

ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES



***IN VITRO* EFFICACY STUDY OF SOME SELECTED
MEDICINAL PLANTS AGAINST *LEISHMANIA spp.***

BY

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By

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ACRONYMS

ATCC:	American Type Culture Collection
CC ₅₀ :	50% Cytotoxic concentration
CL:	Cutaneous Leishmaniasis
DCL:	Disseminated Cutaneous Leishmaniasis
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
EC ₅₀ :	50% Effective concentration
EI:	Electron impact ionization
EO	Essential oil
FCS:	Fetal calf serum
GC:	Gas Chromatography
gp63:	Glycoprotein 63
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIFCS:	Heat Inactivated Fetal Calf Serum
IC ₅₀ :	50% inhibitory concentration
IL-:	Interleukin
IMS:	Ion mobility spectrometry
INF-(γ):	Interferon -gamma
KI:	Kovats index
LC ₅₀ :	50% lytic concentration
LPG	Lipophosphoglycan
MCL:	Mucocutaneous Leishmaniasis

MIC:	Minimum Inhibitory Concentration
MS:	Mass Spectrometry
NNN:	Novy-MacNeal-Nicolle
NIST:	National Institute of Standards and Technology
PBS:	Phosphate-buffered saline
PCR:	Polymeric Chain Reaction
PKDL:	Post Kal-azar Dermal Leishmaniasis
RNA:	Ribonucleic acid
RPM:	Revolution per minute
RPMI:	Roswell Park Memorial Institute
SD:	Standard Deviation
SI:	Selectivity Index
Spp:	Species
THP:	Human acute leukemia monocyte cell line
TyrR:	Trypanothione Reductase
VL:	Visceral Leishmaniasis

ABSTRACT

In this study, 112 gradient extracts in petroleum ether, chloroform and methanol as solvents and three essential oils obtained from the root and aerial parts of ten Ethiopian medicinal plants belonging to eight families were screened for their *in vitro* leishmanicidal activities against clinical isolates of *L. donovani* and *L. aethiopica*. Those that showed anti-promastigote activity were further tested on promastigote and axenic amastigote forms of the parasites. Toxicity study was done on human monocytic leukemia (THP-1) cell line and erythrocytes *in vitro*. Assays were done using simple microscopic cell counting, fluorescence or absorbance measurements as parameters. Phytochemical investigation was done on the plant extracts using chemical test method.

Results of the preliminary screening test indicated that 78 extracts (69.6 %) had significant growth inhibitory effect (> 90%) on at least one *Leishmania* spp. at the test concentrations used. The non-polar and medium polar fractions were more active on both promastigote and axenic amastigote forms than the polar ones. Forty eight (61.5%) and 55 (70.5%) of the extracts had EC₅₀ values less than 10 µg/ml against axenic amastigotes of *Leishmania (L.) aethiopica* and *Leishmania (L.) donovani* respectively. Extracts with EC₅₀ < 3 µg/ml were obtained from petroleum ether extracts of *Artemesia abyssinica* (leaves), *Croton macrostachys* (berries and leaves), *Echnops kebericho* (tuber), *Erythrina brucei* (stembark) and chloroform extract of *Echnops kebericho* (tuber). Cytotoxicity and hemolysis tests indicated that 71(91%) and 76 (97.4%) extracts respectively were safe (SI > 1) at EC₅₀ values. The most cytotoxic extracts on THP-1 cells (SI < 1) were obtained from *Albizia schimperiana* (CC₅₀ = 1.05 ± 0.03 to 13.01 ± 0.49 µg/ml) where as strongly haemolyzing

extract (SI < 1) were obtained from polar fractions of *Albizia schimperiana* (stem bark & root bark) and *Aloe* (stem). The anti-leishmanial effect of essential oils on promastigote (MIC 0.0097 to 0.1565 μ l/ml) and axenic amastigote (EC₅₀'s 0.018 \pm 0.006 to 53.36 \pm 7.65 nl/ml) of *L. donovani* and *L. aethiopica* was comparable or at times higher than that of the standard anti-leishmanial drugs. The oils also had low to moderate toxicities (SI= 0.8 to 44) on THP-1 cell line (CC₅₀'s 0.40 \pm 0.01 to 350 \pm 5.0 μ l/ml) and erythrocytes (LC₅₀'s 0.70 \pm 0.27 to 2.62 \pm 1.0). Essential oil from *Echinops kebericho* had the highest activity against *Leishmania* and also highest cytotoxicity on THP-1 cells. Essential oil from *Artemisia abyssinica* showed the highest haemolytic effect. The phytochemical screening demonstrated presence of alkaloids, polyphenols, tannins, flavonoids, saponins, anthraquinones, coumarins, terpenes, phytosterols and withanolides in different fractions and most of these class of phytochemicals were previously reported to have anti-leishmanial activity.

Findings from this study revealed that extracts from *Artemisia abyssinica*, *Croton macrostachys*, *Echinops kebericho* and *Erythrina brucei* and all the essential oils have potential anti-leishmanial activities and this warrants fractionation and isolation of active constituents for further *in vitro/in vivo* evaluation.

1. INTRODUCTION

1.1 Leishmaniasis

1.1.1 Causative agents, vectors and reservoir hosts

Leishmaniasis is a general term used for vector-borne parasitic diseases caused by several species of *Leishmania* that can infect humans and other mammals (Gilles, 1999). Human leishmaniasis are caused by about 20 *Leishmania* species that belong to the genus *Leishmania* (WHO, 1990; WHO/TDR, 2004b). *Leishmania spp.* are morphologically similar and exist in two forms: the flagellated promastigote (12 to 20 μm in length) that develop in the intestine of sand fly and amastigote (length 2.5 to 6.8 μm and diameter 1.5 to 4.5 μm) with a rudimentary flagella which is an obligate intracellular form that only develop in mammalian macrophages and rarely in other cell types (monocytes, dendritic cells and fibroblasts) (Gilles, 1999; Chan-Bacab and Pena-Rodriguez, 2001) (Figure 1).

The vectors of *Leishmania* parasites are tiny 2-3mm long female sandflies that are distributed throughout intertropical and temperate regions of the world. They belong to two genera: *Phlebotomus* (in the Old World) and *Lutzomia* (in the New World) (Figure 1). Only 60 of about 600 sand flies species are vectors for *Leishmania* (Mehlhorn, 2004). The main reservoir hosts for *Leishmania* are domestic animals (e.g dogs, cats and horses), peridomestic animals (e.g mice and rats) and wild animals (e.g rodents, hyraxes, sloths, bats, opossums, kangaroos, wolves and foxes) (WHO, 1990)

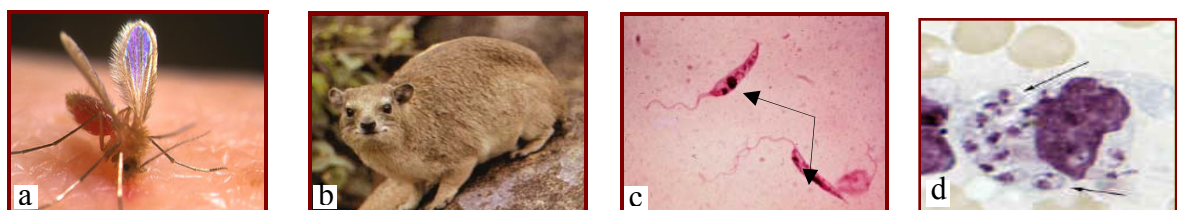


Figure 1: (a) Phlebotomine sandfly: (b) rock hyrax: (c) Promastigotes and (d) amastigotes.

(Source: Red book online, 2006; WHO/TDR, 2004b; <http://www.kalanet.eu/kalanet/Introduction.html>; <http://www.hombori.org/francais/photography/index>.)

1.1.2 Clinical forms of leishmaniasis

Leishmaniasis is a complex disease. Depending on strain (s) of the parasite involved in pathogenesis and the immune response established by the host it can cause clinical symptoms that range from mild self-limiting cutaneous lesion to fatal visceral disease. (Davies *et al.*, 2003).

a) Localized cutaneous leishmaniasis (LCL)

Localized cutaneous leishmaniasis is caused by *L. tropica*, *L. major* and *L. aethiopica* in the Old World and *L. mexicana* complex and the *Viannia* subgenus in the New World (Davies *et al.*, 2003; Gilles, 1999). The disease cause lesions (or ulcers) on the face, nose, forehead, and lower limbs (Figure 2a) that it usually heals on its own leaving a scar. A severe, non-healing and relapsing disease called recidivans can also occur due to *L. tropica* (Davies *et al.*, 2003; Vidyashankar and Agrawal, 2007). LCL cases due to *L. infantum*, *L. chagasi* and *L. donovani* (parasites known to cause VL) were also reported (WHO, 1990; Gilles, 1999).

b) Diffuse cutaneous leishmaniasis (DCL)

The diffuse cutaneous leishmaniasis cause disseminated nodules, plates or lumps, on the face, arms and legs (Figure 2b). DCL due to *L. aethiopica* in Ethiopia, Kenya and Yemen never heal spontaneously and relapses after treatment (Desjeux, 1996). *L. mexicana* and *L. amazonensis* cause DCL in Caribbean and Brazil (Davies *et al.*, 2003; Mehlhorn, 2004).

c) Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (Espundia) produces extensive disfiguring destruction of mucosa and cartilage of the mouth, nose, ear and pharynx leading to a severe mutilation of the face (Figure 2c). The disease may get worse if secondary bacterial or fungal infection occur (Davies *et*

al., 2003; Vidyashankar and Agrawal, 2007). MCL occur due to *L. aethiopica* in the Old World and *L. brasiliensis* complex in the New World. Cases due to *L. donovani*, *L. major* and *L. infantum* were reported (Desjeux, 1996; Paredes *et al.*, 2003)



Figure 2: Clinical forms of Leishmaniasis (a) Cutaneous; (b) Diffuse Cutaneous;

(c) Mucocutaneous; (d) Visceral and (e) Post kala-zar dermal Leishmaniasis

(Source:http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html;<http://www.who.int/leishmaniasis/en/>;http://www.bact.wisc.edu/foodsafety/parasite/l_postvisc.jpg;http://www.infektionsbiologie.ch/seiten/modellparasiten/seiten/leishmania/steckbrief_leish.html)

d) Visceral leishmaniasis (VL)

Visceral leishmaniasis also called “Kala-azar” is a systemic disease typically caused by *L. donovani* and *L. infantum* (in Europe, Middle East, India, and Africa) and *L. chagasi* (in Central and South America) (Mehlhorn, 2004). The disease is characterized by irregular fever, weight loss, enlargement of spleen and liver, anemia, leukopenia, skin pigmentation and weakness associated with parasite invasion of spleen, liver, bone-marrow, lungs, oral mucosa, larynx, oesophagus, stomach, small intestine, skin and sex cells (Gilles, 1999) (Figure 2d). The disease may be asymptomatic and self-resolving but usually becomes chronic and may be fatal if left untreated or despite treatment (Chan-Bacab and Pena-Rodriguez, 2001; Mehlhorn, 2004, Red book online, 2006). Rare VL cases due to *L. tropica* and *L. mexicana* were reported (Megilli *et al.*, 1993; Kreutztes *et al.*, 1993).

Some visceral leishmaniasis patients may develop dermal manifestation (depigmented macule, papules, nodules, or infiltrative plaques) called post kal-azar dermal leishmaniasis (PKDL)

during or shortly after treatment of VL caused by *L. donovani* (Figure 2e). It may also develop on individuals who do not have previous history of VL or its treatment. PKDL is common in India, Bangladesh and China, less frequent in Africa (regularly in Sudan and Kenya) and rare in the new world (Gilles, 1999).

1.1.3 Epidemiology and geographical distribution of leishmaniasis

1.1.3.1 Global situation

Leishmaniasis prevails in 90 tropical and subtropical countries in Central America, South America, Southern Europe, Asia (excluding South-East Asia), Middle East, and Africa (East & North Africa, with some cases elsewhere) where approximately 350 million people live (82% of which are developing countries) (Davis *et al.*, 2003; CDC-Yellow book, 2008) (Figure 3). The disease affects 12 million people world wide and causes an estimated death of 60,000 due to VL each year. The cutaneous forms account for 50-75% of all new cases with 1-1.5 million annual incidence where as half a million cases (25%) of visceral leishmaniasis occur each year (CDC-Yellow book, 2008; Red book online, 2006; Donald, 2003). The risk factors for the disease are socioeconomic factors (primitive housing, low hygienic situation and houses near vector breeding sites), population movements (for agricultural developments, military activity etc.) and environmental changes. Emergence of HIV/AIDS has also increased reactivity of asymptomatic or previously healed leishmaniasis cases. Currently Leishmania-HIV co-infections have been reported from at least 35 countries (WHO, 1990; WHO, 2007b).

1.1.3.2 Ethiopian situation

All forms of leishmaniasis currently occur in the country. Cutaneous leishmaniasis case was first described in Ethiopia by Martogillo in 1913 and DCL by Destobies, Schaller and Serie in 1960

(Ashford *et al.*, 1973). The Ethio-Kenya and Ethio-Sudan border suffered an endemic of kala-azar in 1938-42 (Editors report, *Eth. Med. J.*, 1965).

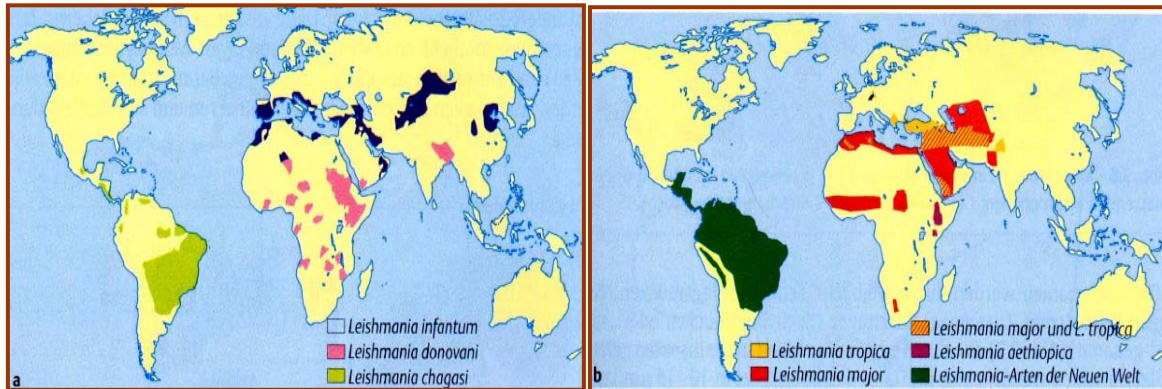


Figure 3: Distribution of (a) visceral and (b) cutaneous and mucocutaneous leishmaniasis

(Source: http://www.izb.unibe.ch/res/roditi/Lehr/2007/07_Leish.pdf)

In Ethiopia visceral leishmaniasis is distributed throughout the lowlands and semi desert areas (below altitude 1500m) with varying degree of endemicity. So far VL cases have been reported from at least 40 localities with annual burden estimated to be 4,500-5,000 (Anonymous, 2006). VL in Ethiopia is caused by *L. donovani* with eight *Phlebotomus* sandfly spp. *P. martini*, *P. orientalis*, *P. celiae*, *P. alexandri*, *P. dubosqi*, *P. saevus*, *P. sergenti* and *P. rodhaini* as main vectors. However animal reservoirs were not specifically documented (Hailu *et al.*, 2006).

Cutaneous leishmaniasis in Ethiopia is dominantly caused by endemic *L. aethiopica* species and rarely by *L. tropica* and *L. major*. It is widely distributed at altitudes between 1500-2700m almost everywhere in the country (Hailu *et al.*, 2006; Price and Fitzherrbert, 1965). The vectors; *P. longipis*, *P. pedifer* and *P. dubosqi* (Hailu *et al.*, 2006; Gebre-Michael *et al.*, 1993) and reservoir hosts; *Procavia habessinica* and *Heterohyrax brucei* (Ashford *et al.*, 1973) were reported for *L. aethiopica*. DCL and MCL are found in areas where other CL types occur (Bryceson and Leithead, 1996; Barnetson *et al.*, 1978).

1.1.4 Transmission, pathogenesis and life cycle of leishmaniasis

Leishmaniasis is transmitted while *Leishmania* infected female sandflies take blood from healthy people. Two transmission ways are possible; zoonotic-in case when parasite is transferred from infected non-human reservoir host to healthy individuals (accidental hosts) or anthroponotic-where humans are the sole reservoir hosts (Anonymous, 2006). Congenital, parenteral and sexual transmissions are also suspected (Gilles, 1999, Vidyashankar and Agrawal, 2007; Davies *et al.*, 2003).

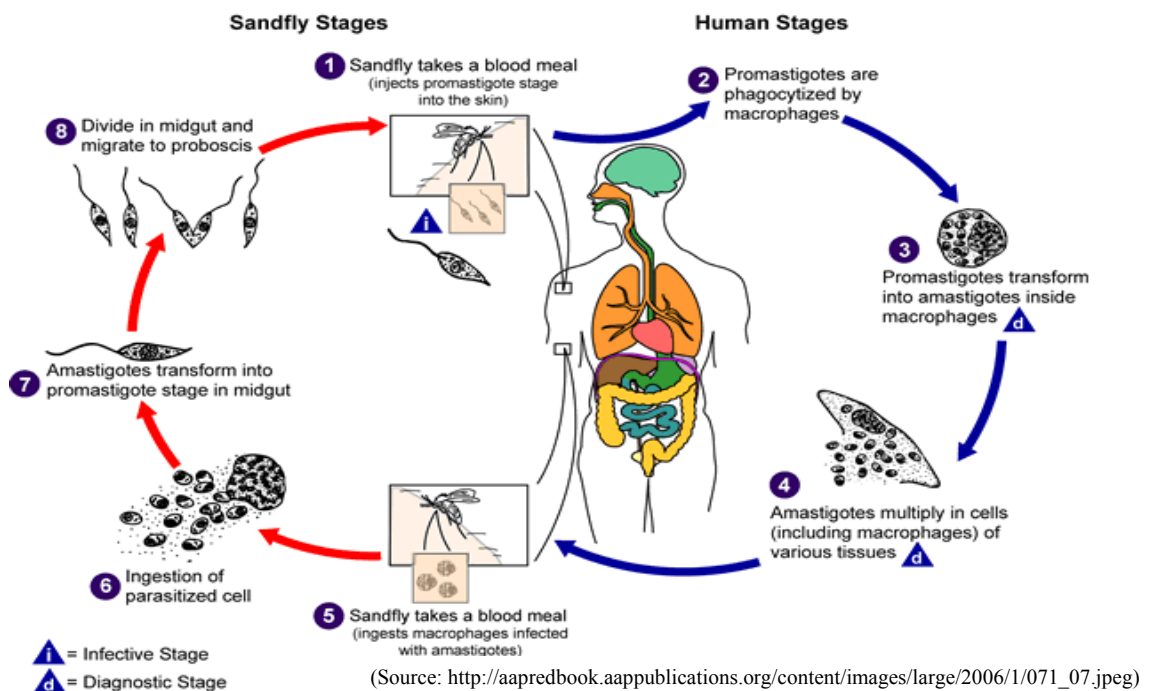


Figure 4: Life cycle of the *Leishmania* parasite.

