**
Promastigote	**Hexane**	**24**	12.4	23.0	37.6	49.9	67.0	86.0
		48	18.5	39.5	53.5	62.3	83.0	100.0
		72	38.7	53.9	67.2	78.3	97.7	100.0
		96	64.2	71.5	87.5	100.0	100.0	100.0
	Chloroform	**24**	14.9	23.4	32.0	41.8	57.9	77.5
		48	18.3	31.3	44.4	63.9	74.4	83.0
		72	24.4	36.3	47.4	64.5	81.6	89.3
		96	28.5	39.0	50.0	66.1	81.9	90.1
	Acetone	**24**	15.9	23.4	36.3	43.3	54.2	67.5
		48	18.3	24.8	36.3	58.5	68.6	73.7
		72	25.8	41.6	53.5	62.1	70.8	76.5
		96	41.4	54.7	61.0	71.7	82.1	91.9
	Methanol	**24**	29.8	38.0	49.0	55.8	69.4	87.1
		48	38.3	42.8	56.6	61.6	76.7	100.0
		72	45.4	59.5	65.4	71.7	87.7	100.0
		96	54.9	67.4	77.5	86.0	98.0	100.0

*ND – Not determined

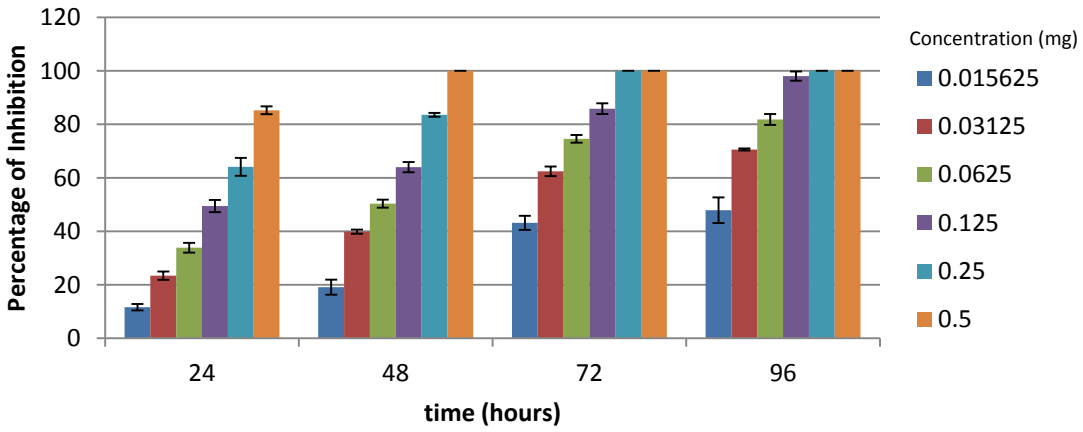

Fig. 4.5a: Effect of hexane fraction on *L. donovani* amastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

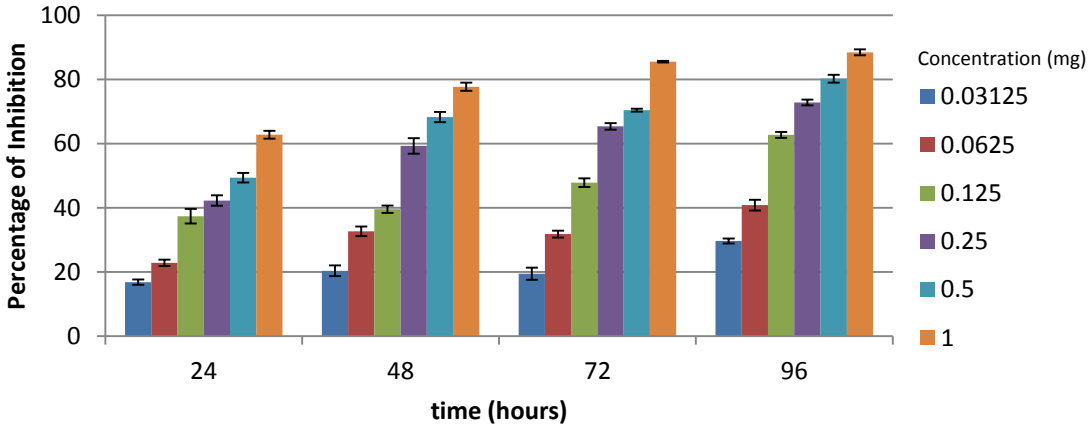

Fig. 4.5b: Effect of chloroform fraction on *L.donovani* amastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

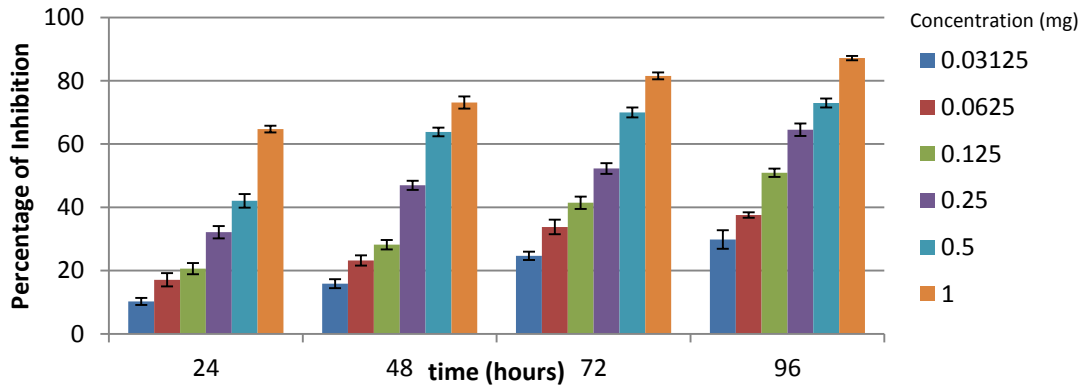

Fig. 4.5c: Effect of acetone fraction on *L. donovani* amastigotes over 96 hours.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

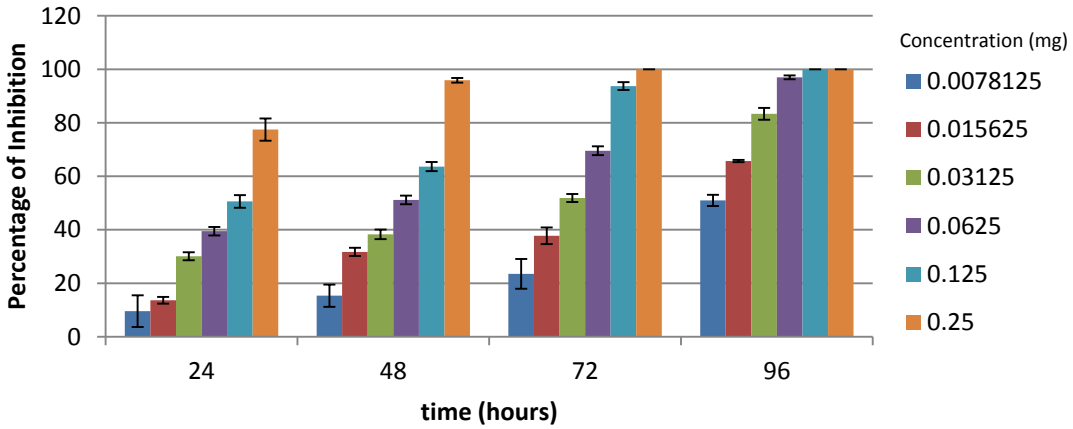

Fig. 4.5d: Effect of methanol fraction on *L. donovani* amastigotes over 96 hrs.

Bars on the top of each bar graph represents standard deviation (±SD) of percentage of inhibition in triplicate experiment.