Once the sandfly has been infected, the amastigote migrates to the alimentary canal of the insect where it attaches to local epidermal cells. The parasites mature and differentiate into motile promastigotes and move to the pharynx and/or the proboscis of the sandfly. Upon a subsequent blood meal, the promastigotes are injected into the blood stream of the victim. Then they are

phagocytized by macrophage and reside in the parasitophorous vacuole where they transform back into amastigote. Amastigotes then replicate and released back into blood stream to infect macrophages within the skin, viscera, blood tissues etc. to disseminate the disease locally or systemically based on distinct *Leishmania* spp. involved. The parasites are equipped to evade the digestive enzymes present in the vacuole and they also have a membrane bound molecule, lipophosphoglycan (LPG) that ensures intracellular survival of parasite (Sacks and Perkins, 1985; Cunningham, 2002; Mehlhorn, 2004).

1.2 Prevention and control of leishmaniasis

Prevention and control measures target at breaking one or more elements in the parasite life-cycle. This can be achieved by spraying houses and outdoor insect resting and breeding sites with insecticide. Also prompt diagnosis, treatment and if this is not possible elimination of the reservoir host may be useful (Davies *et al.*, 2003; WHO/TDR, 2004b). Condition under which man becomes *Leishmania* infected is dependent on ecology and varies with time and place. Therefore, protection from sandfly bite can be achieved by avoiding outdoor activities, using protective clothing and insecticide treated nets and insect repellents (CDC-Yellow book, 2008; WHO/TDR, 2004b; Davies *et al.*, 2003).

Protection against leishmaniasis can also be achieved by vaccination. Vaccination against cutaneous leishmaniasis was a common traditional practice in the Middle East and the Soviet Union. Healthy individuals were immunized by deliberately exposing them to sandflies bites or scratched tissues from active lesions from CL patients. Intradermal inoculation of live *Leishmania* in healthy individuals to produce mild, self-healing cutaneous lesions called Leishmanization. However, due to occurrence of severe or persistent cutaneous lesions and

difficulty in standardizing the virulence of vaccine the method is currently in use only in Uzbekistan (Khamesipour, *et al.*, 2006; Dumoneil *et al.*, 2001; WHO/TDR, 2004a). Several other first generation vaccines (consisting fractions of the parasite or whole killed *Leishmania* with/without adjuvants) and second generation vaccines (from recombinant molecules, *Leishmania* antigens and sandfly salivary immunomodulators) were studied. Many other trials are also still ongoing. Results indicate variable safety and efficacy of these agents. But no effective vaccine has yet emerged for both CL and VL (Dumonteil *et al.*, 2001; WHO/TDR, 2001; Davies *et al.*, 2003).

1.3 Treatment of leishmaniasis

1.3.1 Chemotherapy of leishmaniasis and its therapeutic limitations

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

1.3.1.1 Drugs approved for treatment of leishmaniasis

a) Pentavalent antimonials

Pentavalent antimonials are the first anti-leishmanial agents introduced in 1945. Two of these drugs, Pentostam® and Glucantime®, contain antimony-sugar polymeric compounds sodium stibogluconate (1) and meglumine antimonate (2) as main constituents respectively. They are first-line drugs for VL, LCL cases except due to *L. aethiopica*, MCL due to *L. braziliensis* and DCL due to *L. amazonensis* (Laurence *et al.*, 2006; Gilles, 1999).

Antimonials are thought to inhibit trypanothione reductase, a key enzyme that mediates redox balance in trypanosomes. Both drugs are parenterally administered at 20 mg/kg/day for 20-28

days. They are not orally active (Laurence *et al.*, 2006; Gilles, 1999; Donald, 2003). Intra-leisional administration was also found effective (Paris *et al.*, 1993). Long term parenteral administration, variable efficacy against VL, CL and MCL cases and adverse effects such as pain at the site of injection, stiff joints, gastrointestinal problems, cardiotoxicity, hepatic and renal insufficiency are some of the factors that limite their widespreed use (Croft *et al.*, 2006 a&b; Donald, 2003; Croft and Coombs, 2003; Singh, 2006). Resistance has also become an increasing clinical problem in India and Bangladesh. Resistance mechanisms include decreased activation, increased trypanothione levels and increased efflux of the drug (Croft *et al.*, 2006a; Donald, 2003).

b) Pentamidine

Pentamidine (3) is a cationic aromatic diamine discovered in 1937 as a hypoglycemic compound. It is also toxic to large number of protozoa and some fungi. Pentamidine isethionate (Pentacarinat®) is used as a second-line drug for treatment of VL, LCL and DCL since 1952 (Laurence *et al.*, 2006; Gilles, 1999; Croft and Coombs, 2003).

Pentamidine interferes with ribosomal aggregation and biosynthesis of DNA, protein and many enzymes by reacting with negatively charged biomolecules such as membrane phospholipids, enzymes, RNA, and DNA (Laurence *et al.*, 2006). The drug is administered intravenously at doses 2-4mg/kg daily or every other day for 12-15 days. Higher doses (>4 mg/kg/day) can lead to hypotension, tachycardia, stomach upset, anemia, severe headaches, skin eruptions, abnormal liver function, hypoglycemia, and renal dysfunction (Laurence *et al.*, 2006; Donald, 2003). Resistance was reported in India due to increased drug efflux (Croft *et al.*, 2006 a & b; Donald, 2003).

c) Amphotericin B

Amphotericin B (4) is a polyene macrolide antibiotic extensively used as antifungal. It is also in use for treatment of leishmaniasis as second-line drug, since 1960s. Amphotericin B deoxycholate (Fungizone®) is used for treatment of VL, CL and MCL unresponsive to other drugs. AmBisome® was also approved in 1997 for treatment of VL. Other novel amphotericin B formulations are also being considered (Croft *et al.*, 2006a & b).

Leishmania has membrane sterol similar to fungi and the drug then form complexes preferentially to these sterols leading to porous parasite membrane permeable to entry and loss of ions. The drug is administered intravenously at a dose of 0.5-1.0 mg/kg daily or every other day for 8 weeks (as Fungizone®) or 3 mg/kg/day for 21 days (as AmBisome®) (Donald, 2003; Croft *et al.*, 2006b). Major adverse reaction to amphotericin B is nephrotoxicity. Acute side effects such as fever, chills, muscle spasms, vomiting, headache, hypotension, and anaphylaxis occur less in lipid complex preparations (Croft *et al.*, 2006a; Donald, 2003; Croft and Coombs, 2003). Resistance to amphotericin B is emerging probably associated with changes in *Leishmania* membrane sterols (Croft *et al.*, 2006b).

d) Paromomycin

Paromomycin (Aminosidine®, Humatin®) (6) is aminoglycoside used in oral treatment of amoebiasis, cryptosporidiosis and giardiasis and topically for trichomoniasis. Its anti-leishmanial activity was identified in 1960s. The drug was approved in India in 2006 for treatment of VL (WHO, 2007a; Donald, 2003). Topical paromomycin formulations were also found effective for CL cases (Croft *et al.*, 2006 a & b; Croft and Coombs, 2003).

Paromomycin inhibit protein synthesis binding to polysomes and causing misreading and premature termination of translation (Donald, 2003). It is administered parenterally at dose of 16-20 mg/kg daily for 21 days or 12–20 mg/kg/d combined with antimonials for 20 days (Croft *et al.*, 2006a; Donald, 2003). Toxic effects such as nephrotoxicity and ototoxicity are associated with exceeding the recommended doses and concomitant use of other agents with similar toxicities (Donald, 2003). Resistance cases were reported on *L. aethiopica* isolates and *L. donovani* promastigotes associated with decreased drug uptake (Croft *et al.*, 2006a).

e) Miltefosine

Miltefosine (Impavido®) (5) is an alkylphosphocholine analog originally developed as an anticancer agent. Its anti-leishmanial activity was first discovered in mid-1980s. The drug is registered in India, Colombia, Germany, Nepal and Bangladesh for treatment of VL. It is being considered for CL cases, immunocompromized patients and canine leishmaniasis.

The mechanism of action of miltefosine is not fully understood. However, experimental results suggest drug's effect on mammalian protein kinase C, phosphatidylcholine biosynthesis, lipid metabolism, cell signaling and calcium homeostasis (Croft, *et al.*, 2005). Miltefosine is given at a daily oral dose of 50-200 mg/Kg for 28 days (Donald, 2003). Major adverse reactions include gastrointestinal distress, increased hepatic transaminases and serum creatinine levels, teratogenicity and blood hemolysis (parenteral) (Croft *et al.*, 2006a; Donald, 2003; Croft and Coombs, 2003). Resistance has also been reported related to decreased drug uptake and efflux mechanisms (Croft *et al.*, 2006a; Donald, 2003).

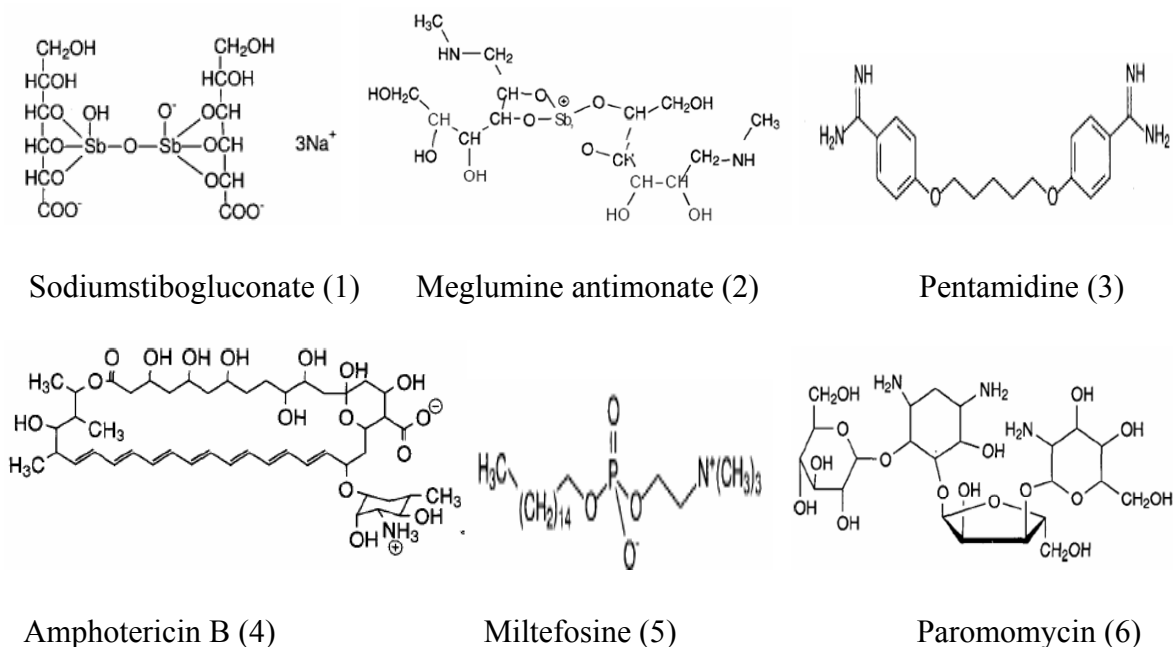


Figure 5: Structure of drugs approved for treatment of leishmaniasis.

1.3.1.2 Anti-leishmanial drugs in clinical trial

a) Azoles

Azoles are other anti-fungal drugs that had effects on different *Leishmania* species. Fluconazole (7) cured 79 % of *L. major* infections whereas ketoconazole (8) was found to have some activity against *L. mexicana* (Piscopo and Mallia, 2006) and Itraconazole (9) on MCL cases. The orally active triazole, posaconazole (10) cured cutaneous and visceral leishmaniases cases at a dose of 60 mg/kg/day (Antony and Lukasz, 2005; Laurence *et al.*, 2006). The anti-leishmanial effect of azoles is by inhibition of cytochrome P450-mediated 14 α -demethylation of lanosterol of the parasite (Antony and Lukasz, 2005).

b) Sitamaquine

Sitamaquine (Lepidine®) (11) is orally active 8-aminoquinoline derivative with spectrum of antiprotozoal activity. The anti-leishmanial activity of this compound was first identified in the 1970s. The drug show variable effect at a daily dose of 1.75-2 mg/kg for 28 days (Croft *et al.*, 2006b; Antony and Lukasz, 2005). The drug is assumed to involve in the parasite "futile redox cycling" system (Croft *et al.*, 2006b) and its N-alkylation metabolites are responsible for activity (Antony and Lukasz, 2005; Croft *et al.*, 2006b; Jha *et al.*, 2005).

c) Allopurinol

Leishmania fails to biosynthesize purine nucleotides and they rely on the host's nucleotide sources. The purine analogs allopurinol (12) and its riboside analogs were reported to have antileishmanial activity in 1980s (Donald *et al.*, 1979). These drugs have been clinically tested for VL and CL, alone and combined with antimonials and results were promising. The drugs inhibit enzymes in purine salvage pathway inhibiting ribonucleoside triphosphate formation and incorporation into RNA. However, their involvement in the purine salvage pathway and pharmacokinetic properties have limited use in treatment of human leishmaniasis. Currently, allopurinol is only in use for canine leishmaniasis (Donald, 2003; Croft and Coombs, 2003).

1.3.1.3 Anti-leishmanial drugs in experimental and pre-clinical stages

a) Anti-microbial peptides

Antimicrobial peptides are cationic substances toxic to microbial membrane. More than 500 peptides were isolated from members of animal and plant kingdom. Many of these anti-microbial peptides were tested *in vitro* on *Leishmania* promastigotes and *in vivo* on *Leishmania* infected dogs and results proved their therapeutic effectiveness. Some of these active peptides include

skin polypeptide YY, dermaseptin S4 derivatives, cecropin A, gomesin, magainin 2, temporins A and B, mussel defensins and cecropin A-melittin (CA(1-8) M (1-18)) and cecropin A-melittin (Antony and Lukasz, 2005).

b) Licochalcone A

Licochalcone A (13) is a flavonoid isolated from the root of traditional Chinese plant (*Glycerrhiza inflata*) and its synthetic oxygenated derivatives showed reasonable oral efficacy in experimental models of VL and CL. The derivative, 35m4ac also suppressed 97% of liver amastigotes in *L. donovani* infected hamsters at 20 mg/kg/d for 6 days i.p. The compounds interfere with mitochondrial function of *Leishmania* (Chen *et al.*, 1994; Croft and Coombs, 2003).

c) Saponins (PX-6518)

Saponins isolated from methanol extract of the leaves of Vietnamese plant *Maesa balansae* had an IC₅₀ of 0.04 µg/ml against intracellular *L. infantum* amastigotes and a CC₅₀ of 1 µg/ml (on murine macrophage) and >32 µg/ml (on human fibroblasts (MRC-5) cell line. PX-6518 administered at 1.6 mg/kg s.c for 14 days produced comparable efficacies with single daily dose (20 mg/kg) of sodium stibogluconate (Pentostam®) (Maes *et al.*, 2004a; Croft and Coombs, 2003). Maesabalide III, from the same plant reduced 94.2 % liver amastigotes in infected hamsters given at 0.8 mg/kg/d for 28 days. The result was comparable to a single daily dose (5 mg/kg) treatment with amphotericin B (Maes *et al.*, 2004b).

d) Tricyclic antidepressants

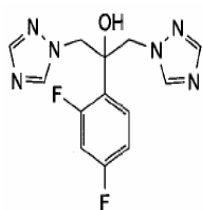
Analogs of tricyclic compounds such as *N*-acylpromazines, 2-substituted phenothiazines and trisubstituted promazines selectively inhibit trypanothione reductase (TryR) in computer generated model of TryR, docking experiments and experiments on recombinant trypanothione reductase (Donald, 2003). Chlorpromazine given orally at 20 mg/kg/d for 14 days reduced parasite burden in *L. donovani* infected hamsters by 64.2-67.9% (Pearson, 1984). Phenothiazines were also found to inhibit activity of multidrug resistance (MDR) transporters Calc-AM and calcein unique to *Leishmania* promastigotes (Chan, 1998).

e) Tamoxifen

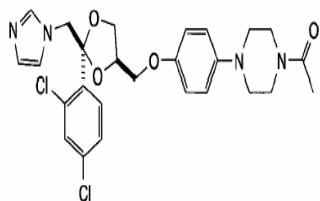
Tamoxifen (15) is a triphenylethylene oestrogen receptor modulator used in the prevention and treatment of breast cancer. It affects growth of promastigote and amastigote forms of *L. amazonensis* with IC₅₀ values of 16.4 ± 0.2 and 11.1 ± 0.2 mM respectively. The drug also had effect on *L. (V.) braziliensis*, *L. major*, *L. chagasi* and *L. donovani* with IC₅₀'s ranging from 9.0 to 20.2 mM. Tamoxifen induces rapid and long-lasting alkalization of the parasitophorous vacuole best at pH 7.5 (Miguel *et al*, 2007).

f) Omeprazole

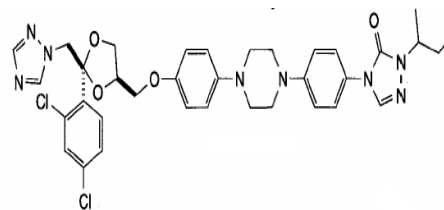
Omeprazole (16), a benzimidazole widely used in the treatment of peptic ulcer was also found to inhibit growth of intracellular amastigote forms of *L. donovani* by 50 % at a concentration of 50 µM. The drug was found to inhibit *Leishmania* membrane enzyme (P-type K⁺/H⁺-ATPase) important in pH homeostasis and maintenance of proton motive force across the membrane. The drug worked effectively at pH 5.5 (Jiang *et al*, 2002).



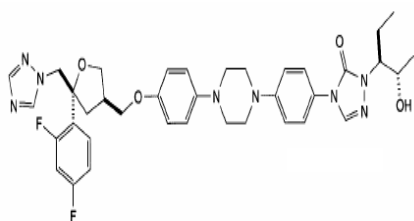
Fluconazole (7)



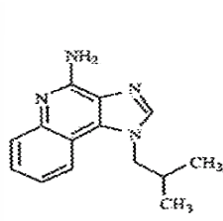
Ketoconazole (8)



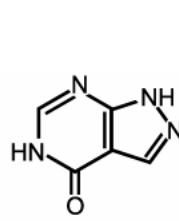
Itraconazole (9)



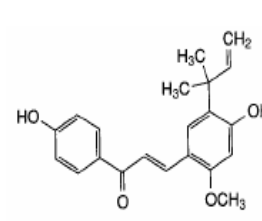
Posaconazole (10)



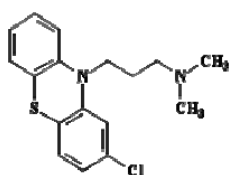
Sitamaquine (11)



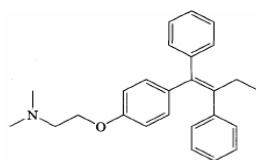
Allopurinol (12)



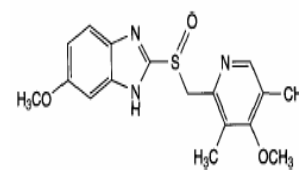
Licochalcone A (13)



Chlorpromazine (14)



Tamoxifen (15)



Omeprazole (16)

Figure 6: Structure of antileishmanial drugs in experimental and clinical trial stages .