Fig. 4.5 a-d: Effect of different fractions on amastigotes over 96 hours: (a). hexane fraction (b). chloroform fraction (c). acetone fraction and (d). methanol fraction

Table 4.4b: Percentage growth inhibition of *L. donovani* amastigotes by various concentrations of *Bombax ceiba* fractions over 96 hours.

			Concentration (mg/mL)							
L. donovani	**Fractions**	**Time (hrs)**	**0.00781**	**0.01563**	**0.03125**	**0.0625**	**0.125**	**0.25**	**0.5**	**1**
Amastigote	**Hexane**	**24**	ND	11.6	23.4	33.9	49.4	64.1	85.2	ND
		48	ND	19.1	39.9	50.3	64.0	83.5	100.0	ND
		72	ND	43.2	62.4	74.6	85.8	100.0	100.0	ND
		96	ND	47.9	70.6	81.8	98.0	100.0	100.0	ND
	Chloroform	**24**	ND	ND	16.8	22.9	37.4	42.3	49.4	62.8
		48	ND	ND	20.4	32.7	39.6	59.3	68.3	77.7
		72	ND	ND	19.5	31.8	47.8	65.4	70.4	85.5
		96	ND	ND	29.7	40.8	62.7	72.8	80.2	88.4
	Acetone	**24**	ND	ND	10.2	17.1	20.6	32.2	42.1	64.7
		48	ND	ND	15.9	23.2	28.2	47.0	63.8	73.1
		72	ND	ND	24.7	33.8	41.4	52.3	70.0	81.5
		96	ND	ND	29.8	37.6	50.9	64.5	73.0	87.2
	Methanol	**24**	9.6	13.6	30.1	39.5	50.6	77.5	ND	ND
		48	15.4	31.7	38.3	51.2	63.6	95.9	ND	ND
		72	23.5	37.8	51.9	69.6	93.7	100.0	ND	ND
		96	51.0	65.7	83.3	97.0	100.0	100.0	ND	ND

*ND – Not determined

4.3 Cytotoxicity of miltefosine, crude extract and fractions

Further cytotoxicity of the crude extract and the fractions was carried out to access the toxicity of the components in RAW 264.7 macrophages cell line. Mitochondrial reductase reduces the yellow tetrazolium salt – MTT to blue formazan within cells. Only intact mitochondria can function to carry out this reaction, making the measurement of optical density of the blue formazan formed as an estimate of cell viability. In the assay, CEE and all the fractions were non toxic to the cell with the respective cytotoxicity values far higher than their anti-leishmanial IC_{50} values (IC_{50}^{a} and IC_{50}^{b}).

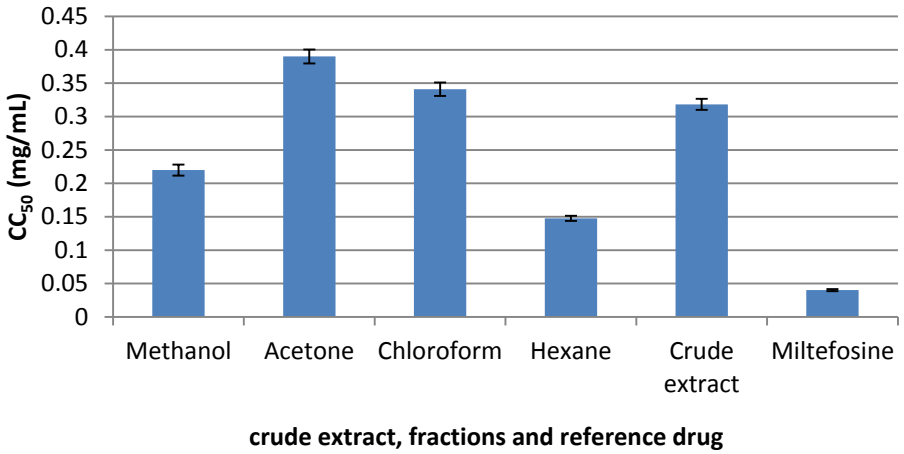

Fig. 4.6: CC_{50} value of crude extract and fractions on RAW 264.7 macrophage cells after 72 hrs.
Bars on the top of each bar graph represent standard deviation (±SD) of percentage of inhibition in triplicate experiment.

Cytotoxicity (CC_{50}) of the crude ethanolic extract (CEE) was determined to be 318 ± 8.15 µg/mL and that of n-hexane, chloroform, acetone and methanol was found to be 147.63 ± 3.90, 340.95 ± 10.06, 390.01 ± 10.40, 219.88 ± 8.31 µg/mL respectively. CC_{50} of miltefosine was found to be 40.12 ± 0.16 µg/mL. Among the fractions, acetone fraction was least cytotoxic followed by chloroform, methanol and n-hexane fraction. Also, acetone and chloroform fractions were found to be less cytotoxic than the CEE, while hexane and methanol fractions were more cytotoxic than the CEE. However, to note, reference drug - miltefosine was cytotoxic than CEE or any of the fractions. Cytotoxicity values (CC_{50}) of the extract and fractions are shown in Table 4.5 and Fig. 4.6.

4.4 Efficacy comparison on the basis of selectivity indices

The cytotoxicity of CEE and fractions on RAW 264.7 macrophage was compared with the activity against promastigotes and axenic amastigotes of *L. donovani*, by using the selectivity index (SI) to compare the efficacy of the treatments. On the basis of selectivity index, among the extract and fractions, methanol fraction was found to be the most

selective against both the promastigote (SI^a = 2.45) form and amastigotes form (SI^b = 3.74), followed by the crude ethanolic extract (SI^a = 2.43; SI^b = 2.53). While n-hexane, chloroform and acetone fractions were found to be 1.40, 2.36 and 2.04 times selective for promastigotes and 2.40, 1.89 and 1.38 times selective to amastigotes respectively, which are still higher than 1 indicating their safety/nontoxicity towards the cell line. Reference drug miltefosine was 9.73 times more selective for amastigotes than the RAW 264.7 cells and 3.56 times more selective for the promastigotes.

Table 4.5: *In vitro* antileishmanial activity (IC_{50}) and cytotoxicity (CC_{50}) of *Bombax ceiba* Linn. flowers CEE and its fractions against *L. donovani* promastigotes and axenic amastigotes.

Extract fraction/drug	Promastigotes IC_{50} (µg/mL)[a]	Axenic amastigotes IC_{50} (µg/mL)[b]	Cytotoxicity CC_{50} (µg/mL)	Selectivity Index	
				$(SI)^a$	$(SI)^b$
Methanol fraction	89.62 ± 0.55	58.73 ± 1.89	219.88 ± 8.31	2.45	3.74
Acetone fraction	191.64 ± 11.94	283.32 ± 6.82	390.01 ± 10.40	2.04	1.38
Chloroform fraction	144.25 ± 2.64	180.37 ± 2.73	340.95 ± 10.06	2.36	1.89
n-hexane fraction	105.12 ± 7.99	61.39 ± 1.34	147.63 ± 3.90	1.40	2.41
Crude ethanolic extract	131.24 ± 12.54	125.96 ± 3.62	318.34 ± 8.15	2.43	2.55
Miltefosine	11.27 ± 0.52	4.12 ± 0.13	40.12 ± 0.16	3.56	9.74

Results are expressed as mean ± SD of three independent experiments.
$IC_{50}{}^a$ – Concentration of test sample that inhibited 50% growth of promastigotes.
$IC_{50}{}^b$ – Concentration of test sample that inhibited 50% parasite growth of axenic amastigotes.
CC_{50} – Concentration of test sample that inhibited 50% decrease in cell proliferation.
$(SI)^a$ – Selectivity Index for promastigotes: CC_{50} of test sample against RAW 264.7 cells/IC_{50} of test sample against promastigotes.
$(SI)^b$ – Selectivity Index for amastigotes: CC_{50} of test sample against RAW 264.7 cells/IC_{50} of test sample against axenic amastigotes.

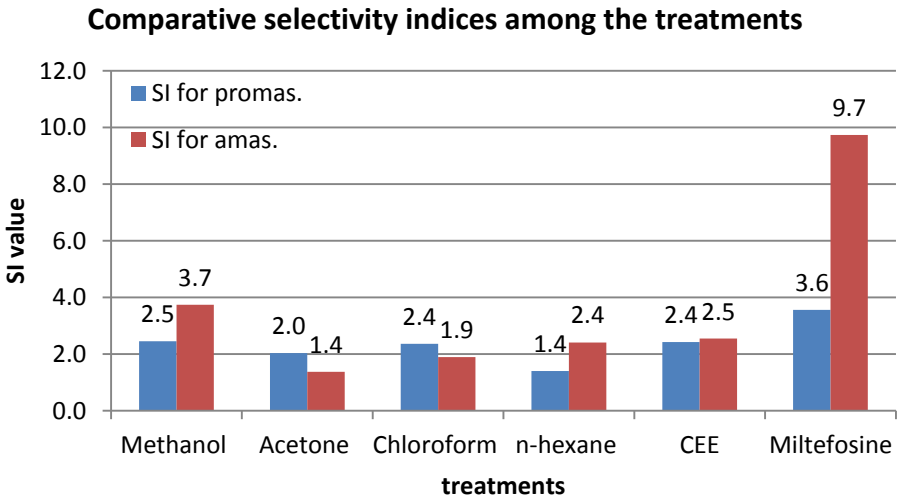

Fig. 4.7: Comparative selectivity indices of CEE, fraction and miltefosine on RAW 264.7 cells after 72 hours.

From selectivity indices, like the reference drug miltefosine; methanol, hexane fraction and CEE were more selectively acting on the amastigotes (clinical form) than the promastigotes

(infective form). These effects are based on the chemical constituents in the respective fractions and extracts. Possibly, similar constituents like the reference drug are present in the methanol and hexane fractions which have direct toxic effect on the axenic amastigotes than the promastigotes. A comparative cytotoxicity (Fig.4.6) and antileishmanial activity along with the respective selective index values is shown in the Table 4.5.

4.5 Thin layer chromatography and R_f value

Thin layer chromatography of the CEE demonstrated the presence of number of compounds, present in the extract (Fig. 4.8). The TLC plates showed numerous blue spots under uv source, possibly due to presence of various groups of aromatic compounds or with extended conjugation systems that are uv active. When same TLC plates were stained in iodine chamber displayed compounds (unsaturated and aromatic compounds) with high affinity for iodine and also a 5% H_2SO_4 spray on the plate further showed compounds that were charred due to oxidation of compounds leaving deposits of carbon on acid spray. Various spot of compounds were seen on uv, iodine and/or 5% H_2SO_4 demonstrating the presence of various groups of compounds present in the crude ethanolic extract (Fig. 4.8). Since, the separation of compounds are based on polarity, high polar compounds could be seen at the bottom of the plate near the sample spot applied while low polar compounds could be seen at the top of the plate near the solvent raise attained. The compounds visualized as spots on the TLC plates could be further purified using other chromatographic techniques like column chromatography; gas chromatography or high performance liquid chromatography for isolation of the individual compounds and a further bioassay can be carried out with these compounds. An active potential antileishmanial compound or a lead to such compound may enter the drug discovery process.

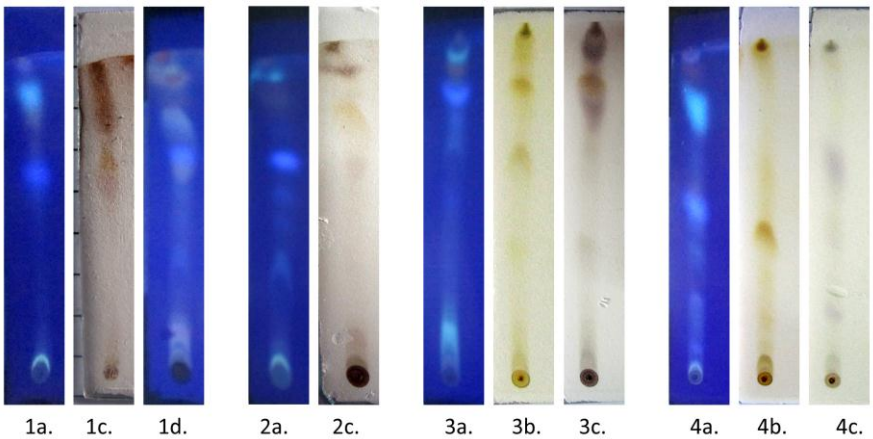

Fig. 4.8: Thin layer chromatography of crude ethanolic extract (CEE) for demonstration of presence of number of compounds. Solvent systems: (1). 100% ethyal acetate; (2). Hexane:Ethylacetate (2:8); (3). Methanol:Ethylacetate (2:8); (4). Methanol:chloroform (1:9).

a – visualized under uv (254nm); b – developed in iodine chamber; c – developed with 5% H_2SO_4; d – visualized in uv after being developed with 5%H_2SO_4.

Retardation factor (R_f) values of various compounds present in the crude ethanolic extract were calculated. A combined table showing R_f values of various spots on the Plates 1-4 is shown below (Table 4.6).

Table 4.6: Retardation factor (R_f) values of spots visualized on the TLC plates 1-4.

Plate no. 1		Plate no. 2		Plate no. 3		Plate no. 4	
100% ethyl acetate		Hexane:Ethylacetate (2:8)		Methanol:Ethylacetate (2:8)		Methanol:chloroform (1:9)	
Spots	R_f value	Spots	R_f value	Spots	R_f value	Spots	R_f value
1	0.054	1	0.052	1	0.064	1	0.061
2	0.137	2	0.324	2	0.133	2	0.105
3	0.339	3	0.457	3	0.362	3	0.227
4	0.601	4	0.549	4	0.628	4	0.365
5	0.756	5	0.647	5	0.745	5	0.453
6	0.875	6	0.734	6	0.803	6	0.519
7	0.911	7	0.821	7	0.867	7	0.641
8	0.946	8	0.902	8	0.904	8	0.807
		9	0.965	9	0.952	9	0.895
						10	0.956

Plate no. 1 to 4 correspondingly refers to fig. 4.7 - 1 to 4. All the spots developed under uv, iodine and/or with H_2SO_4 were marked.

4.6 Statistical comparison on antileishmanial effects of CEE, fractions and reference drug

One way analysis of variance between the mean IC_{50} values of promastigote and amastigote between different treatment fractions and reference drug indicate that their activities are significantly different (P value is < 0.0001). An unpaired t-test (two tailed) analysis between the fractions also showed significant differences in their antilieshmanial activity. However, a comparative antileishmanial activity of miltefosine vs. crude extract/fractions at their 50% (CC_{50}) cytotoxic concentration analyzed with unpaired t-test (two tailed) to determine if a significant difference occurs between the miltefosine and crude extract/fractions expressed interesting result.