1.3.2 Immunotherapy of leishmaniasis

Ability of *Leishmania* to survive and establish infection in humans is by impairment of host macrophage signaling pathways and modulation of macrophage cytokine production. An immunomodulator drug can be rationally used in the treatment of leishmaniasis.

a) Interferon- γ (IFN- γ)

Interferon- γ activates phagocytes and induces generation of oxygen metabolites that are toxic to a number of microorganisms including *Leishmania*. It also enhance activity of antimonials in the treatment of VL and CL by reducing their dose required to kill the parasite and thereby their side

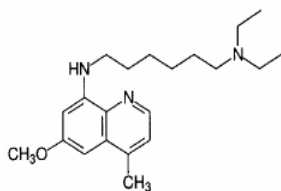
effects. Interferon (IFN)- γ alone reduced *L. donovani* load in splenic aspirates (Croft and Coombs, 2003; WHO/TDR, 2004a).

b) Imiquimod

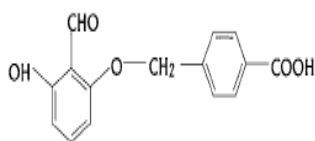
Imiquimod (Aldara®) (17) is an imidazoquinoline derivative which is an ingredient of the topical cream used for treatment of actinic keratosis, superficial basal cell carcinoma and genital warts since 1997. It stimulates a local immune response at the site of application by inducing production of cytokines such as interferon- α , tumor necrosis factor- α and interleukins (IL-1 β , IL-6 and IL-8). In combination with antimonials, the drug showed 90 % cure rate on CL cases refractory to antimonials alone (Firooz *et al*, 2006, Antony and Lukasz, 2005; Croft, *et al.*, 2006a, Laurence *et al*, 2006).

c) Tucaresol

Tucaresol (18), a substituted benzaldehyde used for oral treatment of cytomegalovirus, murine colon adenocarcinoma and malignant melanoma. The drug also showed effect on experimental mouse VL models. The drug enhances T-helper cell activity, with the induction of increased IL-2 and IFN- γ levels (Smith *et al.*, 2000; Croft and Coombs, 2003).



Imiquimod (17)



Tucaresol (18)

Figure 7: Structure of immunomodulatory drugs in clinical trials I on leishmaniasis

1.3.3 Treatment of local cutaneous leishmaniasis

Several methods were practiced for treatment of local cutaneous leishmaniasis cases. These include using heat (such as radiant heat, hot materials and water, infrared heat lamps and electrical heat pads), cryotherapy (freezing either with liquid nitrogen or carbon dioxide), irradiation of lesions (covered with photosensitizing compounds) and carbon dioxide laser. All these methods showed variable efficacies (Chan-Bacab and Pena-Rodriguez, 2001; Gilles, 1999).

1.4 Herbal products in medical health practices

1.4.1 Herbal drugs: Historical perspective, current status and future trends

The history of herbal medicine is rather old and dates back to the time when the early man became conscious of his environment. Since then, medicinal plants have been used virtually in all cultures as a source of medicine (Lanfranco, 1999). According to medical records, Hippocrates (late fifth century B.C.) listed some 300-400 medicinal plants in his *Materia Medica*. The famous Athenian biologist-botanist Theophrastus (370-287 B.C.) included about 500 medicinal plants in his *Historia plantarum* (Tyler *et al.*, 1976; Cowan, 1999). A medicinal plant catalog, *De Materia Medica* describing 600 plant species from Mediterranean region was prepared by Dioscorides (60 A.D.). The most distinguished physician of the Middle Ages, Galen (131 A.D.), used herbs to treat different diseases. About 30 healing plants are described in the Holy Bible (Cowan, 1999).

Many conventional drugs were obtained from plants which were initially used in crude form in traditional or folk healing practice (Mourice *et al.*, 1999). Beginning with the discovery of pure compounds from plants, the art of exploiting drugs from natural products became part of the molecular sciences. Fats and oils, nitrogen compounds, terpenoids and other plant secondary metabolites were among active compounds discovered and exhibited diverse bioactivities such as

antioxidant, antiinflammatory, antitumor, antimutagenic, anticarcinogenic, antiprotozoal, antifungal, antibacterial or antiviral. Some of the drugs included aspirin (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), taxol (from Pacific yew tree) and morphine (from opium poppy) (Vickers and Zollman, 1999). Today, natural products (their derivatives and analogs) still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing *ca.* 25% of them (Chin *et al.*, 2006). However, rapid advance in synthetic organic chemistry, combinatorial chemistry and advent of high throughput screening techniques made the process of finding new drugs focus on bulk screening of natural and synthetic compound libraries rather than new plant metabolites. Currently 250,000 to 500,000 species of higher plants are estimated to be present on earth. But only small proportions (1-10%) of these are used either as foods or medicine both by humans and other animals. Only few (<10%) of these has been systematically investigated for the presence of bioactive compounds (Cowan, 1999). Diversity in class of compounds that plants contain still makes them indispensable sources of novel and effective drugs (Paterson and Anderson, 2005).

In Ethiopia about 95% of the population relies on traditional herbal medicine for treatment of various ailments (Demissew and Dagne, 2001; Gidey, 2001). The Ethiopian flora is also estimated to contain between 6,500 and 7,000 species of higher plants of which about 12% are endemic (Demissew and Dagne, 2001; Gidey, 2001). Therefore scientific evaluation of these plants may provide modern medicine with lead compounds for the development of new drugs.

1.4.2 Herbal anti-leishmanial agents

Several researches were made on antileishmanial activity of crude extracts or chemically defined compounds derived from plants *in vitro* against promastigotes and amastigotes or *in vivo* against *Leishmania* infected animals. Some of these works are reviewed as follows.

Methanol extracts from *Gongronema latifolia*, *Dorstenia multiradiata*, *Picralima nitida*, *Cola attiensis*, and *Desmodium gangeticum* were observed to be active at 50 µg/ml or less against VL isolates (Iwu *et al.*, 1992).

Taddese *et al.* (1993) studied antileishmanial activity of methanol and chloroform extracts of *Vernonia amygdalina* (Asteraceae) *in vitro* against promastigote and amastigote forms of *L. aethiopica* and cytotoxicity on THP-1 cell line. Results indicated that the chloroform and methanol extracts had an ED₅₀ of 13.3-18.5 µg/ml and 45.8-74.4 µg/ml respectively on promastigotes and amastigotes. Cytotoxic doses on THP-1 cell lines were 19.6 µg/ml and 243.4 µg/ml for chloroform and methanol extracts respectively.

El Tahir *et al.* (1998) tested crude methanol extract of *Azadirachta indica*, *Maytenus senegalensis* and *Eucalyptus globulus* against promastigotes of *L. major in vitro*. The extracts had IC₅₀ values of 11.5, 55.0 and 78.0 µg/ml respectively. Similar study by Khalid *et al.* (2005) on *Allium sativum* and *Azadirachta indica* revealed their antileishmanial effects with LC₅₀ of 4.94 µg/ml and 10.2 µg/ml respectively.

The plants *Baccharis trimera*, *Cymbopogon citratus*, *Matricaria chamomela*, *Mikania glomerata*, *Ocimum gratissimum*, *Piper regnelli*, *Prunus demestica*, *Psium guajava*, *Sambucus canadensis*,

Stryphnodendron adstringens, *Tanacetum parthenium* and *Tanacetum vulgare* showed significant growth inhibitory effects (49.5-99.0%) on promastigote and axenic amastigote forms of *L. amazonensis* *in vitro* at 100 µg/ml with no haemolytic effect (Luize *et al.*, 2005).

Chan-Bacab and Pena-Rodriguez (2001) and Rocha *et al.* (2005) critically reviewed the antileishmanial activity of crude extracts and chemically defined molecules isolated from higher plants and microorganisms. The bioactive compounds belong generally to alkaloids, (mono-, sesqui-, di- and tri-) terpenes, lactones, quinoids, flavonoids, steroids, lipids, iridoids, oxygenated heterocycles, benzenoids, carbohydrates, lignans, proteids, coumarins, phenylpropanoids, depsides and sulfur compounds.

Antileishmanial activity of Xantholides isolated from *Xanthium macrocarpum* (Asteraceae) was evaluated on *L. infantum* and *L. mexicana* promastigotes *in vitro*. The compounds had growth inhibitory activities ranging from $3.6 \pm 7\%$ to $40 \pm 8\%$ (Lavaurelt *et al.*, 2005).

Crude hydromethanol and methanol extract of plants used in Gabonese traditional medicine were evaluated for their antileishmanial activities and cytotoxicities. The hydromethanol extract of leaf of *Ganophyllum giganteum* (IC₅₀ 1.3 µg/ml), methanol extracts of the leaves of *Polyalthia suaveolens*, *Dioscorea preussii*, *Augouardia letestui* and stem bark of *Cola lizae* had the highest effect (IC₅₀ <5 µg/ml) on *L. infantum* and with low toxicities (Lamidi *et al.*, 2005).

Methanol extracts of medicinal plants used in treatment of infectious and inflammatory disorders in Brazil were evaluated on *L. amazonensis* and *L. chagasi* promastigotes. Extracts from *Vernonia polyanthes* and *Ocimum gratissimum* were most active against *L. amazonensis* (IC₅₀ 4 µg/ml) and *L. chagasi* (IC₅₀ 71 µg/ml) respectively (Braga *et al.*, 2007).

Fokialakis *et al.* (2007) screened some plant extracts on the *L. donovani* promastigotes and mammalian kidney fibroblast (Vero) *in vitro*. The dichloromethane extract of *Eryngium ternatum*, *Origanum dictamnus* and *Origanum microphyllum* and the methanol extract of *Eryngium amorginum* had IC_{50} 's <10 $\mu\text{g}/\text{ml}$ without any cytotoxicity.

The antileishmanial activity of plants used in traditional management of parasitic infections in Mali was screened against promastigote and amastigote forms of *L. major*. Extracts from *Sarcocephalus latifolius*, *Zanthoxylum zanthoxyloides*, *Entada africana*, *Bobgunnia madagascarensis*, *Pseudocedrela kotschyi* and *Psorospermum guineense* had significant antimastigote effects (Kouassi *et al.*, 2007).

Mori *et al.* (2008) screened crude methanol extract and compounds isolated from wood of *Cordia fragrantissima* on *Leishmania spp.* The crude extract had MIC of 12.5 $\mu\text{g}/\text{ml}$ against *L. major* promastigotes. Compounds cordiaquinol C, I, J and K, Cordiachrome A, B and C and Alliodorin isolated from this extract also exhibited leishmanicidal activity on promastigote forms of *L. major*, *L. panamensis*, and *L. guyanensis* with IC_{50} values ranging from 1.4 to 81.4 $\mu\text{g}/\text{ml}$. The test compounds were all toxic to cells lines (COS-7 & HuH-7).

1.5 Review of plant species included in this study

1.5.1 *Albizia schimperiana* Oliv. (Fabaceae)

Albizia schimperiana “cd, Sesa” is an evergreen tree that grows at an altitude of 1600-2600m. It belongs to the family Fabaceae which consists of 650 genera and about 18,000 species distributed in most part of the world. The genus *Albizia* includes some 100-150 species throughout the tropics (Hedberg and Edwards, 1989). *Albizia spp.* is used in traditional treatment

of various infections. *A. gummifera* is used against malaria (Muregi *et al.*, 2007a), trypanosomiasis (Senn, 2006), bacterial infections and stomach pain. *A. anthelmintica* is also used as purgative and anthelmintic (Anonymous, 2007e). *A. coriaria* had activities on promastigote and amastigote forms of *L. major* at 1mg/ml to 1pg/ml (Tonui *et al.*, 2007).

1.5.2 *Aloe* spp. (Aloaceae)

The family Aloaceae consists of succulent perennial herbs or woods included in seven genera and 650 species. The genus *Aloe* contains 360 species in Africa, Madagascar and Arabia. 36 *Aloe* species “_f, Eret” occur in the Ethiopia and 18 of this are endemic (Edwards *et al.*, 1997, Demisew, 1996). In Ethiopia *Aloe* spp. are used in traditional treatment of rheumatic pain, haemorrhoid, wound, hypertension and diabetics (Amare, 1976: Abate, 1989). Gel of *A. vera* is used in skin protection, minor burns, abrasions, chancre sores, inflammatory situations, gastrointestinal infections, viral infections, cancer and as immunomodulator (Singh *et al.*, 2000; Davis, 1993). *A. vera* leaf exudate showed leishmanicidal activity on promastigotes (IC₅₀ of 100-180 µg/ml) and axenic amastigote (IC₅₀ 6.0 µg/ml) forms of *L. donovani* (Dutta *et al.*, 2006). *A. kedogensis* killed promastigotes and amastigotes of *L. major* at 1mg/ml to 1µg/ml (Tonui *et al.*, 2007). *Aloe* species contain alkaloids, anthraquinoides, pre-anthraquinones, anthrones, chromones, coumarins, bi-anthraquinons, and pyrones (Dagne, 1996b).

1.5.3 *Artemisia abyssinica* Bip ex A. Rich (Asteraceae)

With nearly 400 species mostly in northern temperate regions of the world, the genus *Artemisia* constitutes one of the 60 genera in the family Asteraceae/Compositae. Two of these species *A. abyssinica* “β̄, Chikugn” and *A. abisinithum* “>]+, Ariti” are endemic to Ethiopia (Anonymous, 2007d). *Artemisia* spp are used in traditional treatment of intestinal problems, bronchitis and

other inflammatory situations, cold and fever, anorexia, colic, infectious diseases (bacterial, protozoal), headache, amenorrhoea and dysmenorrhoea (Amare, 1976; Abegaz, 1996; Teixeira, 2004 ; Kayser *et al.*, 2006). Methanolic extract of *A. aucheri* inhibits multiplication of 25 % of *L. major* promastigotes at 750 µg/ml (Sharif *et al.*, 2006). *Artemisia* spp produce sesquiterpene lactones, coumarins and acetylenes as main metabolites. They are also source of essential oils (Teixeira, 2004).

1.5.4 *Croton macrostachys* Hochest. ex A.Rich (Euphorbiaceae)

Croton macrostachys “wd”, Bisana” is deciduous tree 3-25 m high that grows at altitudes 200-2000m. It belongs to family Euphoraceae that includes 312 genera and 8,100 species. The genus *Croton* includes 800 species distributed mostly in the New World. 50 of this spp. grow in Africa. The plant is native to Eritrea, Ethiopia, Kenya, Nigeria, Tanzania and Uganda (Hedberg *et al.*, 1995). *Croton* species have long history in traditional medicine for treatment of cancer, diabetes, digestive problems, dysentery, hypercholesterolemia, fever, hypertension, malaria, inflammation, pain, wound, ulcer, bleeding, cough, weight-loss and infectious diseases (helminthic, bacterial) (Salatino *et al.*, 2007; Amare, 1976; Abate, 1989). In Ethiopia *C. macrostachys* is used in treatment of malaria, leishmaniasis, taeniasis and cancer. The plant also exhibited antileishmanial and immune stimulating activity (Fessahaye *et al.*, 1993). Linalool-rich essential oil from the leaves of *C. cajucara* had an LD₅₀ of 8.3 ng/ml and 8.7 ng/ml against promastigote and amastigote of *L. amazonensis* (Socorro *et al.*, 2003). *Croton* spp generally contain diterpenoids, triterpenoids, alkaloids, flavonoids, lignoids and proanthocyanidins. Volatile oils containing monoterpenoids, sesquiterpenoids, and some shikimate-derived compounds were reported in the genus (Salatino, *et al.*, 2007). *C. mecrostacys* contains crotepoxide, lupeol and quaternary alkaloids (Menberu, 1981).

1.5.5 *Echinops kebericho* Mesfin (Asteraceae)

The genus *Echinops* belongs to family Asteraceae/Compositae and comprises over 120 species. *E. kebericho* “ku`E, kebericho” and 11 other spp. are known to occur in Ethiopia (Erko, 2004) and some are known in traditional herbal medicine. *E. kebericho* is useful in the traditional treatment of migraine, diarrhea, heart pain, typhoid fever, cancer and bacterial & helminthic infections. *E. ellenbeki* treats hemorrhoid (Hymete *et al.*, 2005; Erko, 2004). *Echinops* species were reported to contain alkaloids, saponins, phytosterols, polyphenols, carotenoids, sesquiterpene (alcohols/lactones), lignans, acetylenic & thiophene compounds and essential oil (Abegaz, 1996; Hymete *et al.*, 2005 & 2006; Tene *et al.*, 2004).

1.5.6 *Erythrina brucei* Schwein f. (Fabaceae)

Erythrina brucei “ፕፍ, korich” is a tree that grows 15-20m high. The genus *Erythrina* consists of some 112 species in the tropics and subtropics (Hedberg and Edwards, 1989). *Erythrina* species have folk medicinal uses in treatment of insomnia, malaria, venereal disease, asthma, toothache, wound, helminthes, schizophrenia and dermatological problems (Abate, 1989). Reports are also there on anti-plasmodial (Yenesew *et al.*, 2003 & 2004; Andayi *et al.*, 2006; Rukachaisirikul *et al.*, 2007b), anti-fungal (Yenesew, 2005), anti-bacterial (Yenesew, 2005; Rukachaisirikul *et al.*, 2007a & b) and cytotoxic (Cui *et al.*, 2007) effect of crude extracts and compounds isolated from different *Erythrina* spp. *E. spiciosa* also showed some growth inhibitory effects on *L. amazonensis* and *Trypanosoma cruzi* (Luize *et al.*, 2005). Alkaloids, pterocarpan, chalcones, flavonoids, flavanones, isoflavones, isoflavonoids, prenylated flavanones, triterpenes and steroids were isolated from different African *Erythrina* species (Abdullah *et al.*, 1979; Yenesew *et al.*, 2003, 2004 & 2005; Andayi *et al.*, 2006, Cui *et al.*, 2007).

1.5.7 *Justicia schimperiana* Hochst. ex Nees (Acanthaceae)

Justicia schimperiana “cM, SenseI” is a plant that belongs to family Acanthaceae which consists of 250 genera and 2500 species. This genus consists of 600 species distributed in tropical and temperate regions (Hedberg *et al.*, 2006). *Adhatoda vasica* (now called *Justicia visca*) has been known for more than 2000 years in India and is still in use for treatment of cold, cough, chronic bronchitis, asthma, insomnia, smooth muscle spasm, helminthes, diarrhea, glandular tumor, rheumatic pain and other inflammatory situations (Anonymous, 2007c). In Ethiopia *J. schimperiana* is used in treatment of scabies, malaria, fever, asthma and other inflammatory situations, excessive pellagra and constipation (Mekonnen *et al.*, 2006; Assefa *et al.*, 2006; Gedif and Hahn, 2003 & 2004). *A. vasica* contains vasicoline, adhatodine, vasicolinone, anisotine and other alkaloids (Anonymous, 2007c).

1.5.8 *Phytolacca dodecandra* L’herit (Phytolaccaceae)

Phytolacca dodecandra “ÉÉ, Endod” is a semi-succulent straggling or scrambling shrub with height 10m or more. It belongs to family Phytolaccaceae which includes about 16 genera and 100 species. The genus *Phytolacca* contains about 35 species in tropical and subtropical regions (Hedberg *et al.*, 2000). In Ethiopia, different parts of *P. dodecandra* are used alone or in combination with other plants for the treatment of pruritus, eczema and ascariasis, malaria, rabies, sore throat and rheumatic pain, Jaundice, gonorrhoea and syphilis. It also has molluscicidal, antifungal and spermicidal effects (Harold *et al.*, 1993; Hedberg *et al.*, 2000; Amare, 1976). Proteins from *P. americana* and the butanol extract of *P. dodecandra* had effect on *L. infantum* (Chan-Bacab and Pena-Rodriguez, 2001) and *L. enriettii* (Rocha *et al.*, 2005) respectively. *P. dodecandra* contain saponins lemmatoxin A, B and C (Harold *et al.*, 1993).

1.5.9 *Piper capense* Linn. f. (Piperaceae)

Piper capense “ØU’, Timiz” is a shrubby herb 1-2m high belonging to a family Piperaceae which contains 8 genera and 3000 species. The genus *Piper* includes about 2000 species. The plant is widely distributed in Southern and Western Ethiopia, Sierra Leone, Angola, Zimbabwe and Mozambique (Hedberg *et al.*, 2000). Fruits of *Piper sarmentosum* have antibacterial, antineoplastic, antiprotozoal, antitubercular, antipsychotic and hypoglycemic activities (Anonymous, 2007b). Crude extracts and isolated compounds (chalcones, alkaloids, prenylated diphenols) from different *Piper* spp. (*P. aduncum*, *P. galioides*, *P. regnellii*, *P. cubeba* and *P. retrofractum*) had effect on various *Leishmania* spp. (Fournet *et al.*, 1996; Chan-Bacab and Pena-Rodriguez, 2001; Rocha *et al.*, 2005)

1.5.10 *Rhamnus prenoides* L’ Herit (Rhamnaceae)

Rhamnus prinoides “Ñ@j, Gesho” is a shrub or a small, dense, thick, bushy evergreen tree that grows up to 9 m long at altitudes of 2100m in Botswana, Eritrea, Ethiopia, Lesotho, Namibia, South Africa, Swaziland, Uganda and Kenya (Hedberg and Edwards, 1989). It belongs to the family Rhamnaceae which includes 49 genera and 900 species. The genus *Rhamnus* includes 150 species. The plant is used in traditional treatment of pneumonia, gonorrhoea, rheumatism, stomach ache, malaria and fungal infections (Koch *et al.*, 2005; Muregi *et al.*, 2007a & b; Abegaz, 1996; Tesema, 1980). *R. prenoides* contain unsaturated fatty acids, alkaloids, anthracene and naphthalene derivatives, flavonoids, anthraquinones, emodin anthrones, rhaninisin, mono-, di- and tri-acetylated rhamnosyl glycosides of emodin and emodin-anthrone (Bezabih and Abegaz, 1996; Tesema, 1980)

2. OBJECTIVES

2.1 General objective

To isolate extracts and essential oils from some selected Ethiopian medicinal plants, analyze essential oils for their chemical constituents and evaluate antileishmanial activity and cytotoxicity profile of these extracts and essential oils *in vitro*.

2.2 Specific objectives

- To gradient extract the root and aerial parts of selected medicinal plants.
- To extract essential oils from selected morphological parts of three medicinal plants those are rich in their oil content.
- To evaluate antileishmanial activity of gradient extracts and essential oils on promastigote and axenic amastigote form of *L. aethiopica* and *L. donovani* isolates *in vitro*.
- To carry out an *in vitro* cytotoxicity test on the gradient extracts and essential oils on Human Leukemia Monocyte (THP-1) cell line and Sheep blood.
- To determine chemical constituents of essential oils screened for antileishmanial activity using GC/MS method.
- To screen phytochemical constituents of each gradient extract tested for biological activity.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

3.1.1.1 Reagents and solvents

Reagents and solvents used in this work were; chloroform, (ReAgent ,Cheshire, UK), methanol and petroleum ether (100⁰C-120⁰C) (BDH Lab. Sup., Poole, England), formaldehyde (Merck, Darmstadt, Germany), resazurin sodium salt, dimethyl sulfoxide, hydrochloric acid and triton X-114 (all from Sigma Che. Co., St. Louis, USA).

3.1.1.2 Culture medium

RPMI-1640, and medium-199 (all from Gibco, Invitrogen Co., UK), foetal calf serum, nutrient agar, penicillin-streptomycin soln., D(+)-glucose, calcium chloride dihydrate , potassium chloride, disodium hydrogen phosphate , sodium bicarbonate and potassium dihydrogen phosphate (all from Sigma Che. Co., St. Louis, USA), sodium chloride (Carbo Erba Reagenti, Imbalati, Milano) were supplied to make complete culture mediums.

3.1.1.3 Reference drugs

Paromomycin sulphate (aminosidine sulfate®, Gland pharma, Hydrabad, India), amphotericin B (Fungizon®, Bristol-Myers Squibb, Rueil-Malmaison, France), sodium stibogluconate (Albert David LTD., Kalkata, India), pentamidine isethionate (Sigma Che. Co., St. Louis, USA) were included as reference drugs (positive control).

3.1.2 Materials

The following supplies were used ; polystyrene tissue culture flasks (Corning), 24-well and 96-well cell culture plates (flat bottomed) (Falcon & Corning), micro pipettes (Pipetman ultra), multichannel pipettes (Hamilton), Rota vapor (Model: RV05-ST), Soxhlet apparatus, clevenger type hydrodistillation apparatus (equipped with heating mantle, 3L round bottomed flask, condenser (Jacketed coil) and 3ml graduated receiver (Dean and Strack)), oven (Gallerikamp), UV cabinet, haemocytometer (Improved Double Neubauer type), eppendroff tube, light microscope (normal and inverted types) (Motic & Olympus), thermoshake (Gerhardt), heating mantle (Barnstead), autoclave (Pristage), class II biosafety cabinet (Labconco), analytical balance (Model:AB204-S), microwell plate reader (PerkinElmer), carbondioxide (CO₂) incubator (Thermo Electro), centrifuge (Hettich/ Thermo Electro), digital water bath (Memmet), vortex (Model: whirl VIB2), pipette tips (Oxford), pipette tips rack (Thermo Electro), yellow cups (Corning), microslides (Sunbeam), syringes (20 and 25 guages), membrane filter units (0.22µ) (Schleicher & Schuell), aluminium foil, parafilm, amber glass vials, crucible dishes, clamps, metal stands and spatulla .

3.1.3 Test strains, cell line and animals

The following *Leishmania* spp., human cell line and animals were included in this study. *L. aethiopica* isolate (CL/064/06) and *L. donovani* isolate (VL/15327/06) (both from Leishmania Diagnostic and Research Laboratory collection, Faculty of Medicine, Addis Ababa University), reference strains of *L. aethiopica* (MHOM/ET/82/117-82) and *L. donovani* (MHOM/SD/68/1S) (both kindly donated from Dr. Aldert Bart in Department of Medical Microbiology, University of Amsterdam Academic Medical Center (UvA.AMC), Netherlands), Human Monocytic Leukemia

(THP-1) cell line (ATCC-TIB-202) (donated from Mrs. Marga Goris of Royal Tropical Institute, Amsterdam and Dr. Markos Abebe of Armauer Hansen Research Institute (AHRI)) and Sheep blood.

3.2 Experimental Part

3.2.1 Collection and preparation of plant specimens

Plants were collected from Addis Ababa (Yeka & Kolfe-Keraniyoo sub cities), Jimma Zone (Seka & Dedo Woredas, both 370 Km SW of A.A), Sebeta (24 Km SW of A.A) and Inchini (85 Km W of A.A) between October 31, 2006 and January 13, 2007. Identification of each plant was made and voucher specimens were deposited at the National Herbarium, Department of Biology, Science Faculty, Addis Ababa University. The root (bark) and other aerial parts (leaf, stem (bark), flower (optional) & berries/seed (Optional)) of each plant were air-dried under the shade and powdered to suitable size separately. List of these plants with their families, voucher numbers, respective morphological partes used, site of collection and traditional uses of mentioned plants or other species in the same genus are indicated in Table 1. Picture of these plants are shown in Annex 1.

3.2.2 Preparation and storage of the extracts

30-50 g of powdered plant parts were gradient extracted using three organic solvents (petroleum ether, chloroform & methanol) in a soxhlet extractor. Extracts were concentrated in vacuo and then dried in hot air oven at 25°C for 48hrs. The resulting dried masses were weighed, powdered, packed in a glass vial and stored at -20°C until used. Percentage yield of extractives in the solvents used is presented in Table-2.

Essential oils were extracted from fresh leaves of *A. abyssinica* (150g), berries of *C. macrostachys* (430 g) and tubers of *E. kebericho* (400 g) using Clevenger type hydrodistillation apparatus. Distillation was carried for 3 hrs after the mixture started boiling. The oils were carefully separated; yields were calculated and finally stored in an opaque glass vial in a refrigerator (at -20 °C) until used.

3.2.3 Preparation of stock solutions

Each plant extract and pentamidine isethionate were dissolved in DMSO to a final stock concentration of 40 mg/ml and 25 µg/ml respectively. A stock solution of amphotericin B (5 µg/ml) was prepared in phosphate-buffered saline (PBS). Solution of sodium stibogluconate (100 mg/ml) and paromomycin sulphate (500 mg/ml) were also used. The standard drug solutions were all used as positive controls for anti-leishmanial activity screening and they were kept either at 4°C (amphotericin B, sodium stibogluconate and paromomycin) or -20°C (extract and pentamidine) until used.

3.2.4 Isolation and Characterization of *Leishmania* parasites

Leishmania isolates were obtained by taking samples from active cutaneous lesion and splenic aspirates of cutaneous and visceral leishmaniasis cases from Sendafa (N/Shoa) and Gondar respectively. *Leishmania* promastigotes were isolated from positive cultures previously raised in Lock's treated Novy-MacNeal-Nicolle (NNN) medium containing antibiotic solution (penicillin 100 U/ml and streptomycin 100 µg/ml) for one week at room

3.2.5 Culture conditions

3.2.5.1 Promastigote culture

The *L. aethiopica* and *L. donovani* isolates were grown in tissue cultured flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HIFCS) and 100 IU penicillin/ml-100µg/ml streptomycin solution at 22°C for *L. aethiopica* and 26°C for *L. donovani* following method described by Habtemariam S.(2003) and Ephos *et al*, (1999) respectively.

3.2.5.2 Cell line culture

The human leukemia monocyte THP-1 cells were cultured in RPMI-1640 supplemented with 10% HIFCS, 100U/ml penicillin, 100µg/ml streptomycin and 0.25 µg/ml amphotericin B in humidified, 5% CO₂ incubator at 37°C as described by Habtemariam S.(2003).

3.2.5.3 Axenic amastigote culture

The axenic culture were raised based on method described by Habtemariam S. (2003) (for *L. aethiopica*) and Saar *et al*. (1998) (for *L. donovani*) with minor modifications. Briefly promastigotes in late stationary phase were centrifuged and re-suspended in medium 199 containing Hank's balanced salts, supplemented with 20% heat inactivated fetal calf serum (HIFCS), 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and the pH adjusted to 5.5 (for both strains) using 1.0 N HCl. The cells were then incubated at 31°C (for *L. aethiopica*) or 37°C (for *L. donovani*) in humidified 5% CO₂ incubator for seven days.

Table –1 Plant species; voucher no, areas of collection and uses of the medicinal plants included in this study.

Scientific name (Family)	Voucher No	Time of collection	Parts used	Local name	Site of collection	Claimed and proved uses of the plant and members of the family
<i>Albizia schimperiana</i> Oliv. (Fabaceae)	YT-002/07	Jan 13, 2007	L, SB&RB	cd, Sesa	Jimma (Seka) ⁺	Purgative, antihelmintic (tapeworm), antifungal <u>antitrypanosomal</u> , <u>antileishmanial</u>
<i>Aloe spp.</i> (Aloaceae)	YT-003/06	Nov 8, 2006	L, S & R	_f, Eret	Addis Ababa ^K	Burns, antinflammatory, antifungal, <u>antileishmanial</u> , immune modulating, hypoglycemic
<i>Artemisia abyssinica</i> Bip ex A.Rich (Asteraceae /Compositae)	YT-004/06	Oct 31, 2006	L, S & R	Bl', Chikugn	Inchini [*]	Antihelmintic, bronchitis, cold, <u>malaria</u> , fever, nosebleeds, loss of appetite, colic, headache, earache, intestinal problems, <u>antileishmanial</u>
<i>Croton macrostachys</i> Hochest. ex A.Rich (Euphorbiaceae)	YT-006/06	Dec 23, 2006	B, L, SB & RB	wd", Bisana	Addis Ababa ^Y	Cough, wounds, tapeworm, purgative, skin rash, venereal diseases, malaria, immune modulating <u>antileishmanial</u>
<i>Echnops kebericho</i> Mesfin (Asteraceae/Compositae)	YT-007/06	Dec 12, 2006	FH, L, S & R	ku Œ, Kebericho	Inchini [*]	Migraine, typhoid fever, heart pain, hemorrhoid, diarrhea, <u>antihelmintic</u> , antitumor, fungicidal, antimutagenic, <u>antibacterial</u> , mosquito repellent, Insomnia, <u>antiplasmodial</u> , toothache, <u>antibacterial</u> , burns, gonorrhea, antinflammatory, antihelmintic, muscle relaxant, schizophrenia, <u>antifungal</u>
<i>Erythrina brucei</i> Schwein f. (Fabaceae)	YT-008/06	Dec 23, 2006	F, S, L SB & RB	¢ ¸, Korch	Addis Ababa ^Y	<u>Antinflammatory</u> (Asthma), cough, antibacterial, fever, antihelmintic, scabies, malaria, laxative, insecticide
<i>Justica schimperiana</i> Hochst. ex Nees (Acanthaceae)	YT-001/06	Dec 23, 2006	F, L, SB & R	c"cm, Sensel	Addis Ababa ^Y	<u>Antinflammatory</u> (Asthma), cough, antibacterial, fever, antihelmintic, scabies, malaria, laxative, insecticide
<i>Piper capense</i> Linn.f. (Piperaceae)	YT-012/07	Jan 11, 2007	B, L, S, & R	ØU', Timiz	Jimma (Dedo) ⁺	Coughs, antibacterial, antineoplastic, rheumatism, toothache, antimalarial, antipsychotic, <u>antiamoebic</u> flu, <u>hypoglycemic</u> , <u>antileishmanial</u>
<i>Phytolacca dodecandra</i> L'herit (Phytolacaceae)	YT-011/06	Dec 23, 2006	B, L, S & R	"ÊÉ, Endod	Addis Ababa ^Y	Anthelmintic, <u>molluscicide</u> , cathartic, malaria rheumatism, syphilis, gonorrhea, rabies, pruritus <u>antileishmanial</u> , <u>antifungal</u> , <u>antibacterial</u> .
<i>Rhamnus prenoides</i> L'herit (Rhamnaceae)	YT-013/06	Dec 23, 2006	B, L, SB & RB	Ñ@j, Gesho	Sebeta [*]	Blood purifier, pneumonia, gonorrhea, <u>malaria</u> , rheumatism,syphilis, gonorrhea, <u>antifungal</u>

Y: Yeka subcity, K: Kolfe keraniyo subcity, S: seed, B: Berries, F: Flower, FH: Flower head, L: Leaf, S: Stem, SB: Stem bark, R: root: RB: Root bark, * : 24Km SW of AA * : 85Km W of AA , + : 370 Km SW of AA
The underlined activities are experimentally proved ones (references are indicated in literature review part of these plants, pp 22-27).

3.2.6 Biological assays

All biological assays were performed in Leishmaniasis Diagnostic and Research Laboratory, Faculty of Medicine, Addis Ababa University. In each assay, the highest concentration of DMSO was adjusted not to exceed 1.0% of the complete culture medium, a concentration that showed no growth inhibitory effect. Reference strains of *L. aethiopica* and *L. donovani* were also treated with standard anti-leishmanial drugs to see any drug susceptibility differences between the reference strains and clinical isolates. Assays were done based on methods described by Ephros *et al.* (1999), Habtemariam (2003), Hoet *et al.* (2004) and Tiunan *et al.* (2005 a & b) with slight modifications (such as medium type, volume of medium used in the assay and initial extract concentration)

3.2.6.1 Anti-promastigote assay

3.2.6.1.1 Preliminary antileishmanial activity screening (Determination of percent inhibitory concentration)

In 96-well microtitre plate containing 100 µl of complete culture medium, each extracts were added in triplicate wells to achieve a final concentration of 800 µg/ml. Then 100 µl of suspension of parasites (3.5×10^6 promastigotes/ml of *L. aethiopica* and *L. donovani*) obtained from a logarithmic growth phase of previous culture (3.2.5.1) were added to each well and contents of the plates were then maintained at 22°C (for *L. aethiopica*) and 26 °C (for *L. donovani*) for three days with a daily determination of cell density, motility and morphology using an inverted microscope. After 72 hrs of incubation, parasites were killed with 2% formalin and number of cells was counted with a hemocytometer using an optical microscope. The antileishmanial activities (% inhibitions) were expressed as mean \pm SD of triplicate measurements compared with that of the control after a 72 hrs. Paromomycin, amphotericin B, pentamidine and sodium

stibogluconate at standard concentrations were used as positive control where as medium alone and 1% DMSO was included as negative controls.

3.2.6.1.2 Determination of minimum inhibitory concentration (MIC)

In 96-well microtitre plate extracts were serially diluted to twice final test concentrations of 12.5 to 400 µg/ml (for extracts) and 0.0244 to 25.0 µl/ml (for essential oils) in 100 µl culture medium with each test concentration in duplicate. Then 100 µl suspensions containing 3.5×10^6 promastigotes/ml in a logarithmic phase were added to each well. Contents of the plates were then maintained at 22°C (for *L. aethiopica*) and 26°C (for *L. donovani*) under a 5% CO₂ incubator. The cell density, motility and morphology for each treatment were determined daily with an inverted microscope and the anti-leishmanial activity was expressed as the MIC values after a 72 h incubation. Biological tests were performed three times, with each test concentration in duplicate. Paromomycin sulphate, amphotericin B, pentamidine isethionate and sodium stibogluconate were used as positive control where as medium alone and 1% DMSO were included as negative controls.

3.2.6.2 Anti-amastigote assay

In a 96-well microtitre plate, test substances were serially diluted to final test concentrations of 0.8 to 400 µg/ml (for extracts) and 0.000228 to 0.50 µg/ml (for essential oils) in 50µl culture medium and 50µl suspensions containing 2×10^7 cells/ml axenic amastigotes were added to each well. Contents of the plates were then incubated in humidified atmosphere at 31°C (for *L. aethiopica*) or at 37°C (for *L. donovani*) under a 5% CO₂ for 72hrs. After 68 h of incubation, 10µl of fluorochrome resazurin solution (12.5µg dissolved in 100 ml of PBS (pH=7.2)) was added into each well and the fluorescence intensity was measured after a total incubation time of 72h using

Victor³ Multilabel Counter at excitation wavelength of 530nm and emission wavelength of 590nm. The EC₅₀ values for each extract was evaluated from sigmoidal dose–response curves using computer software Graphpad prism 3.0 and values expressed as mean \pm SD of triplicate experiments with each test concentration in duplicate. Assays with standard anti-leishmanial drugs and negative controls (medium alone and 1% DMSO) were also performed to have reference values. Also the background fluorescence intensity of each extract, essential oil and reference drug was measured. (See Annex 3 for plate layout)

3.2.6.3 Cytotoxicity assays

3.2.6.3.1 Cell line (THP-1) viability assay

Drugs were serially diluted in 96-well plates as indicated in section 3.2.6.2 and 50 μ l suspensions of 1×10^6 THP-1 cells was added to each well. Contents of the plates were then incubated in a 5% CO₂-air mixture at 37°C for 72 h. Assays were performed as in 3.2.6.2 and the cell viabilities were evaluated from sigmoidal dose–response curves using Graphpad prism 3.0 software as mean \pm SD of triplicate experiments with each test concentration in duplicate. Standard antileishmanial drugs and negative controls (medium alone and 1% DMSO) were used in this assay to have reference values.