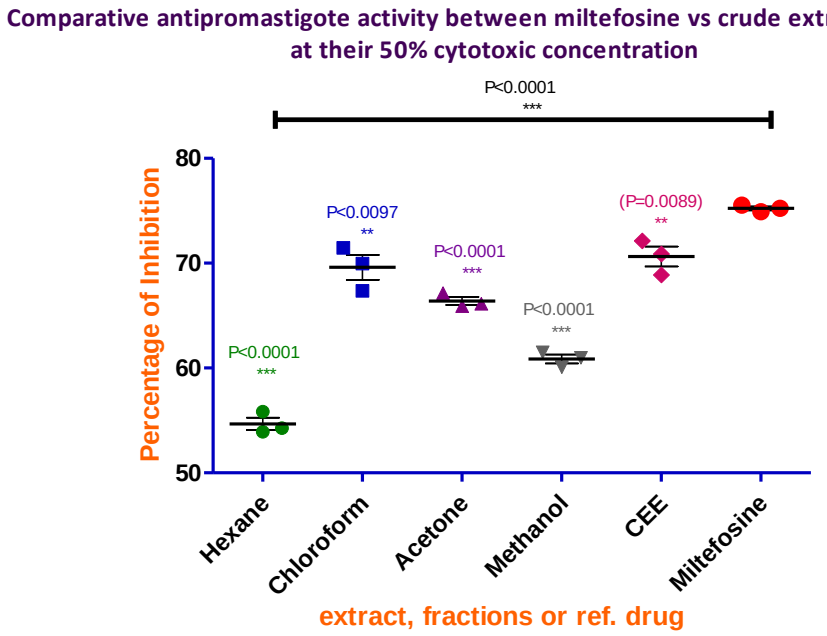

Fig. 4.9a: Comparative antipromastigote activity between miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration.

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

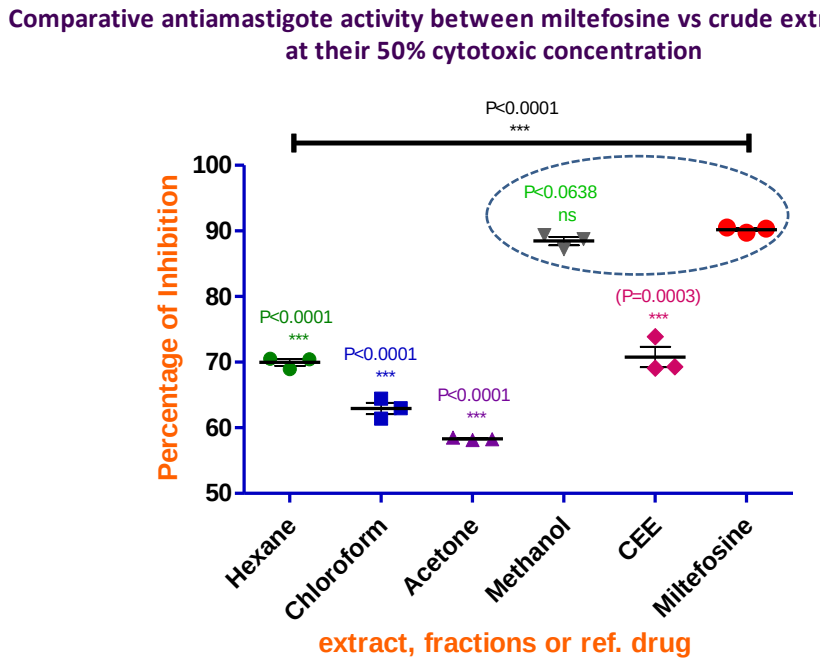

Fig. 4.9b: Comparative antiamastigote activity between miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration, showing antileishmanial activity (anti-axenic amastigote) activity not significantly different between methanol and miltefosine

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

Table 4.7 shows the comparative antileishmanial activity of miltefosine vs. crude extract/fractions at their 50% cytotoxic concentration. The aim of the analysis was to determine if crude extract or fractions have similar antileishmanial activity as reference drug – miltefosine. According to this analysis, only the most promising fraction – methanol fraction was found to have similar antileishmanial activity against amastiogtes with P value 0.0638. At CC_{50} of miltefosine, PI of amastigotes by methanol fraction was 88.46 ± 1.107 vs. 90 ± 0.409 PI of miltefosine at its CC_{50}. It can be stated from this observation that methanol fraction can be as effectively used as miltefosine as there is no significant difference between their inhibitory potential against amastigotes – which is again the clinically relevant form of the parasite. Graphical representation of comparative antileishmanial activity between miltefosine vs. crude extract/fractions at the CC_{50} has been shown in Fig. 4.9a and Fig. 4.9b.

Table 4.7: Comparative statistical analysis on antileishmanial activity of miltefosine vs. *Bombax ceiba* components at their 50% cytotoxic concentration.

Drug/extract /fraction	**CC_{50} conc.**	**Promastigotes**		**Amastigotes**	
		PI at CC_{50}	Significance (P value)	PI at CC_{50}	Significance (P value)
Methanol	219.88 ± 8.31	60.86 ± 0.7207	*** (P<0.0001)	88.46 ± 1.107	ns (P=0.0638)
Acetone	390.01 ± 10.40	66.40 ± 0.6758	*** (P<0.0001)	58.33 ± 0.1937	*** (P<0.0001)
Chloroform	340.95 ± 10.06	69.60 ± 2.076	** (P=0.0097)	62.94 ± 1.516	*** (P<0.0001)
n-hexane	147.63 ± 3.90	54.67 ± 1.022	*** (P<0.0001)	69.96 ± 0.8763	*** (P<0.0001)
CEE	318.34 ± 8.15	70.64 ± 1.636	** (P=0.0089)	70.78 ± 2.709	*** (P=0.0003)

*** - Extremely significant (P value <0.001)
** - Very significant (P value 0.001 to 0.01)
* - Significant (P value 0.01 to 0.05)
ns - Non significant (P value >0.05)

Chapter V
Discussion

Visceral leishmaniasis has been noted as the second most serious infectious disease after malaria (ScienceDaily, 2011) in the world and till date there is neither effective gold standard diagnostic tool nor drug to treat. Nepal is one of the endemic countries of this fatal disease. In this circumstance, this Himalayan country, rich by its medicinal herbs seeks research work exploring the efficacy of those available potential medicinal plants. This research work is an attempt to exploring the efficacy of those available potential medicinal plants active against leishmaniasis. One of the major approach of interest in discovery and development of new drugs is the natural product research (Rates, 2001). The knowledge of active compounds identified form the selected medicinal plants would come to modern medicine after their efficacy and toxicological information (Iwu et al., 1994). This is one among the various other approaches to obtain a herbal medicine or an isolated active compound (Rates, 2001). A huge amount of literature from all over the world have demonstrated in various plant studies that plant extracts exhibit anti-leishmanial activity (Delorenzi et al., 2001; Rocha et al., 2005; Tiuman et al., 2005; Santos et al., 2008; Singh et al., 2008; Martin-Quintal et al., 2009; Sharma et al., 2009; Sane et al., 2010; Singh et al., 2010). Particularly, this work has tried to find the potential activity of flower of the plant, *Bombax ceiba* as having medicinal value against this infectious disease.

B. ceiba Linn. has been used since long for wide range of disease and has been validated by literatures describing their medicinal values in dysentery, menorrhagia, skin troubles, haemorroids, chronic inflammation, ulceration of bladder and kidney, splenomegaly etc. (Adhikari et al., 2007; Chakraborty and Charkraborty, 2010). In context of ethno-botanical use of the plant; the traditional healers of Chhattisgarh Plains have been using the flowers of this plant for the treatment of enlarged spleen (Adhikari et al., 2007; Verma et al., 2009; Chakraborty and Charkraborty, 2010; Verma et al., 2011). Splenomegaly is a hallmark of visceral leishmaniasis, characterized by extensive parasitization of the splenic cells (Yurdakul et al., 2011). The *in vitro* antileishmanial activity results obtained from this work support the traditional use of the flower of the plant, which being used in case of splenic disorders (splenomegaly) as in case of visceral leishmaniasis. This is ever first report of *B. ceiba* flower having antileishmanial activity.