3.2.6.3.2 Haemolysis test

A 4% suspension of sheep blood was prepared by adding 2ml of freshly defibrinised whole blood to 48ml of sterile 5% glucose solution. 200 μ l of red-blood-cell suspension was added to eppendorf tube and different concentrations of the extracts (50-1000 μ g/ml), essential oils (0.00114-2.5 μ l/ml) and reference drugs (156.25-5000 μ l/ml for sodium stibogluconate, 1000-31.25 μ l/ml for paromomycin and 50-1.56 μ g/ml for amphotericin B and pentamidine) were

added, gently mixed, and incubated at 37°C for 2hrs. Samples were centrifuged at 1,000 g for 10 min, the supernatants were transferred to 96 well plate and absorbance of the supernatant was measured at 540 nm using Victor³ Multilabel Counter. Haemolytic effects were expressed as percentage of the absorbance of the positive control and the 50% lytic concentrations (LC₅₀) were determined from sigmoidal dose–response curves with Graphpd prism 3.0 software, expressed as Mean \pm SD. The negative control was the red blood cell suspension alone or with 1.0 % DMSO and the positive control was Triton X-114 (5 μ l/ml). These tests were all performed in duplicate on three separate occasions.

3.2.7 GC/MS analysis and phytochemical screening

3.2.7.1 GC/MS analysis of essential oils

The GC/MS analyses of essential oils were performed at The Plant Bio-center, Norwegian University of Science and Technology (Norway) using a Varian star 3400cx gas chromatograph equipped with a fused silica Chrompack WCOT CP-Sil-5 capillary column (30m x 0.32mm i.d. with a film thickness of 0.25 μ m) and automatic injector in splitless mode coupled with a Varian Saturn 3 mass spectrometer (ionization voltage 70 eV). The oven temperature was held at 40⁰C, then programmed from 40 to 220⁰C at a rate of 3⁰C/min, and then held at 220⁰C for 3 min. The carrier gas was helium (5psi) at 50 ml/min through the injector and a 30cm/sec through the column. The injector temperature was 220⁰C for the analysis. The MS detector was set at 175⁰C and a mass range of 40-400 (m/z) was recorded. All mass spectra were acquired in EI mode. The compounds in the volatile fractions were identified using a combination of mass spectrum data base search (IMS Terepene Library, 1989; NIST MS Data base, 1992 and 1998), relative retention index (ESO Database of essential oils, 1999) and comparison of mass spectra with that

in literature (Adams, 1995). Quantitative analysis (in %) was performed by peak area normalization measurements (TIC=Total ion count).

3.2.7.2 Phytochemical screening of gradient extracts

All extracts used for preliminary screening of anti-leishmanial activity were also subjected to phytochemical screening. The phytochemical screenings were performed following methods described in Debela (2002), Mojab *et al.* (2003), Akinyemi *et al.* (2005) and Parekh *et al.* (2007).

a) Alkaloids

1.5ml of 10% HCl was added to about 0.5 mg of the extracts in a test tube. The mixture was heated for 20 minutes. It was cooled and filtered. To 1ml of the filtrate 5 drops each of Mayer's and Dragendorff's reagents were added. Formation of cream and orange colored precipitates respectively indicated the presence of alkaloids in the extracts.

b) Saponins

Froth test: An aqueous solution of 0.5 mg of the extract in a test tube was vigorously shaken for 2 minutes. Foam which persisted for 30 minutes and doesn't disappear upon warming was taken as indication of the presence of saponin in the extract.

c) Polyphenols (Phenolic compounds)

3 drops of a mixture of 1 ml each of 1% FeCl₃ and 1% K₃Fe(CN)₆ were added to 2 ml of the aqueous solution of the extracts. Formation of green or blue color was taken as an indication of the presence of polyphenols.

c) Flavonoids

- i) Shibata's reaction:** 3ml of an aqueous solution of the extract was warmed with three pieces of magnesium turning and mixed with 3 drops of concentrated HCl. An orange pink colouration was taken as indication of the presence of flavonoids.
- ii)** To 2 ml of aqueous solution of the extract 4 drops of 2% lead acetate solution was added. Development of yellow or orange color confirms the presence of flavonoids.

e) Coumarins

2ml of 10% ammonia solution was added to 5ml concentrated alcoholic solution of the extracts. The occurrence of an intensive fluorescence under UV light indicates the presence of coumarin derivatives.

f) Terpenoids (Ketonic)

1ml of 2, 4-dinitrophenylhydrazine solutions (0.5g dissolved in 100ml of 2M HCl) was added to 2ml aqueous solution of the extract. Formation of yellow-orange coloration indicates the presence of a ketonic terpenoids.

g) Anthraquinones

Borntrager's test: 5ml of the extract was dried and shaken with 3ml petroleum ether. The filtrate was added to 2ml of a 25% ammonia solution. The mixture was shaken and formation of a red coloration was taken as indication of the presence of free anthraquinones.

h) Tannins

- i)** 3 drops of 5% ferric chloride solution was added to 1ml of the extract solution in water. A greenish or blue coloration or precipitation was taken as indication of the presence of tannins.

ii) 1ml of 1% gelatin solution was added to 1ml clear filtrate of plant extracts dissolved in ethanol and treated them with 2% NaCl solution. Precipitation which disappears with addition of excess gelatin confirms the presence of tannin.

i) Phytosterols and Withanoids

i) Five drops of 3% vanillin in conc. H_2SO_4 was added to a concentrated chloroform solution of extracts. Formation of a rose or reddish brown color indicates the presence of withanoids or phytosterols.

ii) **Liebermann's Burchard test:** 1ml of the extract was dissolved in 0.5ml of acetic anhydride and cooled well in ice. The mixture was mixed with 0.5ml of chloroform and 1ml of concentrated H_2SO_4 was then carefully added by means of pipette. Formation of a reddish-brown ring between the junctions of the two phases was taken as an indication of the presence of steroids.

4. RESULT AND DISCUSSION

4.1 Yield for plant extraction

4.1.1 Fractional extraction of the plants

Thirty seven different parts of ten species of plants that belong to eight different families were successively extracted with petroleum ether, chloroform and methanol in a soxhlet extractor. These extracts after removal of the solvents were used for the anti-leishmanial, cytotoxicity and hemolytic activity tests. The dried methanol extracts had powdery appearance and some were colored. The dried chloroform and petroleum ether extracts were mostly greenish, brownish or yellowish and oily/resinous in consistency. The yield from different morphological parts of the investigated plant species in the three solvents is presented Table 2.

4.1.2 Essential oil extraction from the plants

Essential oils were extracted from the leaves of *A. abyssinica*, berries of *C. macrostachyus* and tubers of *E. kebericho* using Clevenger type essential oil extraction apparatus and the yields were 0.60%, 0.038% and 0.05% respectively (Table 3). The essential oils obtained were all yellow in color.

Table 2 Percentage yields of the gradient extracts of the powdered plant materials

Scientific name	Part used	% Yield for gradient extracts		
		Pet. Ether	Chloroform	Methanol
<i>A. schimperiana</i>	Leaf	2.80	1.89	14.74
	Stem bark	0.78	1.60	12.59
	Root bark	1.22	1.56	20.08
<i>Aloe spp.</i>	Stem	1.30	0.89	18.04
	Root	1.09	0.46	9.86
<i>A. abyssinica</i>	Leaf	8.17	2.43	9.73
	Stem	3.46	1.07	6.46
	Root	1.67	0.60	7.32
<i>C. macrostachys</i>	Berries	6.43	1.45	22.59
	Leaf	2.66	2.10	16.00
	Stem bark	2.89	0.21	2.83
	Root bark	6.87	1.77	7.47
<i>E. kebericho</i>	Flower head	5.08	0.74	11.33
	Leaf	8.35	0.85	8.95
	Stem	1.55	0.23	6.00
	Root (tuber)	5.78	0.42	9.66
<i>E. brucei</i>	Flower	3.23	1.36	18.89
	Seed	10.16	1.22	14.38
	Leaf	4.56	3.03	12.00
	Stem bark	1.09	3.41	8.83
	Root bark	1.80	1.56	5.73

Table 2 Contiued

Scientific name	Part used	% Yield for gradient extracts		
		Pet. Ether	Chloroform	Methanol
<i>J. schimperiana</i>	Flower	1.92	1.75	3.00
	Leaf	2.92	2.08	27.00
	Stem bark	1.80	0.83	27.30
	Root	1.51	0.61	22.24
<i>P. capense</i>	Fruit	12.26	1.03	10.71
	Leaf	8.21	1.84	13.47
	Stem bark	2.17	0.84	5.64
	Root	4.18	0.44	3.45
<i>P. dodecandra</i>	Berries	4.20	0.96	29.00
	Leaf	4.20	1.09	9.54
	Stem	1.13	0.40	8.05
	Root	0.93	0.29	22.22
<i>R. prenoides</i>	Berries	10.3	36.60	35.00
	Leaf	4.00	1.46	32.00
	Stem bark	2.55	1.05	27.50
	Root bark	1.06	5.20	28.64

Table-3 Yield for extraction of essential oil from claimed medicinal plants

Scientific Name	Part used	Amount used for extraction (g)	Oil Color	% Yield (v/w)
<i>A. abyssinica</i>	Leaf	150	Pale yellow	0.60
<i>C. macrostachys</i>	Berries	400	yellow	0.038
<i>E. kebericho</i>	Root (tuber)	400	yellow	0.050

4.2 Biological assays

All species of plants included in this study were selected on the basis of data obtained from literature, traditional local or systemic uses and chemical composition of these plants or other members in the same genus. Some of the infections/disorders treated by candidate plants or the genus to which they belong include viral, bacterial, fungal and protozoal (including leishmaniasis) infections, inflammation, fever and cancer. Leishmaniasis resemble these infections, particularly in terms of pathogenetic mechanisms involved and potential drug targets such as sterol biosynthesis pathways and glycolysis, cysteine peptidases, trypanothione metabolism, microtubules, dehydrofolate reductases, protein kinases and topoisomerases (Croft and Coombs, 2003; Donald, 2003). Fever and inflammatory responses are among initial and long period clinical symptoms of one or several clinical forms of these infectious diseases.

Anti-leishmanial activity studies of drugs and plant products are usually done on two stages of the parasite; promastigote and amastigote stages. *In vitro* assays on the promastigote form of the parasite are easier because they can be easily cultivated and maintained under *in vitro* conditions.

Since promastigotes significantly differ from amastigotes in terms of their biochemistry and sensitivity to standard and experimental drugs, assays done on promastigotes have only an

indicative value of the possible leishmanicidal activity of a drug or plant extract tested. However, antipromastigote assays are good indicators of cytotoxicity in bioassay guided fractionation of plant extracts. Therefore preliminary evaluation of drugs on promastigotes needs to be complemented with assays on axenic or intracellular forms of amastigote.

In order to establish whether the *in vitro* activity of a drug or a plant extract is due to its general cytotoxic activity or selective toxicity against the *Leishmania* parasite; usually drugs/plant extracts are evaluated against non-parasitized macrophages or cell lines like THP-1 (Chan-Bacab and Pena-Rodriguez, 2001). They could also be tested on erythrocytes for possible haemolytic effects (Tiuman *et al.*, 2005b).

4.2.1 Anti-promastigote activities

4.2.1.1 Preliminary anti-leishmanial activity screening

This preliminary screening assay was done to select extracts showing high bioactivity for a thorough study of the antileishmanial and toxicity profile. The concentration of extracts, types of negative control and reference drugs used in this study were set based on information obtained from literature and the optimization experiments conducted in this study.

In this study, a total of 112 extracts (37 each for petroleum ether, chloroform and methanol and 1 leaf exudate) obtained from different morphological parts of ten Ethiopian medicinal plants belonging to eight families were used for preliminary screening of anti-leishmanial activities on promastigote forms of clinical isolates of *L. donovani* and *L. aethiopica*.

The results illustrate that extracts from all species of plants (all morphological parts except the seed of *E. brucei*) included in the present study were found to be active on at least one of the two *Leishmania* spp. Complete growth inhibitory activities were observed for the non-polar and less polar fractions (i.e. 36 petroleum ether and 34 chloroform extracts) compared to the relatively polar fraction (i.e. 7 methanol extracts and the leaf exudates of Aloe) at 400 µg/ml. The non-polar fractions with weak growth inhibitory activities were the petroleum ether extract of seeds of *E. brucei*, the chloroform extracts of seeds and leaves of *E. brucei* and leaves of *P. dodecandra*. Methanol extracts obtained from the leaf, stem bark and root bark of *A. schimperiana*, stem of *Aloe*, tuber of *E. kebericho* and stem bark and root bark of *E. brucei* showed strong growth inhibitory activities. Therefore only these 78 relatively more active extracts and three essential oils were used for further studies of anti-leishmanial and cytotoxicity studies.

4.2.1.2 Anti-promastigote activities (MIC)

Minimum Inhibitory Concentration (MIC) values for the 78 selected extracts and three essential oils obtained from the leaf of *A. abyssinica*, berries of *C. macrostachyus* and tubers of *E. kebericho* were determined against clinical isolates of *L. aethiopica* and *L. donovani in vitro*. Anti-leishmanial effect of the reference drugs, sodium stibogluconate, amphotericin B, paromomycin and pentamidine isethionate was also determined for comparison on both clinical isolates and reference strains of *L. aethiopica* and *L. donovani*.

MICs of these extracts and essential oils was determined by investigating morphological and motility changes of drug treated parasites under light microscope following 72 hours of incubation. Results of this experiment are illustrated in Table 4. Among the extracts tested 56 (71.79 %) and 60 (76.92 %) of them had MIC values between 200-400 µg/ml or higher against *L.*

aethiopica and *L. donovani* respectively. Also 17 (21.79 %) and 12 (15.38 %) extracts showed MIC values between 50 -100 µg/ml against *L. aethiopica* and *L. donovani* respectively. Five most active extracts with MIC values of 25 µg/ml were obtained from petroleum ether extracts of stem bark of *Albizia schimperiana* and chloroform extracts of the leaves, stem bark and root bark of *Albizia schimperiana*, and flower of *Erythrina brucei*. All the essential oils also had MIC values between 0.0097 and 0.1565 µg/ml against *L. aethiopica* and 0.0765 to 0.1565 µg/ml against *L. donovani*. Essential oil of *Echnops kebericho* had the highest activity on both species. Standard anti-leishmanial drugs had MIC values between 0.0244 µg/ml (for Amphotericin B) and >10,000 µg/ml (for Sodium stibogluconate). The promastigote stages the two *Leishmania* species had slightly different susceptibilities to paromomycin.

Generally the non-polar and less polar fractions were found to be more active than the polar fractions and the petroleum ether fractions had stronger activity compared to chloroform fractions. The methanol fractions were the least active indicating that anti-promastigote activities decrease with increasing polarity. These results were also in agreement with the preliminary anti-leishmanial activity screening results.

When the anti-leishmanial activities of these plant extracts against promastigote forms of the two strains were compared to activities of two standard anti-leishmanial drugs (sodium stibogluconate and paromomycin), almost all of them had better activities (lower MIC values) but most of these had much lower activities compared to amphotericin B and pentamidine. However all the essential oils showed better activities compared to any of the standard drugs tested (including amphotericin B and pentamidine). The negative controls (1% DMSO and RPMI-1640 medium alone) were devoid of any antileishmanial activity.

Most of the extracts showed relatively higher activities against promastigote forms of *L. aethiopica* than on *L. donovani*. These differences in drug susceptibility between the two species could be associated with slight differences in their membrane composition and metabolic characteristics between the two species; and also by the extent to which they are exposed to different chemical substances and culture conditions (Glew *et al.*, 1988; Croft *et al.* 2006 b; Singh, 2006).

4.2.2 Anti-amastigote activities

Promastigotes and amastigotes clearly differ in their morphology, bioenergetics (including the utilization of fatty acids, enzymes of fatty acid oxidation, glycolytic enzymes and pathways and glycosomes), gene expression and protein phosphorylation (eg. proteinases and nucleases) and expression of membrane proteins (including gp63, LPG, and a metalloproteinase (Glew *et al.*, 1988; Callahan *et al.*, 1997). As a result, susceptibility of promastigotes and amastigotes to standard (or experimental) anti-leishmanial drugs and plant extracts may differ (Sereno and Lemesre, 1997; Callahan *et al.*, 1997)

Promastigotes can be transformed into amastigotes *in vitro* in two ways; by infecting macrophages or monocytic cell lines derived from mouse (e.g J774) or human (e.g THP-1, U937, HL-60) or axenically by adjusting medium conditions (like % HIFCS, temperature and pH), to mimic the environment in the parasitophorous vacuole (Zilberstein, 1994; Sereno and Lemesre, 1997; Teixeira, *et al.*, 2002)

Axenically grown amastigotes resemble intracellular amastigotes in their ultrastructural, biological, biochemical, and immunological properties and they are used for *in vitro* drug

screening procedures. So far protocols for axenic cultivation of leishmania parasite have been described for *L. pifanoi* (Pan, 1993), *L. mexicana*, *L. amazonensis* and *L. chagasis* (Teixeira et al., 2002), *L. braziliensis* (Bates, 1993), *L. donovani* (Al-Bashir et al., 1992; Saar et al., 1998), *L. infantum* and *L. tropica* (Limoncu et al., 1997; Sereno et al., 1998) and *L. (V). panamensis* and *L. (V). guyanensis* (Puentes et al., 2000). For *L. aethiopica* temperature elevation alone or together with lowering the pH to 5.5 and 5% CO₂-air mixture could result in differentiation of promastigotes to amastigotes (Habtemariam, 2003; Getti *et al.*, 2006).

The axenic amastigote based assay has advantages and disadvantages compared to the conventional macrophage based (intracellular amastigotes) drug screening. The axenic amastigotes generally are simple to cultivate, harvested within a few days and are reliable for rapid and large scale drug screening experiments. However, for drugs which may have immune stimulating characteristics the macrophage based assay is a good indicator of drug effect. Macrophages can also play roles in accumulating certain drugs and their metabolism influencing local concentration and intracellular activities of these drugs. Infact the pentavalent antimony (Sb⁵⁺) in antimonials (prodrugs) are converted to toxic trivalent antimony (Sb³⁺) in macrophages. In some strains slight differences in drug susceptibility between axenic amastigotes and intracellular amastigotes were also reported (Sereno and Lemesre, 1997; Callahan, *et al.*, 1997).

The 78 extracts and the three essential oils included in the anti-promastigote assay were screened for their effect on axenic amastigote forms of *L. aethopica* and *L. donovani in vitro*. Four standard antileishmanial drugs (sodium stibogluconate, paromomycin, pentamidine and amphotericin B) were also tested for comparison. The 50% effective concentrations (EC₅₀) of the extracts and essential oils were evaluated from fluorescence characteristic of resazurin added to

drug treated cultures. Respective EC₅₀ values of each extract, essential oil and reference drug is presented in Table 4.

A number of assay methods are available to study proliferation and viability of *Leishmania* and other cell types. These include simple microscopy and haemocytometer based assays (Tiuman *et al.*, 2005b; Luize *et al.*, 2005), trypan blue assay (Socorro, *et al.*, 2003), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Tadesse *et al.*, 19993; Mariana *et al.*, 2005; Miguel *et al.*, 2007; Mori *et al.*, 2008), tritiated [³H] thymidine incorporation assay (Tadesse *et al.*, 19993) and flow cytometry based assay (Giorgio *et al.*, 2000; Kamau *et al.*, 2001). In most of these assays, cell samples are prepared following certain steps such as fixation, DNA denaturation, washing and lysing, replacement of drug and medium etc. and they do not allow continuous monitoring of cells. Some of these assays also involve handling radioactive substances. In general these assaying methods are extremely useful when the experimental conditions are well standardized.

On the other hand the Alamar blue® (Resazurin) (7-hydroxy-3H-phenoxazin-3-one 10-oxide) assay permits a simple, rapid, reliable, sensitive, cost-effective method for continuous monitoring of cell cultures. It circumvents problems associated with other assays and help to accurately and quantitatively measure proliferation of *Leishmania* (Habtemariam, 2003; Hoet *at al.*, 2004; Maes *et al.*, 2004a) and variety of cell types (human, animal, bacteria, mycobacteria, fungi) (Anonymous, 2007a). The dye (resazurin) employed in this method is soluble and stable in culture medium and non-toxic to cells. The dye also does not affect the secretory abilities of cells. However optimization of experimental conditions such as temperature, pH, cell density and dye concentration are required (Anonymous, 2007a). Metabolically active cells (growing cells) in

the culture medium result in reduction of the oxidized, nonfluorescent and blue redox indicator (resazurin) to the reduced, fluorescent and red form of the dye (resorufin) (Figure 6). Continuous cell growth maintains a reduced environment while inhibition of growth by any chemical substance maintains an oxidized environment. In this assay cell viability is monitored by measuring fluorescent signal at excitation wavelength of 530-560 nm and emission wavelength of 590 nm or measuring absorbance at 570 nm and 600 nm. The absorbance and fluorescence intensities produced are proportional to the number of viable cells (Anonymous, 2007a).

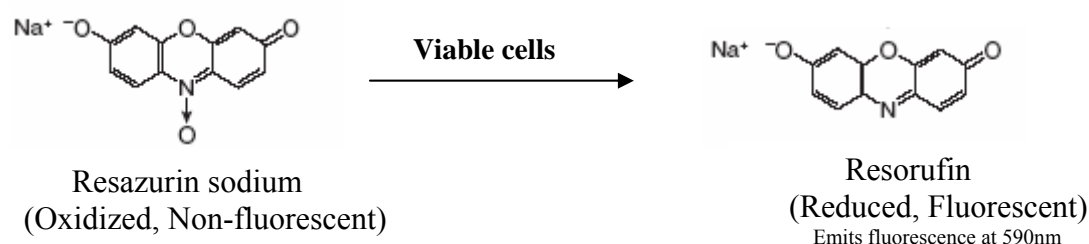


Figure 8: Conversion of resazurin to resorufin by metabolically active cells

Following incubation of *Leishmania* cells at appropriate temperature, rounded and amastigote-like parasites were formed with loss of flagella and cell clumping appeared within 24 h but these changes were not complete for a week as some motile parasites with intermediate forms and short flagella were detected on microscopic observations. Metabolic differences, such as ability to readily reduce resazurin dye were revealed for promastigotes and axenic amastigotes during the experiments.

Among the extracts tested 27 (34.6 %) and 20 (25.6 %) of them had EC_{50} values between 10-50 $\mu\text{g/ml}$ against *L. aethiopica* and *L. donovani* respectively. Also 48 (61.5 %) and 55 (70.5 %) extracts had EC_{50} values less than 10 $\mu\text{g/ml}$ against *L. aethiopica* and *L. donovani* respectively. The most active ones against both organisms were ($EC_{50} < 3 \mu\text{g/ml}$) petroleum ether extracts of leaves of *A. abyssinica*, berries and leaves of *C. macrostachyus*, tuber of *E. kebericko*, stem bark

of *E. brucie* and chloroform extract of tuber of *E. kebericho*. The EC₅₀ values for essential oils were between 0.24 ± 0.01 and 7.94 ± 5.58 nl/ml against *L. aethopica* and 0.50 ± 0.07 and 42.00 ± 2.80 nl/ml against *L. donovani*. Essential oil from *E. kebericho* showed the highest activity against both species. Standard anti-leishmanial drugs i.e amphotericin B, pentamidine, paramomycin and sodium stibogluconate had EC₅₀ values of (0.018 ± 0.006 & 0.047 ± 0.008), (0.63 ± 0.01 & 0.70 ± 0.06), (3.88 ± 0.92 & 7.34 ± 0.87) and (53.36 ± 7.65 & 41.80 ± 7.15) against *L. donovani* and *L. aethopica* respectively.

In this study the non-polar and less polar fractions were found to be more active than the polar fractions and these observations are in agreement with the preliminary anti-leishmanial activity screening result and report on anti-leishmanial activity of similar fractions and defined class of compounds from different plant species in previous works (Mariana *et al.*, 2005; Rocha *et al.*, 2005). Although several reports have been made on anti-leishmanial potential of methanol extracts of different plants (El Tahir *et al.*, 1998; Lamidi *et al.*, 2005; Braga *et al.*, 2007; Mori *et al.*, 2008), only 7 of the 37 methanol extracts (18.9 %) included in this study showed good anti-leishmanial activity. Moreover important phytochemical classes of compounds previously reported to have anti-leishmanial activities were detected in this fraction (Chan-Bacab and Pena-Rodriguez, 2001; Rocha *et al.*, 2005).

When the anti-leishmanial activities of these plant extracts against promastigote forms of the two strains were compared to standard anti-leishmanial drugs most of them had better activities than sodium stibogluconate. Six extracts obtained from petroleum ether fraction of leaves of *A. abyssinica*, berries and leaves of *C. macrostachyus*, tuber of *E. kebericho*, stem bark of *E. brucie* and chloroform fraction of tuber of *E. kebericho* were found to be more active than

paromomycin. Almost all the essential oils had better activities compared to the standard drugs amphotericin B and pentamidine against both *Leishmania* species. Moreover the observed anti-leishmanial activities (EC_{50}) of Pentamidine and Paramomycin were in agreement with previous reports (Gebre-Hiwot et al, 1992; Tadesse et al, 1993; Croft et al., 2006b) on similar strains. However that of amphotericin B was lower than reported values and sodium stibogluconate was higher (Habtemariam, 2003; Mikes and Steverding, 2000; Tadesse et al, 1993; Gebre-Hiwot et al, 1992). In fact for these two drugs (amphotericin B and sodium stibogluconate), EC_{50} values were reported for brands other than what was used in this work and this may explain the observed variation. Comparative experiments made on susceptibility of promastigote and axenic amastigote forms of reference strains and isolates of the two *Leishmania* spp to standard anti-leishmanial drugs also showed significant differences between these isolates and reference strains. The negative controls (1% DMSO and RPMI-1640 medium) alone were found to be devoid of any anti-leishmanial activity.

Some of the candidate plants and other species of plants that belong to the same genus have already been investigated for antileishmanial activity on different *Leishmania* species in other studies. The plants include *Albizia coriaria* (Tonui et al., 2007), *Aloe vera* (Dutta et al, 2006), *Aloe kedogensis* (Tonui et al., 2007), *Artemesia aucheri* (Sharif et al., 2006), *Croton cajucara* (Socorro et al., 2003), *Croton macrostachys* (Fessahaye et al., 1993), *Erythrina spiciosa* (Luize et al., 2005), *Phytolacca americana* (Chan-Bacab and Pena-Rodriguez, 2001), *Phytolacca dodecandra* (Rochaa et al., 2005) and different *piper* spp. (*P. aduncum*, *P. galioides*, *P. regnellii*, *P. cubeba* and *P. retrofractum*) (Fournet et al., 1996b; Chan-Bacab and Pena-Rodriguez, 2001; Rocha et al., 2005). Moreover, it is the lipophilic extracts of some of these plants (particularly of piper species) that showed significant antileishmanial effect.

Table 4: Minimum inhibitory concentration (MIC) and EC₅₀ values for gardient extracts and essential oils (*A. Abyssinica*, *C. macrostachyus* and *E. kebericho*) screened against promastigote and axenic amastigote forms *L.donovani* and *L. aethiopica* *in vitro*.

Study plant	Type of extract	MIC values (µg/ml)		EC ₅₀ values (µg/ml) for	
		Promastigote		Axenic amastigote	
		<i>L. donovani</i>	<i>L. aethopica</i>	<i>L. donovani</i>	<i>L. aethopica</i>
<i>A. schimperiana</i>	L ^P	200	50	24.42 ± 0.90	13.92 ± 3.20
	L ^C	25	25	20.68 ± 3.31	4.09 ± 4.57
	L ^M	200	200	14.86 ± 7.09	74.10 ± 13.71
	SB ^P	25	25	8.45 ± 0.57	8.40 ± 1.78
	SB ^C	25	25	25.80 ± 1.21	9.71 ± 1.46
	SB ^M	25	50	15.41 ± 0.49	24.08 ± 4.06
	RB ^P	50	50	20.32 ± 2.03	29.45 ± 0.60
	RB ^C	50	50	4.64 ± 0.48	21.91 ± 2.70
	RB ^M	200	400	46.45 ± 9.92	>400
<i>Aloe spp.</i>	LE	400	400	54.31 ± 5.48	74.66 ± 1.00
	S ^P	400	200	3.09 ± 0.91	8.75 ± 0.74
	S ^C	400	200	14.68 ± 2.14	11.02 ± 2.47
	S ^M	400 ⁺	400	>400	10.24 ± 1.12
	RB ^P	200	200	2.54 ± 0.54	7.70 ± 0.24
	RB ^C	400	200	10.45 ± 0.18	1.77 ± 0.77
<i>A. abyssinica</i>	L ^P	100	100	2.36 ± 0.10	1.21 ± 0.26
	L ^C	400	400	5.05 ± 0.01	10.82 ± 4.15
	S ^P	200	200	2.08 ± 0.04	5.61 ± 1.85
	S ^C	400	200	6.24 ± 0.17	9.30 ± 2.01
	R ^P	200	100	2.22 ± 0.52	3.38 ± 0.40
	R ^C	400	400	10.64 ± 0.35	10.43 ± 1.92

Table 4: Continued

Study plant	Type of extract	MIC values ($\mu\text{g/ml}$)		EC ₅₀ values ($\mu\text{g/ml}$) for	
		Promastigote		Axenic amastigote	
		<i>L. donovani</i>	<i>L. aethiopica</i>	<i>L. donovani</i>	<i>L. aethiopica</i>
<i>C. macrostachys</i>	B ^P	200	100	2.02 \pm 0.21	1.77 \pm 0.21
	B ^C	400	200	2.39 \pm 0.30	7.43 \pm 4.47
	L ^P	400	200	2.56 \pm 0.03	2.01 \pm 0.70
	L ^C	400 ⁺	400	7.52 \pm 0.05	6.01 \pm 3.95
	SB ^P	200	400	5.20 \pm 1.84	22.83 \pm 5.34
	SB ^C	400	200	2.27 \pm 0.23	3.36 \pm 0.24
	RB ^P	200	200	2.93 \pm 0.33	6.28 \pm 4.00
	RB ^C	200	200	3.40 \pm 0.72	3.14 \pm 1.03
<i>E. kebericho</i>	FH ^P	100	200	4.90 \pm 0.59	8.04 \pm 1.26
	FH ^C	200	200	2.26 \pm 0.66	9.20 \pm 5.50
	L ^P	200	200	7.64 \pm 0.05	7.78 \pm 3.83
	L ^C	100	200	5.87 \pm 1.29	14.26 \pm 13.57
	S ^P	100	200	11.14 \pm 2.22	13.47 \pm 3.68
	S ^C	200	200	3.80 \pm 1.53	5.29 \pm 1.35
	R ^P	50	50	0.66 \pm 0.02	0.74 \pm 0.22
	R ^C	100	200	1.64 \pm 0.82	1.41 \pm 0.29
	R ^M	400	400 ⁺	5.23 \pm 1.74	9.41 \pm 0.91

Table 4: Continued

Study plant	Type of extract	MIC values ($\mu\text{g/ml}$)		EC ₅₀ values ($\mu\text{g/ml}$) for	
		Promastigote		Axenic amastigote	
		<i>L. donovani</i>	<i>L. ethiopica</i>	<i>L. donovani</i>	<i>L. ethiopica</i>
<i>E. brucei</i>	F ^P	200	200	2.26 \pm 0.70	4.80 \pm 1.98
	F ^C	25	25	6.07 \pm 0.00	7.06 \pm 0.69
	L ^P	400	400	3.25 \pm 0.39	9.51 \pm 1.88
	SB ^P	200	200	1.87 \pm 0.15	2.86 \pm 0.65
	SB ^C	200	200	2.57 \pm 0.59	3.41 \pm 0.64
	SB ^M	200	200	19.74 \pm 2.61	18.54 \pm 10.44
	RB ^P	200	200	9.43 \pm 0.12	4.85 \pm 1.21
	RB ^C	200	200	4.72 \pm 2.02	4.26 \pm 0.05
	RB ^M	400	400	22.67 \pm 0.19	27.21 \pm 8.36
<i>J. schimperiana</i>	FH ^P	400	200	19.07 \pm 2.15	21.66 \pm 4.37
	FH ^C	400 ⁺	400	6.74 \pm 5.48	5.78 \pm 2.51
	L ^P	400	200	2.165 \pm 0.06	4.90 \pm 1.10
	L ^C	400 ⁺	400 ⁺	6.65 \pm 5.33	22.34 \pm 1.77
	SB ^P	400	200	3.19 \pm 0.02	3.43 \pm 0.11
	SB ^C	400	400 ⁺	13.02 \pm 0.85	9.01 \pm 0.18
	R ^P	400	400	9.80 \pm 2.88	8.88 \pm 1.17
	R ^C	400	400 ⁺	49.3 \pm 5.43	51.99 \pm 13.03

Table 4: Continued

Study plant	Type of extract	MIC values ($\mu\text{g/ml}$)		EC ₅₀ values ($\mu\text{g/ml}$) for	
		Promastigote		Axenic amastigote	
		<i>L. donovani</i>	<i>L. ethiopica</i>	<i>L. donovani</i>	<i>L. ethiopica</i>
<i>P. capense</i>	B ^P	50	50	6.15 \pm 0.31	11.28 \pm 7.30
	B ^C	200	100	2.61 \pm 0.47	3.64 \pm 0.72
	L ^P	200	200	4.44 \pm 0.71	6.05 \pm 1.29
	L ^C	200	200	5.01 \pm 0.02	6.25 \pm 1.43
	SB ^P	100	100	8.21 \pm 0.04	15.62 \pm 5.68
	SB ^C	200	400	3.48 \pm 1.90	15.93 \pm 9.74
	R ^P	100	50	8.08 \pm 0.105	13.13 \pm 8.08
	R ^C	400	200	11.63 \pm 1.25	29.08 \pm 0.20
<i>P. dodecandra</i>	B ^P	200	400	2.28 \pm 1.59	8.37 \pm 3.01
	B ^C	400 ⁺	400 ⁺	16.61 \pm 2.39	16.12 \pm 0.23
	L ^P	400	400	3.19 \pm 0.70	5.49 \pm 1.59
	S ^P	200	50	6.80 \pm 0.72	20.44 \pm 6.35
	S ^C	400	100	1.62 \pm 0.77	19.02 \pm 11.96
	R ^P	50	100	1.79 \pm 0.89	12.83 \pm 0.50
	R ^C	200	400	5.65 \pm 1.69	8.95 \pm 0.84

Table 4: Continued

Study plant	Type of extract	MIC values ($\mu\text{g/ml}$)		EC ₅₀ values ($\mu\text{g/ml}$) for	
		Promastigote		Axenic amastigote	
		<i>L. donovani</i>	<i>L. aethiopica</i>	<i>L. donovani</i>	<i>L. aethiopica</i>
<i>R. prenooides</i>	B ^P	200	100	5.99 \pm 1.96	7.97 \pm 0.62
	B ^C	400	400	31.69 \pm 11.79	44.09 \pm 11.49
	L ^P	400	400	3.16 \pm 1.17	5.68 \pm 1.39
	L ^C	400 ⁺	400	11.52 \pm 5.32	25.47 \pm 11.10
	SB ^P	200	200	6.85 \pm 2.95	6.85 \pm 2.14
	SB ^C	400 ⁺	400	7.05 \pm 1.38	15.21 \pm 4.28
	RB ^P	50	50	11.57 \pm 4.29	17.51 \pm 1.52
	RB ^C	100	200	0.85 \pm 1.03	9.10 \pm 1.01
<u>Essential oils</u>					
<i>A. abyssinica</i>	L	0.1565 [*]	0.1565 [*]	42.00 \pm 2.80 [∇]	7.94 \pm 5.58 [∇]
<i>C. macrostachyus</i>	B	0.0765 [*]	0.1565 [*]	20.00 \pm 2.10 [∇]	6.66 \pm 0.99 [∇]
<i>E. kebericho</i>	R	0.0765 [*]	0.0097 [*]	0.50 \pm 0.07 [∇]	0.24 \pm 0.01 [∇]
<u>Reference drugs</u>					
Sodium Stibogluconate		>10,000	>10,000	53.36 \pm 7.65	41.80 \pm 7.15
Amphotericin B		0.0244	0.0244	0.018 \pm 0.006	0.047 \pm 0.008
Paromomycin		2775	925	3.88 \pm 0.92	7.34 \pm 0.87
Pentamidine Isethionate		8.33	8.33	0.63 \pm 0.01	0.70 \pm 0.06
<u>Negative controls</u>					
RPMI medium		0.00	0.00	0.00	0.00
1% DMSO solution		0.00	0.00	0.00	0.00

FH-Flower head, F-Flower, L-Leaf, SB-Stem bark, S-Stem, RB-Root bark, R-Root, B-Berries, SD-Seed (P)-Petroleum ether extract, (C)-Chloroform extract,(M)-Methanol extract, * = Unit ($\mu\text{l/ml}$), ∇ =Unit (nl/ml)

4.2.3 Cytotoxicity

Large percentage of the world population has no access to conventional drugs and relies on traditional herbal medications. To complement this growing interest in alternative therapies and guarantee safe therapeutic use of these medicinal plants, their toxicity should be investigated. Toxicity assays indicate degree of safety of herbal drugs and results from such assays are important for further *in vivo* studies. In this research, cytotoxicity and blood haemolysis tests were done *in vitro* to assess safety of the plant extracts included in this study.

All the 78 extracts and the three essential oils previously screened *in vitro* for anti-leishmanial activities were included in this cytotoxicity assay. The assay was performed on human monocyte leukemia cell line (THP-1) with increasing concentrations of extracts and essential oils. After 72 h of incubation, cell viability was assessed with resazurin and the 50% cytotoxic concentrations (CC₅₀) were reported as mean \pm SD from three separate experiments in duplicate. Four standard anti-leishmanial drugs were also used as positive references. The cytotoxicity and selectivity index (SI) (CC₅₀ for THP-1/ EC₅₀ for amastigotes) are indicated in Table 5.

The results showed that 62 extracts (79.48 %) had selectivity index greater than five against both strains with CC₅₀ values greater than 21.52 ± 2.80 . CC₅₀ values for seven extracts were between 21.46 ± 1.64 and 133.22 ± 73.08 $\mu\text{g/ml}$ with selectivity index ranging from 1.5 to 4.7. All extracts from *A. schimperiana* except petroleum ether extract of its leaf and stem bark showed severe cytotoxicity (with CC₅₀ values ranging from 1.05 ± 0.03 to 13.01 ± 0.49 $\mu\text{g/ml}$) each with selectivity index less than one. A selectivity index greater than one generally indicates selectivity against the parasites where as a value less than one shows selectivity against host cells. The CC₅₀ values for essential oils ranged between 0.40 ± 0.01 and 350 ± 5.0 nl/ml with selectivity index values ranging from 0.8 to 44. Essential oil of *E. kebericho* showed the highest cytotoxicity.