The biological activity of plant extracts are attributed to diverse group of the secondary metabolites or quinones, alkaloids (isoquinoline, indole, naphthylisoquinoline, benzylisoquinoline, quinoline, steroidal, benzoquinolizidine, diterpene and pyrimidine-*β*-carboline), terpenes (iridoids, monoterpenes, sequisterpenes, diterpenes, saponins),

phenolics (chalcones, flavonoids) and other metabolites like acetogenins, antimicrobial peptides etc. and are well known to exhibit antileishmanial activity (Chan-Bacab and Pena-Rodriguez, 2001; Fournet and Munoz, 2002; Kayser et al., 2003; Rocha et al., 2005; Kedzierski et al., 2009; Mishra et al., 2009). Previous phytochemical studies of the different parts of this plant have led to the isolation of terpenoids, napthaquinones, napthol (Vijaya Bhaskar Reddy et al., 2003), polysaccharides (Agrawal et al., 1972), anthocyanins (Niranjan and Gupta, 1973), leupeol, shamimicin (Saleem et al., 2003) and flavonoids form root bark, flowers and stem bark. In a recent study on phytochemical investigation carried out by El-Hagrassi *et al.* of the flowers of the plant have revealed 14 compounds, including cholesterol, stigmasterol, campesterol and alpha-amyrin, while the residual 10 compounds being hydrocarbons, in the n-hexane fraction and methanol fraction with 7 flavones namely - vicenin 2, linarin, saponarin, cosmetin, isovitexin, xanthomicrol and apigenin (El-Hagrassi et al., 2011). Several types of flavonoids have been identified as antiprotozoal principles of the plant extract (Tasdemir et al., 2006). The antileishmanial activity shown by our study in the n-hexane and methanol fractions might also be attributed to these compounds (flavones in n-hexane and methanol fraction as described by El-Hagrassi et al.) singly or in combination.

By soxhlation process the solvent elutes out the phytochemicals present in the plant material (Cowan, 1999). Similar protocol was followed in this experiment and the extraction of the phytochemicals was evaluated for its yield, color and consistency. The CEE was transparent yellow in color, which might be due the elution of various colored plant secondary metabolites along with the soxhlation. Quinones, one of the major groups of plant secondary metabolites are generally colored (Cowan, 1999; Das et al., 2010), and this group of compounds has been reported form the plant (Sreeramulu et al., 2001; Vijaya Bhaskar Reddy et al., 2003). The extraction quality and quantity depends on various factors like the part of plant material used, the relative presence of the phytochemical extracts at the time of harvest and extraction, solvent used, extraction method used, duration of extraction, temperature, nature of solvent, solvent concentration, polarity etc (Cowan, 1999; Tiwari et al., 2011). Extraction with methanol or ethanol can extract nearly all of the plant active constituents against microorganisms (Cowan, 1999; Tiwari et al., 2011), however methanol may be unsuitable for extraction over ethanol for it is more cytotoxic and bioassays may lead to erroneous results due to traces of methanol remained in the extract (Tiwari et al., 2011). Therefore ethanolic extraction was carried out in this experiment.

The crude ethanolic extract of the plant inhibited promastigote growth (IC_{50} of 131.24 ± 12.54 µg/mL), leading us to carry out a bioassay-guided fractionation of the antileishmanial

activity into four fractions based on the polarity from low polar n-hexane, chloroform and acetone to high polar methanol fraction. Among the fractions, methanol and hexane fraction were found to be more active against the parasite than the other fractions, indicating that the active compounds of *B. ceiba* might have been attributed to either the least polar solvent used (n-hexane) or to the highest polarity solvent (methanol) used. This is not surprising as the antiparasitic activity does not distributed throughout the extract but is confined to some particular fractions (Lakshmi et al., 2007).

The methanol fraction showed a greater inhibitory effect against promastigotes (IC_{50} of 89.62 ± 0.55 µg/mL) and amastogotes (IC_{50} of 58.73 ± 1.89 µg/mL) than the other fractions. n-hexane fraction also had appreciable inhibitory activity aginst promastigotes (105.12 ± 7.99 µg/mL) and amastigotes (61.39 ± 1.34 µg/mL). The reference drug miltefosine had much lower 50 % inhibitory concentration values for both forms of the parasite (IC_{50} for promastigote: 11.27 ± 0.52 µg/mL and IC_{50} for amastiogote: 4.12± 0.13 µg/mL) than any of the fractions or the crude extract. The higher IC_{50} values of the fractions or the crude extract might be due to the lower quantities of the active compound in them. However fractionation seemed to have effect on concentrating the active constituents to the methanol and/or hexane fraction since their IC_{50} values are lower than that of the curde extract. Similar results with active fraction being more polar solvent used (butanol fraction) of the ethanol extract of *Tinospora sinensis* against *L. donovani* have described by Singh et. al., (IC_{50} values 41.6 ± 6.5 µg/mL for promastigotes and 17.6 ± 4.1 µg/mL for intracellular amastigotes) (Singh et al., 2008). While in another study carried out by Lakshmi et. al., with the *in vitro* and *in vivo* leishmanicidal activity of *Dysoxylum binectariferum* and its fractions against *Leishmania donovani*, chloroform fraction was found to be active against promastigotes with 91 ± 4.6% parasite inhibiton at 100 µg/mL dose and 58.2 ± 5% at 50 µg/mL dose for intracellular amastigotes, while other fractions being moderately active (hexane) or inactive (butanol) (Lakshmi et al., 2007). Hence, it can also be concluded that the active fractions may vary on the medicinal plant types.

Referring to the dose response curves and time dependent efficacy response graphs it can be conferred that n-hexane and methanol fractions are leishmanicidal; and acetone and chloroform fractions are leishmanistatic at the doses used in this work for up to 96 hours. Also, it seemed hexane fraction was effective at low dose against promastigotes (IC_{100} at 250 µg/mL) and so was methanol fraction against amastigotes (IC_{100} at 125 µg/mL) at low dose. However, comparing the inhibitory potential of the active fractions (hexane and methanol) against the parasite morphological form, with that of the reference drug; both the active fractions and miltefosine have higher inhibitory potential to the amastiogtes than to promastiogotes. May be similar active principles are involved in those fractions to

counter the parasite. Again, antileishmnaial activity of the methanol fraction to the clinically relevant stage - amastigotes is encouraging, deciphering the antileishmanial activity of the flowers of the plant being confined to the compounds present in the same fraction.

Axenic amastigotes have been used to access the antileishmanial activity because this form represents the clinical stage of *L. donovani*. Although, axenic amastigote cultures may not strictly reflect what might happen in amastigote infected macrophages and some extract might not act directly on the amastigote but do enhance the capacities of the macrophage or enhance the immunomodulatory functions of the T cells helping to mount an action possibly Th1 response to destroy the parasite. Thus, this study further looks forward to study the effect of the extract on infected macrophage.

Methanol and hexane fractions of the flowers of the plant have also been described to possess antimicrobial activity and antioxidant activity (El-Hagrassi et al., 2011). In the paper El-Hagrassi et al. has described the n-hexane and methanol fraction being active against *Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Neisseria gonorrhea, Pseudomonas aeruginos, Candida albicnas*, however only the methanol fraction was active against fungal species – *Aspergillus niger* and *A. flavus.* Methanol fraction had a comparable inhibition zone with the standard drug- fluconazole used (11 mm vs 13 mm), which warrants the potential of the methanol fraction having antifungal activity (El-Hagrassi et al., 2011). To note, fluconazole as an oral agent has been shown to be effective against kala-azar (Sundar et al., 1996; Sundar and Rai, 2002).