Standard anti-leishmanial drugs; pentamidine, amphotericin B, sodium stibogluconate and paramomycin had CC_{50} values of $1.86 \pm 0.05 \mu\text{g/ml}$, 13.27 ± 1.8 , $>2,000$ and $>10,000 \mu\text{g/ml}$.

Generally a progressive decrease in the cytotoxicity was observed with increasing polarity of gradient extracts (Table 5). As a result the petroleum ether extracts were having the highest cytotoxicity to the THP-1 cells. These observations are in agreement with the cytotoxicity reports in previous works for similar fractions obtained from different plants (Mariana *et al.*, 2005; Rocha *et al.*, 2005). Although traditional use of a herbal product doesn't fully guarantee its safety and efficacy, some of the study plants such as *R. prenoides* and *P. capense* have been in use for making traditional local beverage and as a spice respectively. Experimental results also guarantee safety of these plants. In fact, it is the water soluble components that are possibly contained in these preparations. Similarly the observed high cytotoxicity profile of two of the anti-leishmanial drugs, amphotericin B and pentamidine were also in agreement with that reported in previous works. Amphotericin B had a CC_{50} value of $13.9 \mu\text{g/ml}$ against J774G8 cells (Tiunan *et al.*, 2005) and $>10 \mu\text{g/ml}$ on THP-1 cells (Habtemariam, 2003). The reported CC_{50} value for pentamidine, $1.4 \mu\text{g/ml}$ on THP-1 cells (Tadesse *et al.*, 1993) was in agreement with that in this experiment, $1.86 \pm 0.05 \mu\text{g/ml}$.

Table 5: Cytotoxicity, hemolytic property and selectivity index of plant extracts and essential oils tested against human leukemia monocyte (THP-1) cell line and red blood cells.

Study plants	Type of extract	Cytotoxicity (CC ₅₀ , µg/ml) Mean + SD	Selectivity index (CC ₅₀ Cell /EC ₅₀ . parasite)		Blood hemolysis LC ₅₀ (µg/ml) Mean + SD
			<i>L. donvani</i>	<i>L. aethopica</i>	
<i>A. schimperiana</i>	L ^P	94.77 ± 29.50	3.9	6.8	>1000
	L ^C	13.01 ± 0.49	0.6	3.2	>1000
	L ^M	10.23 ± 0.92	0.7	0.1	>1000
	SB ^P	133.04 ± 40.07	16	16	>1000
	SB ^C	1.05 ± 0.03	0.1	0.1	238 ± 41.02
	SB ^M	2.06 ± 0.66	0.1	0.1	174.25 ± 22.27
	RB ^P	11.01 ± 1.03	0.5	0.4	660.15 ± 25.95
	RB ^C	1.18 ± 0.16	0.3	0.1	189.7 ± 38.46
<i>Aloe spp.</i>	RB ^M	2.41 ± 0.76	0.1	0.1	675.45 ± 0.45
	LE	133.22 ± 73.08	2.5	1.8	>1000
	S ^P	81.14 ± 20.65	26	9.3	705.10 ± 3.39
	S ^C	84.64 ± 21.92	5.8	7.7	>1000
	S ^M	90.58 ± 8.78	0.2	8.8	275.60 ± 22.41
	R ^P	106.09 ± 8.20	42	14	894.40 ± 18.95
<i>A. abyssinica</i>	R ^C	15.20 ± 3.53	1.5	8.6	>1000
	L ^P	41.65 ± 10.47	18	34	756.20 ± 106.91
	L ^C	113.32 ± 15.61	22	10	>1000
	S ^P	116.43 ± 20.46	56	21	794.85 ± 56.78
	S ^C	117.28 ± 14.50	19	13	>1000
	R ^P	22.41 ± 4.73	10	6.6	754.40 ± 5.23
	R ^C	154.15 ± 30.38	14	15	>1000

Table 5: Continued

Scientific Name	Type of extract	Cytotoxicity	Selectivity index		Blood hemolysis
		(CC ₅₀ , µg/ml)	(CC ₅₀ , Cell /EC ₅₀ , parasite)		LC ₅₀ (µg/ml)
		Mean + SD	<i>L. donvani</i>	<i>L. aethopica</i>	Mean + SD
<i>C. macrostachys</i>	B ^P	42.78 ± 51.67	21	24	>1000
	B ^C	87.36 ± 19.79	37	12	>1000
	L ^P	76.45 ± 25.41	30	38	>1000
	L ^C	>400	53	67	>1000
	SB ^P	192.14 ± 65.72	37	8.4	646.55 ± 47.44
	SB ^C	180.33 ± 61.45	79	54	297.70 ± 89.52
	RB ^P	189.33 ± 62.88	65	30	>1000
	RB ^C	161.65 ± 72.34	48	51	>1000
<i>E. kebericho</i>	FH ^P	142.77 ± 65.27	29	18	>1000
	FH ^C	118.62 ± 49.88	52	13	>1000
	L ^P	115.70 + 7.30	15	15	>1000
	L ^C	41.79 + 3.17	7.1	2.9	>1000
	S ^P	149.20 ± 62.51	13	11	>1000
	S ^C	199.63 ± 47.55	53	38	>1000
	R ^P	31.03 + 4.00	47	42	>1000
	R ^C	140.52 + 39.23	86	100	>1000
	R ^M	>400	76	43	>1000

Table 5: Continued

Scientific Name	Type of extract	Cytotoxicity	Selectivity index		Blood hemolysis
		(CC ₅₀ , µg/ml) Mean ± SD	(CC ₅₀ , Cell / EC ₅₀ , parasite) <i>L. donovani</i> <i>L. aethiopica</i>		LC ₅₀ (µg/ml) Mean ± SD
<i>E. brucei</i>	F ^P	>400	177	83	>1000
	F ^C	21.46 + 1.64	3.5	3	>1000
	L ^P	139.17 + 43.52	47	16	>1000
	SB ^P	152.13 ± 22.07	74	49	>1000
	SB ^C	21.52 + 2.80	8.4	6.3	249.55 ± 15.34
	SB ^M	200.83 + 49.70	10	11	>1000
	RB ^P	48.26 ± 6.65	5.1	10	>1000
	RB ^C	48.98 ± 0.88	10	11	>1000
	RB ^M	>400	18	15	>1000
<i>J. schimperiana</i>	F ^P	230.14 ± 127.14	12	11	861.6 ± 11.34
	F ^C	192.52 ± 68.56	29	33	>1000
	L ^P	91.76 ± 39.16	42	19	>1000
	L ^C	372.75 ± 70.40	56	17	>1000
	SB ^P	129.60 ± 24.70	41	38	238 ± 41.02
	SB ^C	370.63 ± 135.02	28	41	>1000
	R ^P	>400	41	45	>1000
	R ^C	>400	8.1	7.7	>1000

Table 5: Continued

Scientific Name	Type of extract	Cytotoxicity	Selectivity index		Blood hemolysis
		(CC ₅₀ , µg/ml) Mean + SD	(CC ₅₀ , Cell / EC ₅₀ , parasite) <i>L. donvani</i> <i>L. aethopica</i>		LC ₅₀ (µg/ml) Mean + SD
<i>P. capense</i>	B ^P	151.03 ± 60.15	25	13	>1000
	B ^C	126.49 ± 25.13	48	35	336.40 ± 6.08
	L ^P	93.24 ± 18.34	11	6	>1000
	L ^C	232.46 ± 118.39	67	15	>1000
	SB ^P	96.60 ± 4.60	12	7.4	>1000
	SB ^C	>400	34	14	>1000
	R ^P	73.98 ± 22.44	17	12	282.00 ± 14.14
R ^C	153.85 ± 34.28	31	25	816.60 ± 7.91	
<i>P. dodecandra</i>	B ^P	>400	175	48	>1000
	B ^C	>400	24	25	441.30 ± 13.15
	L ^P	115.89 ± 27.76	36	21	546.50 ± 67.17
	S ^P	80.09 ± 19.27	12	3.9	>1000
	S ^C	115.18 ± 32.87	71	6.1	814.55 ± 11.95
	R ^P	155.44 ± 48.88	87	12	>1000
	R ^C	220.40 ± 23.05	39	25	705.05 ± 76.86

Table 5: Continued

Scientific Name	Type of extract	Cytotoxicity	Selectivity index		Blood hemolysis
		(CC ₅₀ , µg/ml)	(CC ₅₀ , Cell / EC ₅₀ , parasite)		LC ₅₀ (µg/ml)
		Mean + SD	<i>L. donovani</i>	<i>L. aethiopica</i>	Mean + SD
<i>R. prenoides</i>	B ^P	67.81 + 27.17	11	8.5	> 1000
	B ^C	130.26 ± 34.67	4.1	3	>1000
	L ^P	244.56 + 117.91	77	43	>1000
	L ^C	119.70 + 11.70	10	4.7	>1000
	SB ^P	347.35 + 98.14	51	51	>1000
	SB ^C	265.92 ± 64.84	38	17	>1000
	RB ^P	>400	35	23	>1000
	RB ^C	167.28 + 34.15	197	18	>1000
<u>Essential oils</u>					
<i>A. abyssinica</i>	L	350 ± 5.0 [∇]	8.3	44	0.70 + 0.27*
<i>C. macrostachys</i>	B	10 ± 4.0 [∇]	5.5	17	2.45 + 0.57*
<i>E. kebericho</i>	R	0.40 ± 0.01 [∇]	0.8	1.7	2.62 + 1.0*
<u>Reference drugs</u>					
Sodium stibogluconate		>2000	48	37	>5000
Amphotericin B		13.27 ± 1.8	282	737	47.71 ± 2.06
Paromomycin		>10000	1362	2577	>1000
Pentamidine Isethionate		1.86 + 0.05	2.7	3.0	>50
<u>Negative controls</u>					
RPMI medium		0.00	-	-	0.00
1% DMSO solution		0.00	-	-	0.00

FH-Flower head, F-Flower, L-Leaf, SB-Stem bark, S-Stem, RB-Root bark, R-Root, B-Berries, SD-Seed (P)-Petroleum ether extract, (C)-Chloroform extract,(M)-Methanol extract, * = Unit (µl/ml), ∇=Unit (nl/ml)

4.2.4 Haemolytic properties

Severe destruction of blood cells and blood forming tissues occur during pathogenesis of visceral leishmaniasis resulting in anemia and leucopenia as major clinical manifestations of the disease. Some of the standard antileishmanial drugs were reported to cause haemolytic anemia (Mahajan and Marwaha, 2007). Phytochemicals like saponins were also found to have strong haemolytic effects (Yang *et al.*, 2005). Therefore blood haemolysis experiments are important to assess possible presence of such side effects for extracts with reference to that of positive control drugs for comparison.

The 78 extracts and three essential oils used for anti-leishmanial activity study were also evaluated for their hemolytic effects as described in section 3.2.6.3.2. The 50% lytic concentration (LC₅₀'s) for extracts, essential oils and reference drugs are presented in Table 5.

Results indicated that 50 of the extracts (64.10 %) had LC₅₀ values higher than 1000 µg/ml and 13 extracts (16.67 %) showed LC₅₀ values between 500 and 1000 µg/ml. Eight extracts (10.25 %) had LC₅₀ ranging from 100 to 499 µg/ml. Generally for 76 of 78 extracts tested (97.44 %), the LC₅₀ values were 10-100 times higher than the EC₅₀'s for promastigote and amastigote forms of both *Leishmania* species. Four extracts of *A. schimperiana* (methanol and chloroform extracts of its stem bark and root bark) and the methanol extract of *Aloe* showed strong hemolytic properties with LC₅₀ values 0.7 to 9.2 times their corresponding EC₅₀'s. Essential oils also had LC₅₀ values between 0.70 ± 0.27 and 2.62 ± 1.0 and the strong haemolytic effect was exhibited by *A. abyssinica* oil (Table 5).

The LC₅₀'s for standard anti-leishmanial drugs; pentamidine, amphotericin B, sodium stibogluconate and paramomycin were >50 µg/ml, 47.71 ± 2.06 µg/ml, >5000 µg/ml and >1000

µg/ml respectively (Table 5). The negative control (1% DMSO) did not cause lysis. Previous reports indicate strong haemolytic activity of amphotericin B with EC₅₀ of 25 µg/ml for 60 min incubation (Tiuman *et al.*, 2005a) and 36 µg/ml for 120 min incubation (Tiuman *et al.*, 2005b) (cf. LC₅₀ for amphotericin B is 47.71 ± 2.06 µg/ml for 120 min in this study)

4.3 GC/MS analysis and phytochemical constituents of the herbal drugs

4.3.1 GC/MS analysis of essential oil

From the essential oils of *A. abyssinica*, *C. macrostachys* and *E. kebericho*, 25, 31 and 39 compounds were identified, representing 85.2, 98.4 and 43.8 % of the total oil respectively. Components of the essential oil of studied plants are shown in Table 6.

As can be seen from the data presented in Table 6, considerable qualitative and quantitative differences exist between essential oil constituents of the three species.

The oil of *A. abyssinica* contained mainly of monoterpene hydrocarbons (44.09 %) followed by oxygenated monoterpene (38.04 %), oxygenated sesquiterpenes (1.89 %) and phenolics (1.16 %). Major constituents of this oil were α-terpinolene (39.98 %), artemisyl acetate (25.40 %), artemisia alcohol (6.46 %) and 1, 8-cineole (1.91 %) which were also reported previously in different quantities from the same plant (Anonymous, 2007d).

The benzyl benzoate comprised 51.46 % of the oil of *C. macrostachys* followed by γ-muurolene (11.98 %), linalool (10.06 %) and α-farnesene (3.21 %). The oil contained phenolic esters (51.46 %), sesquiterpene hydrocarbons (17.57 %), oxygenated monoterpene (10.81 %),

oxygenated sesquiterpenes (8.58 %) and monoterpene hydrocarbons (1.09 %). Linalool was also reported as a major constituent of essential oil of *C. cajucara* (Socorro *et al.*, 2003).

The essential oil of *E. kebericho* also contained monoterpene hydrocarbons; sesquiterpene hydrocarbons, oxygenated sesquiterpenes, oxygenated monoterpenes and phenolics representing 23.99, 15.56, 3.86, 2.80 and 0.59 % of the total oil respectively. β -phellandrene (10.06 %), germacrene (5.38 %), α -selinene (4.13 %), α -pinene (3.63 %) and β -pinene (3.62 %) were the major constituents of the oil. Qualitatively, these results are to some extent in agreement with previous reports of essential oil constituents of *E. kebericho* but no sesquiterpene lactone were detected in this oil (Hymete *et al.*, 2006).

Table 6: Chemical composition (% peak area) of the essential oils obtained by hydrodistillation of *A. abyssinica*, *C. macrostachys* and *E. kebericho*.

Compound	KI	<i>A. abyssinica</i>	<i>C. macrostachys</i>	<i>E. kebericho</i>
2-Methyl-1-butyl acetate	881	0.13	-	-
Santolina triene	909	1.85	-	-
Tricycline	927	0.36	-	0.14
α -Pinene	939	0.06	0.24	3.63
Camphene	954	-	-	0.28
Sabinene	975	1.42	tr.	0.72
β -Pinene	979	-	0.54	3.62
6-Methyl-5-heptene-2-one	986	tr.	-	-
β -Myrcene	991	-	-	0.30
Octanal	999	-	tr.	-

Table 8: Continued

Compound	KI	<i>A. abyssinica</i>	<i>C. macrostachys</i>	<i>E. kebericho</i>
α -Phellandrene	1003	-	-	0.96
α -Terpinolene	1017	39.98	-	-
p-Cymene	1025	0.42	-	0.14
1,8-Cineole	1029	1.91	-	-
β -Phellandrene	1030	-	-	10.84
Limonene	1031	-	0.17	2.25
3-Carene	1031	-	-	0.83
trans-Ocimene	1050	-	-	0.11
cis-Sabinene hydrate	1070	0.15	-	-
Artemisia alcohol	1084	6.46	-	-
Terpinolene	1089	-	0.14	0.15
dehydro-Linalool	1091	0.24	-	-
Linalool	1097	1.86	10.06	-
Heptyl acetate	1115	-	0.23	-
Trans-p-2, 8-menthadiene- 1-ol	1123	0.21	-	-
Artemisyl acetate	1173	25.40	-	-
4-Terpineol	1177	-	0.14	0.24
α -Terpineol	1189	0.35	0.38	-
Cuminyaldehyde	1242	0.21	-	-
Phenyl ethyl acetate	1258	0.56	-	-
α -Copaene	1377	-	tr.	-

Table 8: Continued

Compound	KI	<i>A. abyssinica</i>	<i>C. macrostachys</i>	<i>E. kebericho</i>
Neryl formate	1282	0.65	-	-
Bornyl acetate	1289	-	-	2.24
trans-Verbenyl acetate	1293	-	-	0.16
Geranyl acetate	1381	0.40	-	-
β -Maaleine	1382	-	-	0.20
Isocaryophyllene	1409	-	0.90	1.97
α -Gurjunene	1410	-	-	0.14
β -Caryophyllene	1419	0.06	-	0.38
β -Copaene	1432	-	tr.	-
β -Gurjunene	1434	-	-	0.21
α -Himachalene	1451	-	-	tr.
α -Humulene	1455	-	0.20	tr.
allo-Aromadendrene	1460	-	-	tr.
γ -Gurjunene	1477	-	0.13	-
γ -Muurolene	1480	-	11.98	-
β -Ionone	1489	-	-	1.00
Phenyl ethyl-3-methyl butanoate	1491	0.33	-	-
Valencene	1496	-	-	1.18
Viridiflorene	1497	-	-	0.75
α -Selinene	1498	-	0.14	4.13
α -Farnesene	1506	-	3.21	-

Table 8: Continued

Compound	KI	<i>A. abyssinica</i>	<i>C. macrostachys</i>	<i>E. kebericho</i>
γ -Cadinene	1523	-	1.02	-
Germacrene B	1561	-	-	5.38
Caryophyllene oxide	1583	-	-	0.97
Guaiol	1601	-	-	tr.
τ -Cadinol	1640	-	1.24	0.27
Cubenol	1647	-	0.42	-
α -Cadinol	1654	-	2.04	-
epi- α -Bisabolol	1685	-	0.84	0.09
α -Bisabolol	1686	-	0.70	-
cis,tarans-Farnesol	1701	1.62	0.27	-
cis, cis-Farnesol	1718	-	0.17	-
Benzyl benzoate	1760	-	51.46	0.59
cis-Lanceol	1761	-	-	1.34
cis- α -Santalyl acetate	1779	-	-	0.14
trans- α -Bergamotol acetate	1795	-	-	0.66
Total (%)		83.23	89.04	46.80
Monoterpene hydrocarbons		44.09	1.09	23.99
Oxygenated monoterpenes		38.04	10.81	2.8
Sesquiterpene hydrocarbons		-	17.51	15.56
Oxygenated sesquiterpenes		1.89	8.58	3.83
Phenol, Phenolic ethers and esters		1.16	51.46	0.59

KI =Kovats index on unpolar column CP-Sil 5, tr. =traces (<0.05%)

Essential oils are also among leishmanicidal compounds of plant origin. Essential oil isolated from *Ocimum gratissimum* L. (Lamiaceae) showed dose dependent growth inhibitory effect on *L. amazonensis* at 100-1000 µg/ml (Ueda-Nakamura *et al.*, 2006) and that of *Croton cajucara* (Euphoraceae) inhibited growth of *L. amazonensis* with LC₅₀ of 8.3-8.7 ng/ml (Socorro *et al.*, 2003). Essential oil of *Chenopodium ambrosioides* also had effects with EC₅₀ value of 4.45- 5.1 µg/ml on *L. donovani* and 3.7-4.6 µg/mL on *L. amazonensis* (Monzote *et al.*, 2006, Monzote *et al.*, 2007).