In regards to antifungal medicine, currently the first line drug Amphotericin B is also originally drug for fungus infection. Later, due to its action on ergosterol component of *Leishmania* parasite, it became successful first line drug for the treatment of VL. The previous report of methanol extract of *B. ceiba* flowers (El-Hagrassi et al., 2011) and water extract (highest polar fraction used comparable to methanol fraction in this work) of bark of *B. ceiba* (Anandarajagopal et al., 2011) of having antifungal activity, directs the potential candidacy of the methanol extract of *B. ceiba* be developed as an antileishmnial component. Possibly, similar compounds acting on parasite sterol membrane might have been acting, present in the methanol fraction is responsible for consequently killing the parasites. Also methanol extraction/fraction constitute most polyphenols, flavones, tanins, lactones and phenones are extracted with the methanol (Cowan, 1999) which are potential groups of antileishmanial compounds (Kayser et al., 2003; Mishra et al., 2009).

Among several reasons for the growing interest in developing drugs of plant origin, the main one is that conventional medical treatments can be improved without side effects and

ineffective therapy. Large percentage of world's population has no access to conventional pharmacological treatments, the widespread use of folk medicine and ecological awareness suggest that natural products are harmless. Even though these products have been traditionally used, there is no guarantee of their safety and their efficacy and safety require investigation (Ernst, 1998; Rates, 2001; Tiuman et al., 2005). Therefore, the active specific components separated for the traditionally used herbal plants as adopted by this piece of research work would be useful in the way of new drug development.

Given a strong interest in alternative therapies and the therapeutic use of medicinal plants, cytotoxicity tests with natural products are indispensible. Cytotoxicity test revealed the extract to be safe to the RAW 264.7 cells. In the test, the toxicity induced by the treatments (extract/fraction/drug) towards the RAW cells could be analyzed by MTT assay by the reduction in the mitochondrial dehydrogenase activity. Hence, the production of formazan is an indicative of cell viability. Cytotoxicy induced was studied at their 50% reduction in cell proliferation (CC_{50}). On the basis of the CC_{50}, among all treatments, miltefosine – the current drug of VL – was found the most toxic (CC_{50}: 40.12 ± 0.16 µg/mL) than the CEE and any of the fractions. n-hexane fraction (CC_{50}: 147.63 ± 3.90 µg/mL) induced higher toxicity among all the fractions while acetone fraction induced the lowest toxicity (CC_{50}: 390.01 ± 10.40 µg/mL). While it was observed that methanol and n-hexane fraction induced higher toxicities compared to the CEE (CC_{50} of CEE: 318 ± 8.15 µg/mL vs. CC_{50} methanol fraction: 219.88 ± 8.31 µg/mL and CC_{50} n-hexane fraction: 147.63 ± 3.90 µg/mL), while acetone and chloroform induced lower toxicities compared to CEE (CC_{50} of CEE: 318 ± 8.15 µg/mL vs. CC_{50} acetone fraction: 390.01 ± 10.40 µg/mL and CC_{50} chloroform fraction: 340.95 ± 10.06 µg/mL). As the effective fractions were also determined to be methanol and n-hexane fraction, it might be possible that the effective constituents are also inducing toxicity.

In a similar study done by Vieira et al. (2009) has reported safety of the methanol extract of the plant in Vero cell line, 2.83 folds safe than in our study (750 µg/mL in Vero cell line vs. 219.88 µg/mL in RAW 264.7 cell line) (Vieira et al., 2009; Jain and Verma, 2012); probably the difference in toxicity level being attributed to difference in the test cell lines. This indicates that the use of the flowers of the plant by the traditional communities being considerably safe however further investigation on safety are required before coming to a discrete conclusion about their safety.

To compare the antileishmanial activity shown by the extract and fraction with their associated toxicity, their selectivity indices were calculated. Selectivity indices are the comparative values for their safety towards the cells relative to their antileishmanial activity. The selectivity indices of the CEE and fractions were greater than 1 indicating them being selectivity toxic to the parasite than the RAW 264.7 cells. That is to say, methanol

fraction was 3.74 fold active towards axenic amastigotes than RAW cells. Similarly, CEE – 2.55 fold, n-hexane – 2.41 fold, chloroform – 1.89 fold and acetone – 1.38 fold active towards axenic amastigotes than RAW cells.

Antileishmanials used in the current chemotherapeutics are toxic (gastrointestinal toxicity, ototoxicity, hepatotixic, cardio-toxic etc.) with various adverse drug reactions (myocarditis, severe hypokalaemia, renal dysfunction etc.) (Thakur, 1998; Chappuis et al., 2007; Moore and Lockwood, 2010). A herbal drug which is effective in enhancing immunity or is protective to shield the toxic effect of the currently used current therapeutics, can serve either as an adjunct or in combination therapy. Recently, Ravi et al. demonstrated methanolic extract of the flower of *B. ceiba* being hepatoprotective against toxicity induced by anti-tubercular drugs – Isoniazid (IHN) and Rifampicin (RIF) in experimental rats (Ravi et al., 2010). Similar hepatoprotective activity of immunomodulator-picroliv – a standardized mixture of iridoid glycosides, prepared from the alcoholic extract of the root and rhizome of *Picrorhiza kurroa,* have shown to enhance the antileishmanial activity of the antileihmanial drugs and has been proposed to be used as an adjunct or in combination therapy of kala azar along with antileishmanials (Puri et al., 1992; Mittal et al., 1998; Gupta et al., 2005; Sane et al., 2010; Shakya et al., 2011; Shakya et al., 2011). In another recent study, aqueous flower extract of *B. ceiba* was shown to have protective effect against adriamycin - induced cardio-toxicity and may have potential as a cardioprotective agent. Based on these hepatoprotective and cardioprotective activities, may be extract of *B. ceiba* be also protective against antileishmanial drugs – induced toxicity, severe side effects and immunosuppression and would probably supplement in kala azar therapy. However, these effects need further scientific exploration and validation.

Regarding immunopathology, countering leishmaniasis is attributed to immune response capable of activating macrophages to eliminate those parasites residing inside them. IFN-γ is the most potent cytokine for the induction of anitleishmanial activity in the macrophages, associated with the Th1-cell response which is a major shift to takeover the disease condition (Awasthi et al., 2004; Kedzierski et al., 2009). Various reports on plants products with antileishmanial activity have been described with their mode of antileishmaniasis being immune modulator (Puri et al., 1992; Mittal et al., 1998; Gupta et al., 2005; El-On et al., 2009; Ozer et al., 2010; Sane et al., 2010; Shakya et al., 2011; Shakya et al., 2011). The presence of flavonoids and tannis etc. could contribute to immunostimulatory activities as these constituents are reported to stimulate nonspecific macrophage function. Such findings have been reported with *Desmodium gangeticum* (Singh et al., 2005), *Ocimum gratissimum* (Ueda-Nakamura et al., 2006) and *Tinospora sinensis* (Singh et al., 2008). However, if the methanol extract have truly the potential of

receding splenomegaly caused by VL need to be warranted first in amastigote infected macrophages (intracellular amastigotes) and then to *in vivo* animal models. Thus, further studies on immunomodulatory effect of the extract or the compound are to be followed to confirm the therapeutic potential of *Bombax ceiba.*

Yet another interesting results with further encouragement to our work is, methanol extract of another plant from same family, *B. buonopozense* has shown to be active against trypanosomiasis, a parasitic disease caused by *Trypanosoma brucei brucei* (Mann and Emmanuel, 2012). Also hexane and methanol floral extracts of the *B. buonopozense* has been shown to posses significant antimicrobial activity (Mann et al., 2011).Further more recently, larvicidal activity of leaf extract of *B. malabaricum* against filarial vector *Culex quinquefasciatus* (Hossain et al., 2011) and anthelmintic effect of methanol extract of *B. malabaricum* leaves on *Paramphistomum explanatum* (Hossain et al., 2012) also advocates, the plant contains compounds that are active against helminth parasites and filarial vector.