Constituents of the oils may have a role for the observed high leishmanicidal effect of essential oils. In this regard, the monoterpene, linalool was reported to have potent antileishmanial activity and this may explain the significant anti-leishmanial activity of *C. macrostachyus* (Socorro *et al.*, 2003). Although not specific several phenolic compounds, monoterpenes, sesquiterpenes and their oxygenated derivatives were reported to possess anti-leishmanial activities (Tandon *et al.*, 1991; Chan-Bacab and Pena-Rodriguez, 2001; Habtemariam, 2003; Kayser *et al.*, 2006) and this class of compounds were also detected in the studied oils. Thus the observed strong anti-leishmanial activity of the oils may be attributed to some components of the essential oils.

4. 3. 2 Phytochemical constituents of the plant extracts

Here, 112 gradient extracts were screened with the intention of finding out presence or absence of different class (es) of secondary metabolites in the plant extracts. For most plant extracts major secondary metabolites detected were dependent on type of solvent used for extraction (Table 7).

Numerous studies conducted on certain plant secondary metabolites reported for their potential growth inhibitory effect on different *Leishmania* spp. These include alkaloids (Vennestrom, *et*

al.,1990; Fournet *et al.*, 1993; Fournet *et al.*, 1994; Fournet *et al.*, 1996a; Henriques *et al.*, 2001), lipids and related aliphatics (fatty acids and steroids) (Kayser *et al.*, 2006; Sartorelli *et al.*,2007; Rocha *et al.*, 2005), phenolics and polyphenols (Oliver *et al.*, 2003; Fournet *et al.*, 1996b: Chen *et al.*, 1994; Koide *et al.*, 2002; Bodiwala *et al.*, 2007), coumarins (Brenzan *et al.*, 2007; Iranshahi *et al.*, 2007; Kayser *et al.*, 2006; Chan-Bacab and Pena-Rodriguez, 2001), tannins (Kolodziej *et al.*, 2001), flavonoids and flavonoid-derivatives (Chan-Bacab and Pena-Rodriguez, 2001; Muzitano *et al.*; Tasdemir *et al.*,2006; Kayser *et al.*, 2006; Ericil *et al.*, 2005; Muzitano *et al.*, 2006), anthraquinones (Chan-Bacab and Pena-Rodriguez, 2001), saponins (Delmas *et al.*, 2000; Maes *et al.*, 2004 a & b) and terpenes (terpenoids) (Tandon *et al.*,1991; Chan-Bacab and Pena-Rodriguez, 2001; Habtemariam, 2003; Kayser *et al.*, 2006). Infact plants like *E. kebericho* was selected based on bioactive (antileishmanial) chemical constituents (such as sesquiterpenes and sulfur compounds) which were reported to be contained in them (Abegaz, 1996).

The antileishmanial activity of herbal drugs may not only be attributed to specific chemical compound. As confirmed by the phytochemical screening, one or more of plant metabolites discussed above were contained in all extracts included in this study. Therefore, the observed biological activity and toxicity profile of these extracts can be attributed to either the individual class of compounds present in each plant or to the synergistic effect that each class of compound exerts. There is also chance for existence of certain compounds that antagonize each other. Therefore, further investigations should be carried to resolve this issue.

Most lipophilic (petroleum ether and chloroform) fractions of the study plants had strong growth inhibitory effects on both *Leishmania* species. In fact the phytochemical class of compounds detected in this fraction such as steroids, terpinoids and phytosteroides were reported to have

antileishmanial activities (Kayser *et al.*, 2006; Chan-Bacab and Pena-Rodriguez, 2001). *Leishmania* membrane has high lipid content (Glew *et al.*, 1988) therefore, these extracts may produce effects being incorporated into membranes thereby disrupting their integrity or crossing these membranes affecting other cellular processes. Although several research findings report therapeutic effectiveness of alkaloids, flavonoids, coumarins, polyphenols, tannins, anthraquinones and saponins (class of compounds detected in most methanol fractions) against leishmanial infections but it is these polar fractions which had the least activities against the pathogens in this research. This may be attributed to the fact that, the bioactive polar compounds contained in this fraction are lower in amount. The extraction data (Table 1) and phytochemical screening results (Table 7) indicate that large numbers of classes of compounds are contained in this major fraction lowering the relative amount of these bioactive polar compounds contained in it. Hence, further fractionation of this component may provide solution to the paradox. This can be done by activity guided fractionation and isolation and characterisation of pure compounds.

Table 7: Major classes of secondary metabolites detected on preliminary phytochemical investigation of plant extracts.

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids
<i>A. schimperiana</i>	L ^P	-	-	-	-	+	-	-	-	-
	L ^C	-	-	-	-	+	-	-	-	-
	L ^M	+	++	++	-	-	-	-	+	-
	SB ^P	-	-	-	-	-	-	-	-	++
	SB ^C	+	-	++	+	-	++	-	-	++
	SB ^M	++	+	++	+	-	-	-	+	-
	RB ^P	-	-	-	+++	-	-	-	++	+
	RB ^C	++	-	++	-	-	++	-	+	++
	RB ^M	++	++	++	-	-	+	-	+	-
<i>Aloe spp.</i>	S ^P	-	-	++	-	-	+++	-	-	++
	S ^C	-	-	++	-	-	++	-	-	++
	S ^M	-	++	++	-	-	+	++	-	-
	RB ^P	-	-	-	-	-	+++	-	-	++
	RB ^C	-	-	++	-	-	++	-	-	++
	RB ^M	-	++	++	+	-	-	++	-	-
<i>A. abyssinica</i>	L ^P	-	-	-	-	-	-	-	-	++
	L ^C	-	-	++	-	++	-	-	-	+
	L ^M	-	++	++	++	-	-	-	++	-
	S ^P	-	-	-	-	-	++	-	-	++
	S ^C	-	-	++	+	-	-	-	-	+

Table 7: Continued

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids
<i>C. macrostachys</i>	S ^M	-	-	++	++	-	+	-	++	-
	R ^P	-	-	-	-	-	++	-	-	++
	R ^C	-	-	++	-	++	-	-	-	++
	R ^M	+	++	++	++	-	-	-	++	-
	B ^P	-	-	-	-	-	++	-	-	++
	B ^C	-	-	++	-	-	-	-	-	++
	B ^M	+	+	++	+	-	-	-	-	-
	L ^P	-	-	-	-	-	-	-	-	++
	L ^C	-	-	+	-	-	-	-	-	-
	L ^M	-	++	++	++	-	-	-	++	-
	SB ^P	-	-	-	-	+++	-	-	-	++
	SB ^C	-	-	-	+	-	++	-	-	+
	SB ^M	+	+	++	+	-	-	-	-	-
	R ^P	-	-	-	-	-	++	-	-	++
<i>E. kebericho</i>	R ^C	-	-	-	-	-	+	-	-	+
	R ^M	++	-	++	+	-	-	-	-	-
	FH ^P	-	-	-	-	-	++	-	-	+
	FH ^C	-	-	-	-	-	-	-	-	+
	FH ^M	-	-	++	++	-	-	-	-	-
	L ^P	-	-	-	-	-	-	-	-	+

Table 7: Continued

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids
<i>E. brucei</i>	L ^C	-	-	-	-	-	-	-	-	+
	L ^M	-	+	++	++	-	-	-	++	-
	S ^P	-	-	-	-	-	+++	-	-	++
	S ^C	-	-	-	-	++	++	-	-	+
	S ^M	-	-	++	++	-	-	-	++	-
	R ^P	-	-	-	-	-	++	-	-	++
	R ^C	-	-	-	-	-	++	-	-	+
	R ^M	-	-	++	++	-	+	-	++	-
	SD ^P	-	-	-	-	-	-	-	-	++
	SD ^C	-	-	-	-	-	+	-	-	+
	SD ^M	+++	-	++	+	-	+	-	++	-
	FH ^P	-	-	-	-	-	-	-	-	++
	FH ^C	-	-	-	-	-	-	-	-	+
	FH ^M	+	-	++	++	-	-	-	+	-
	L ^P	-	-	-	-	-	-	-	-	++
	L ^C	-	-	-	-	-	-	-	-	+
	L ^M	-	-	++	++	-	+	-	-	-
	SB ^P	-	-	-	-	-	-	++	-	-
SB ^C	-	-	-	-	-	++	-	-	-	+
SB ^M	+++	-	-	-	++	-	++	-	++	-
RB ^P	-	-	-	-	-	++	++	-	-	++

Table 7: Continued

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids
<i>J. schimperiana</i>	RB ^C	-	-	-	-	-	-	-	-	+
	RB ^M	++	-	-	++	-	+++	-	+++	-
	FH ^P	-	-	-	-	-	-	-	-	++
	FH ^C	-	-	+	-	++	++	-	-	-
	FH ^M	+	-	++	+	+	-	-	++	-
	L ^P	-	-	-	-	++	+	-	-	++
	L ^C	-	-	+	-	++	-	-	-	+
	L ^M	-	++	++	++	+	-	-	-	-
	SB ^P	-	-	-	-	-	++	-	-	++
	SB ^C	-	-	+	-	-	-	-	-	+
	SB ^M	+	-	++	+	++	-	-	-	-
<i>R. prenoides</i>	R ^P	-	-	-	-	-	++	-	-	++
	R ^C	-	-	-	-	-	-	-	-	+
	R ^M	+	+	+	-	++	-	-	-	-
	B ^P	-	-	-	-	++	++	-	-	++
	B ^C	-	-	-	+	-	+	-	-	+
	B ^M	+	+	+	++	++	-	+++	-	-
	L ^P	-	-	-	-	-	-	-	-	++
	L ^C	-	-	+	-	++	-	-	-	-
	L ^M	++	+	++	++	++	++	-	++	-

Table 7: Continued

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids	
<i>P. capense</i>	SB ^P	-	-	-	-	-	-	-	-	++	
	SB ^C	-	-	+	-	++	-	-	-	-	
	SB ^M	+	++	++	++	-	-	+	+	-	
	RB ^P	-	-	-	-	-	-	-	-	++	
	RB ^C	-	-	++	-	-	+	-	-	-	
	RB ^M	+	-	++	+++	-	++	-	++	-	
	B ^P	-	-	-	-	-	-	-	-	+++	
	B ^C	-	-	-	-	-	-	-	-	+	
	B ^M	++	++	++	++	-	++	-	++	-	
	L ^P	-	-	-	-	-	-	-	-	++	
	L ^C	-	-	+	-	-	+	-	-	+	
	L ^M	+	++	++	+	-	+	-	++	-	
	SB ^P	-	-	-	-	-	-	+	-	-	++
	SB ^C	-	-	-	-	-	++	-	-	-	+
	SB ^M	+	++	++	+	-	-	-	-	++	-
R ^P	-	-	-	-	-	-	+++	-	-	+++	
R ^C	-	-	-	-	-	++	-	-	-	+	
R ^M	+	++	++	+	++	++	++	+	++	-	
<i>P. dodecandra</i>	B ^P	-	-	-	-	-	-	-	-	++	
	B ^C	-	-	-	-	-	-	-	-	+	
	B ^M	++	+++	++	++	-	+	-	++	-	

Table 7: Continued

Study plants	Type of extract	Alkaloids	Saponins	Polyphenols	Flavonoides	Coumarins	Terpenes	Anthraquinones	Tannins	Phytosteroles & Withanoids
	L ^P	-	-	-	-	-	-	-	-	+
	L ^C	-	-	-	-	-	-	-	-	+
	L ^M	-	++	++	+	-	-	-	++	-
	S ^P	-	-	-	-	-	+++	-	-	+
	S ^C	-	-	-	-	-	-	-	-	+
	S ^M	+++	+++	++	++	-	+++	-	++	-
	R ^P	-	-	-	-	-	+++	-	-	++
	R ^C	-	-	-	-	-	-	-	-	+
	R ^M	++	+++	++	++	-	+++	+	++	-

FH-Flower head, F-Flower, L-Leaf, SB-Stem bark, S-Stem, RB-Root bark, R-Root, B-Berries, SD-Seed (P)-Petroleum ether extract, (C)-Chloroform extract,(M)-Methanol extract, - = negative, + = fairly positive, ++ = positive, +++ = strongly positive

5. CONCLUSION

Leishmaniasis is a major protozoal health problem worldwide. It is highly endemic in many developing tropical and subtropical countries including Ethiopia. The disease is transmitted mainly by a bite of infected sandfly and rarely with sharp contaminated objects. The disease shows a spectrum of clinical manifestations classified as localized cutaneous, diffuse cutaneous, mucocutaneous, visceral and post kala-azar leishmaniases. Incidence and complexity of the disease has increased since the emergence of HIV/AIDS. The effort to get vaccine has not yet materialized. The existing first and second-line antileishmanial drugs have serious side effects and are expensive. Resistance to existing drugs has also become a severe problem. Therefore, there is an urgent need for effective and inexpensive drugs and vaccine to replace or supplement those in current use.

Plants can make important contributions as valuable sources of new medicinal agents. In this regard scientific evaluation of the rich Ethnobotanical heritage of Ethiopia is expected to provide modern medicine with lead compounds for the development of new drugs. This study aimed at investigating the *in vitro* antileishmanial activity, cytotoxicity and haemolytic effect of gradient extracts and essential oils from ten medicinal plants growing in Ethiopia, using simple microscopy and measurement of changes in absorbance and fluorescence character of culture medium containing parasites or cells treated with extracts. Plants included in the present study were selected based on literature report of their traditional use or scientifically proven activities of plants in the same genus. This report also comprises eight plants (except *C. macrostachys* and *P.dodecandra*) screened for *in vitro* antileishmanial activity for the first time.

Results of this study indicated that lipophilic fractions of most of the studied plants and all essential oils have significant antileishmanial activity and relatively higher cytotoxicity to parasites than the host cells at concentrations used to inhibit growth of the parasites. In conditions where all three gradient extracts were screened for their haemolytic effects, it was the polar fractions that showed the highest haemolytic effects. The lipophilic extracts of *A. abyssinica*, *C. macrostachys*, *E. kebericho* and *E. brucie* had high antileishmanial activity ($EC_{50} < 3 \mu\text{g/ml}$), low cytotoxicity (Selectivity index ≥ 2.9) and weak haemolytic effects. These candidates can therefore be used for further *in vivo* studies on animals that are good models for cutaneous and systemic leishmanial infections. The essential oils of *E. kebericho* also possessed high antileishmanial activity (higher than that of the most potent anti-leishmanial drugs) with moderate cytotoxicity (Selectivity index 5.5-17) and weak haemolytic effect, so can also be tested for cutaneous and systemic leishmanial infections. Regardless of their good antileishmanial effects, the essential oils of *C. macrostachys* and *A. abyssinica* which had the highest toxicity (Selectivity index 0.8-1.7) and haemolytic effects respectively may have serious side effects if taken for systemic leishmanial infections.

In conclusion, based on the observed moderate to high antileishmanial activity and low or absence of cytotoxicity, it is suggested that four of the study plants (*A. abyssinica*, *C. macrostachys*, *E. kebericho* and *E. brucie*) can be used for bioassay-guided fractionation and isolation of bioactive compounds which could serve as new drug lead structures. Isolation of bioactive components can also be carried out with the intention of removal of components with strong cytotoxic and haemolytic effects.

RECOMMENDATIONS FOR FURTHER WORK

1. Some lipophilic extracts and essential oils (*A. abyssinica*, *C. macrostachys*, *E. kebericho* and *E. brucie*) included in this study showed excellent anti-leishmanial activity and low toxicities to the cell line indicating their potential application in treating systemic or topical leishmanial infections. Thus, further fractionation, and isolation of active constituents to be followed by anti-leishmanial and cytotoxicity studies are recommended.
2. The anti-leishmanial activity screening was conducted on two *Leishmania* Spp. The same work should be carried out on large variety of *Leishmania* strains (at least on the four *Leishmania* spp. reported in Ethiopia) in order to have clear idea of the spectrum of anti-leishmanial activity of these plant extracts.
3. The anti-amastigote test was conducted on axenically cultured extracellular form of amastigote and similar tests are recommended to be carried on intracellular amastigote to see effect of macrophages on drug efficacy and possible immune stimulating effects of extracts.
4. The *in vitro* antileishmanial and cytotoxicity profile of the extracts and essential oils of *A. abyssinica*, *C. macrostachys*, *E. kebericho* and *E. brucie* are promising. So further detailed *in vivo* studies are recommended on rodent or primate models.
5. Mostly cutaneous leishmanial infections get worse when secondary bacterial or fungal infections coexist. Therefore, further investigations on effect of the extracts and essential oils of *A. abyssinica*, *C. macrostachys*, *E. kebericho* and *E. brucie* on selected bacterial and fungal dermatophytes is recommended.

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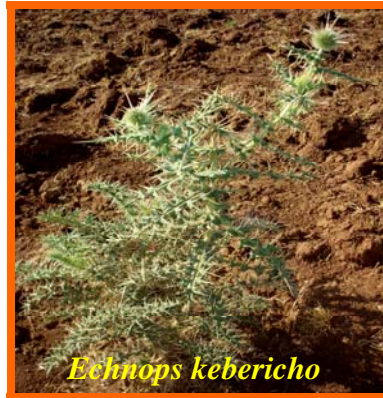
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Annexes

Annex-1 Picture of plants included in this study





Annex-2

Preparation of Phosphate-Buffered Saline (PBS)

Phosphate-buffered saline was obtained by dissolving 80 g sodium chloride, 2 g potassium chloride, 14.4 g disodium hydrogen phosphate (Na_2HPO_4) and 2.4g potassium dihydrogen phosphate (KH_2PO_4) in one liter of distilled water.

Preparation of NNN medium

9.2g Nutrient agar (Defco), 0.6g D-(+) Glucose (anhydrous) (Sigma) and 2.4 g sodium chloride (Sigma) were weighed, mixed and dissolved in 400ml of distilled water by boiling in hot plate with repeated shaking until clear solution was obtained. The mixture was then autoclaved at 121°C for 30 minutes. Sheep blood was then aseptically collected with a sterile bottle containing glass beads and shaken to defibrinate. The defibrinated was then heat inactivated by keeping it in 37°C water bath for 50 minutes and then transformed into 56°C water bath for 20

minutes. The autoclaved ingredients (400ml) and of heat inactivated blood (100ml) were mixed at 50⁰C. 2ml of the mix is dispensed among culture vials, allowed to settle slant and stored at 4⁰C until use.

Preparation of Locke's solution

The ingredients 4.5g sodium chloride ,0.2g potassium chloride, 0.1g calcium chloride ,0.1g sodium bicarbonate and 1.25g D-(+)-glucose(anhydrous) all from sigma were mixed and dissolved in 500ml distilled water. The mixture was autoclaved at 121⁰C for 30 minutes and stored at 4⁰C until use.

Annex-3 Plate layout