Miltefosine, which was used parallel as reference drug had better activity than any of the fractions or the crude extract. Though, the fractions/extract could not exceed the reference drug miltefosine efficacy wise, the toxicity associated with prolonged use of the latter puts limitations on the its use (Sundar et al., 2000). This drug is potentially teratogenic and abortifacient in addition to renal and gastrointestinal toxicity. Further risks associated with non-adherence to the recommenced regimen and longer half life of drug poses it to the risk of widespread emergence of resistance (Perez-Victoria et al., 2006; Chappuis et al., 2007; Matlashewski et al., 2011). Owing to this, when the antileishmanial activity of the miltefosine at its cytotoxic concentration, CC_{50}, was compared to antileishmnaial acitivity at the same concentrations of the fractions and the extract, there was no significant difference in the activity of the methanol fraction and miltefosine against amastigotes (PI of methanol 88.46 ± 1.107 vs 90.19 ± 0.408 of miltefosine at CC_{50} of miltefosine; P=0.0638; ns). Thus any material showing even marginal advantage over existing antileishmanial drug, would call for more detailed investigation in the different stages of development.

Hence these encouraging reports on *Bombax species* on being antiprotozoal, antifungal, antimicrobial, larvicidal and antihelminthic and antileishamanial reports from this work seek a detail investigation and further exploration for further antileishmanial activities. All these findings and reports on various uses of the plant define wide scope of use the plant itself and also of such medicinal plants and natural products. We believe, de-facto small, this work has made a significant contribution in the field of research on such a neglected tropical affliction of the poor – KALA-AZAR.

Chapter VI

Summary

Kala-azar is one of the tropical afflictions caused by protozoan parasite, *Liehmania*, which is fatal if not treated in time. Eight million of 350 million people worldwide at risk of this disease reside in eastern Terai of our country in 13 endemic districts. This disease lacks a proper diagnostic tool and in absence of an effective vaccine of human use till date, lets no option to rely on than the unsatisfactory chemotherapeutic drugs which are either toxic, with severe side effects or are developing resistance.

Phytotherapy and use of medicinal plants for various disorders and ailments dates back from early human civilization and is still in practice by a large number of population, where modern medicines are either not accessible or not used for their harmful effects. It is widely believed that phytotherapy and natural products (traditional medicine) are free from side effects and affordable compared to synthetic drugs. Actually a significant number of modern medicines are developed or discovered form natural products. Among various approaches of screening those natural products for dugs or their leads; tracking the traditional uses and knowledge to locate the target are one of the attractive approaches. Extensive research work can be observed on going worldwide in search of new drugs from plants against various diseases. In context of the same, Nepal is rich in wide diversity of medicinal plants. However, screening of those medicinal plants have been very limited and none to quote in case of such a parasitic disease as kala-azar.

To rationalize our thought of screening medicinal plants for antileishmanial activity, a literature search through the online library sources were carried out and could come up with various medicinal plants that have not been documented for antileishmanial activity. Ethnobotanical uses of those selected potential medicinal plants were reviewed and those plants which have been used for signs and symptoms similar to kala-azar or those they are used in similar parasitic or helminthic or protozoal diseases were considered for an *in vitro* antileishmanial assay. In preliminary screening, *Bombax ceiba* flowers and *Viscum album* twigs were ethanol extracted and assayed against promastigotes. Both plants showed antilieshmanial activity, however the one with higher inhibitory activity (in preliminary antipromatigote assay), i.e. *B. ceiba* was chosen to further detailed investigation.

B. ceiba is a widely used ethnomedicinal plant with its various parts, used in various afflictions and disorders such as diarrhea, dysentery, wound healing, skin troubles, splenomegaly, hemorrhoids, gonorrhea and chronic cystitis etc. Research worldwide have revealed its anti-inflammatory, antibacterial, antiviral, anti-microbial, analgesic, antioxidant, antitumor, hypotensive, hypoglycemic, antiangiogenic and heptoprotective activities. While this work demonstrates its antileishmanial activity of the first time.

Traditional healers in Chattisgarh Plains have been using the preparation of the flowers of the plant in receding splenomegaly, which is a hallmark of Kala-azar. To make an

assessment of the same, an *in vitro* antileishmnaial activity was carried out with an objective of accessing the potential activity of the flower extract against *Leishmania*.

Air dried and fine powered flower of the plant was soxhlated with ethanol for 38 hours. The extract was concentrated and fractionated into n-hexane, chloroform, acetone and methanol fraction on the basis of polarity. The fractions along with the crude ethanolic extract was then assayed for antipromastigote and anti-axenic-amastigote assay. To verify the toxicity of the extract, cytotoxicity of the components were assayed using RAW 264.7 cell line. A TLC was also carried out to screen the number of compounds present in the crude ethanolic extract, however further works are to be carried out for identification of the compound(s) effective against *Leishamnia*.

The crude ethanolic extract of the plant inhibited promastigote growth at IC_{50} of 131.24 ± 12.54 µg/mL. The methanol fraction showed a greater inhibitory effect against promastigotes (IC_{50} of 89.62 ± 0.55 µg/mL) and amastogotes (IC_{50} of 58.73 ± 1.89 µg/mL) than the other fractions. n-hexane fraction also had appreciable inhibitory activity aginst promastigotes (105.12 ± 7.99 µg/mL) and amastigotes (61.39 ± 1.34 µg/mL). It indicating that the active compounds might have been attributed to either the least polar solvent used (n-hexane) or to the highest polarity solvent (methanol) used. The reference drug miltefosine had much lower 50 % inhibitory concentration values for both forms of the parasite (IC_{50} for promastigote: 11.27 ± 0.52 µg/mL and IC_{50} for amastiogote: 4.12± 0.13 µg/mL) than any of the fractions or the crude extract. The higher IC_{50} values of the fractions or the crude extract might be due the lower quantities of the active compound in them. However fractionation seemed to have effect on concentrating the active constituents to the methanol and/or hexane fraction for their IC_{50} values are lower than that of the crude extract. Based on results from the dose response curves and time dependent efficacy response graphs it can be conferred that n-hexane and methanol fractions are leishmanicidal and acetone and chloroform fractions are leishmanistatic at the doses used in this work for upto 96 hours. Also, it seemed hexane fraction was effective at low dose against promastigotes (IC_{100} at 250 µg/mL) and so was methanol fraction against amastigotes (IC_{100} at 125 µg/mL) at low dose. Cytotoxicy test revealed the components were safe with selectivity indices greater than 1 and their antileishmanial activity was more pronounced than being toxic.

B. ceiba flowers have been know to constitute several groups of compounds such as flavonoids, terpenes, quinones and phenolic compounds etc. which are the potential antileishamanial agents. The antileishmnaial activity might also have been attributed to those compound(s). Further, other recent documented activity like being hepatoprotective, anti-inflammatory, antibacterial, antiviral, anti-microbial, analgesic, antioxidant, antifungal, anti-helminthic, larvicidal, antitumor, hypotensive, hypoglycemic etc. further support and encourage further exploration of works on various aspects on this plant.

Chapter VII

Conclusion

Plant diversity is an obvious and potential source of new medicinal agents (Rocha et al., 2005). Rich in all forms of biodiversity – species, genetic and habitat; Nepal is land of more than 900 types of valuable medicinal plants among 7000 medicinal plants found all over the world (Manandhar, 2000). However, these natural resources have not been explored for its medicinal and therapeutic potential against most of the parasitic diseases including leishmaniasis, seeking screening and research work on natural products.

In ethnomedical relevance, various parts of *Bombax ceiba* is being used for diarrhea, dysentery, wound healing, skin troubles, hemorrhoids, gonorrhea and chronic cystitis etc. and typically its flowers are being used in splenomegaly, which is a typical symptom of VL. This present study demonstrated antilishmanial activity of *B. ceiba* flowers against *L. donovani* based on its traditional use for the first time. Hexane and methanol fraction of the ethanolic extract of flowers were found to have potential antileishmanial activity along with absence of cytotoxicity. Further, at CC_{50} of miltefosine, its antiliehsmanial activity was found non-significantly different from methanol fraction against axenic amastigotes.

Scope: These finding encourage us for further investigation and research work on various aspects of antileishmaniasis. Active fractions (hexane and methanol) of *B. ceiba* are required to investigate further for the identification of the active compound which may lead to the discovery of the new or leads to antileishmanial agent. Further, study of mode of action, macrophage effector functions, cytokine assays for accessing immune-stimulating activity of the fraction or the active compound would be the imminent works to be followed up. The prime interest would be analyzing the activity of the extract and fraction *in vivo* in animal model to conform the therapeutic potential of *B. ceiba* flowers as being used by traditional people for countering splenomegaly. Yet another interesting work/scope to be followed up is the study of combined antileishmanial effect of the active fractions in cocktail formulation.

Recommendations:

- There is need of tracing the ethnopharmacological species for screening of natural product in order to come up with potential medicinal components against leishmaniasis.

- Active fractions (hexane and methanol) have potential antileishmanial activity, thus, *in vivo* animal model efficacy testing is to be carried out. Probably these fractions could be a herbal supplement to current unsatisfactory antileishmanials.

- Further purification of the active fractions is recommended to isolate and identify the active compound in the flower against *L. donovani* which could lead to novel antileishmanials.

- Need of extensive study on effective, safe, cheap and non-toxic drug formulations, which would not only increase value to our resources but also is a rational approach to exploit our resources.

"Let's our country be recognized for the origin of herbal medicines

to combat infectious diseases."

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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
TDW:	1 L
Gentamycin:	20 µg/ml
Streptomycin:	100 µg/ml
Penicillin:	100 U/ml
pH:	7.4

PBS (Phosphate Buffer Saline)

NaCl :	8 gm
$Na_2HPO_4.2H_2O$:	1.44 gm
KCl:	0.2 gm
KH_2PO_4:	0.2 gm
pH:	7.3 to 7.4
TDW:	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

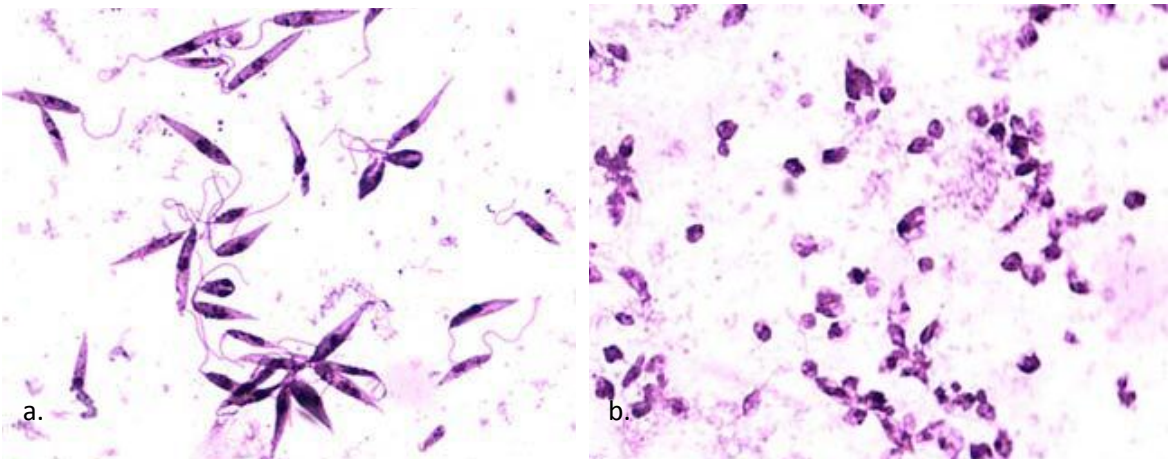

Plate 1: Giemsa stained slides of (a) promastigotes and (b) axenic amastigotes.

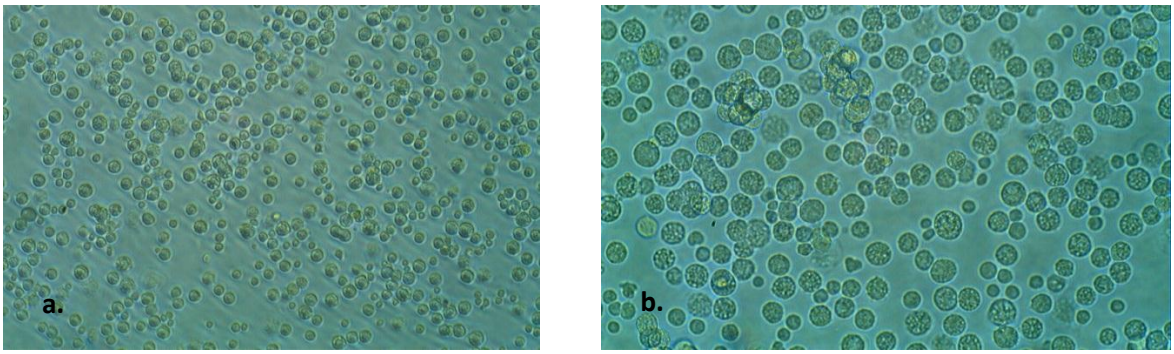

Plate 2: RAW 264.7 cell line, (a) under 20x microscope objective (b) under 40x microscope objective.

Plate 3: (a) Soxhlation with ethanol, (b) Rotatory evaporator, (c) Vacuum dried CEE

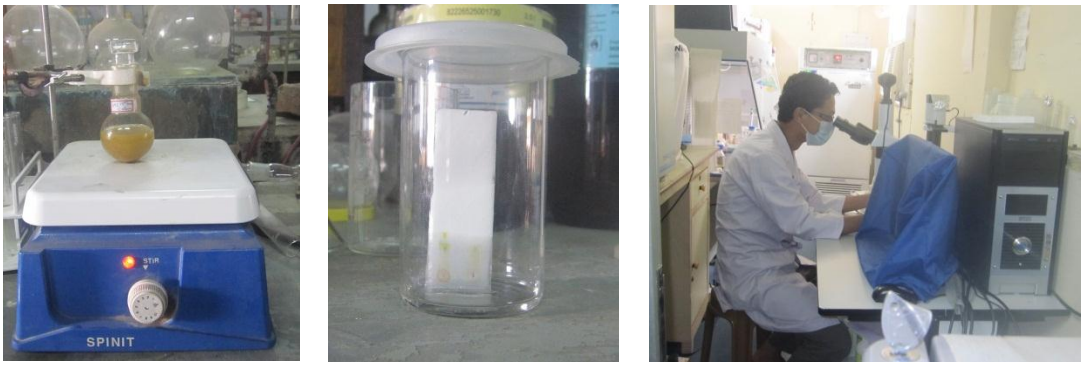

Plate 4: (a) Fractionation of CEE, (b) TLC of CEE, (c) Counting of parasite using hemocytometer