UNIVERSITY OF CAPE COAST

IN VITRO ANTI LEISHMANIAL ACTIVITY OF SOME SELECTED MEDICINAL PLANTS IN GHANA

BY

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Thesis submitted to the Department of Biomedical Sciences of the School Of Allied Health Sciences, University Of Cape Coast in partial fulfillment of the requirement for award of Master Of Philosophy degree in Parasitology

FEBRUARY 2016

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's signature: …….. Date: ……

Name: Alberta Serwah Anning

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature: …… Date: ……...

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ABSTRACT

Leishmaniasis is a parasitic infection that affects mostly tropical and sub-tropical regions of the world and caused by diverse pathogens that belong to the genus *Leishmania*. The pentavalent antimonials developed in 1945 are still first line treatment drugs for both cutaneous and visceral leishmaniasis while amphotericin B is a second line treatment drug. These treatments come with toxic side effects even at effective doses and the lack of vaccine demand the urgent need for new anti leishmanial agents. This study aimed at investigating four plants used traditionally to treat parasitic infections. The collected plant parts were washed, dried, powdered and then extracted using ethanol. Different concentrations of the extracts ranging from 15.6 to 500 µg/mL in 0.1 % DMSO with M199 and a positive control of Amphotericin B were prepared in triplicates in 24-well plates that contained 117,000 parasites/well. The plates were incubated at 25 °C and promastigotes counted on 8, 12, 24 and 48 hours after incubation. Phytochemical screening on all crude extracts revealed the presence of steroids, triterpenoids, tannins, anthraquinons, saponins, alkaloids, flavonoids and glycosides. Of the four plants, *Erythrophleum ivorense* gave the best activity with an IC_{50} of 6.3 µg/mL after 72 hours. This was followed by *C. oxycarpum*, *A. aubryanum* and *A. ahia* respectively. Three compounds have been isolated from *E. ivorense*; erythroivorensin, eriodictyol and betulinic acid, with IC_{50}s of 0.5, 61.8 and 247 µg/mL correspondingly on the promastigotes of *L. donovani*.

Keywords: erythroivorensin, eriodictyol, betulinic acid, Amphotericin B, Pentamidine, leishmanicidal, promasigotes, Cutaneous leishmaniasis

AKNOWLEDGEMENTS

I am thankful to my supervisors, Dr. Elvis Ofori Ameyaw and Dr. Godwin Kwakye-Nuako for agreeing to supervise me and this project. The lessons taught and guidance given from inception to the finish of this research has been one of a kind. I appreciate the opportunity given.

My utmost and heartfelt gratitude to Prof. Johnson Nyarko Boampong (Dean, School of Biological Sciences, UCC), for his support and encouragement throughout my post-graduate education.

My sincere appreciation to Mr Armah of the Department of Pharmacognosy, (Kwame Nkrumah University of Science and Technology), for given me all the needed help during the fractionaton of the extracts and to the Department of Chemistry, (University of Cape Coast), for making available their laboratories for use and for their welcoming assistance. I again appreciate the work of workers from the herbarium, University of Cape Coast for their immense help during the identification and collection of plants. Thank you to Mba of Noguchi Memorial Institute of Medical Research (NMIMR) for providing some parasites for the continuation of this research

Special thanks to Mrs Jemimah Lasim and Ernest Asiamah both of the Department of Biomedical Sciences for being there whenever needed.

A special thank you to my course mates Samuel Boateng, Emmanuel Nyarko, Mohammed Badawie and Eric Ofori, you made me feel at home, God bless you.

DEDICATION

I dedicate this work to my family, especially to the one woman who has always given more than I ask, my mother, Rebecca Osei.

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LIST OF ACRONYMS

ABCD	Amphotericin B Colloidal Dispersion
ABLC	Amphotericin B Lipid Complex
AmB	Amphotericin B
CDC	Centres for Disease Control
CFSPH	Centre for Food Security and Public Health
CL	Cutaneous Leishmaniasis
DALY	Disability-Adjusted Life Years
DMSO	Dimethyl Sulfoxide
EtOAc	Ethyl Acetate
GHS	Ghana Health Service
GOI	Government of India
GMP	Good Manufacturing Practices
LPG	Lipophosphoglycan
MA	Meglumine Antimoniate
MCL	Mucocutaneous leishmaniasis
MDA	Mass drug administration
MOH	Ministry of health
MSF	Médecins Sans Frontières
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
NNN	Novy-mcNeal-Nicolle
NTD	Neglected Tropical Disease
PAHO	Pan American Health Organization
PKDL	Post Kala-azar Dermal Leishmaniasis

PM	Paromomycin
SAR	Structure-Activity Relationship
Sb	Stibogluconate
SSG	Sodium Stibogluconate
TGF	The Global Fund
TRIPS	Trade Related Aspects of Intellectual Property Rights
VL	Visceral Leishmaniasis
WHA	World Health Assembly
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

Background

Leishmaniasis is an important parasitic disease that threatens the lives of millions of people worldwide and is caused by any of the numerous species of *Leishmania* (World Health Organization, 2013). The worldwide increase in the incidence of leishmaniasis has been mainly attributed to a surge in several risk factors that are clearly manmade (Desjeux, 2001). The disease presents in several forms depending on the type of species that is implicated and the immune response of the host (Herwaldt, 1999). In some forms, it is lethal, while in others, it has been described as a cruel mutilator leaving its victims scarred for life (Yanik, Gurel, Simsek, & Kati, 2004).

Anti-leishmanial vaccines are still being developed and as such the current control strategies for leishmaniasis rely on case management case such as detection and treatment, vector and reservoir control. Case management that includes early diagnosis and treatment is essential for both individual patients and for the community. There is reason to believe that the number of cases of leishmaniasis is on the rise (Desjeux, 1996) which could be due to artificial environmental changes which increase human exposure to the sand fly vector (Reithinger et al., 2007). Extracting timber, mining, building dams, widening areas under cultivation, new irrigation schemes, road construction, widespread migration from rural to urban areas and fast urbanization worldwide are

among the main causes for an increased exposure to the sand fly (Reithinger et al., 2007).

Several treatments are available for the various groups of leishmaniasis. These drugs span from the ancient old antimonies to the most current miltefosin. Because most of the treatment drugs are old, their efficacy is limited presenting with several undesirable side effects (Chakravarty & Sundar, 2010; Diro et al., 2014; Lage, et al., 2013), making them far from satisfactory in their action even at the supposed effective doses. Drugs that are recommended for use in the treatment of cutaneous leishmaniasis and visceral leishmaniasis include the pentavalent antimonials which were first introduced nearly seven decades ago (Yardley & Croft, 2000). Over the past 20 years, alternative drugs or new formulations of other standard drugs have been presented and registered for use in some countries, whilst other drugs are on clinical trial for both forms of the disease (Yardley & Croft, 2000). However, serious side effects in the patients, prolonged treatment time, and increased parasite resistance have been draw backs over the years (Chakravarty & Sundar, 2010; Diro et al., 2014; Lage et al. 2013). Therefore, alternative drugs to the antimonials such as amphotericine B, pentamidine, paromomycin, and miltefosine have been recommended, but they also come with some problems and even therapeutic failure (Lage et al., 2013, Machado et al., 2012; Wiwanitkit, 2012).

The use of herbal medicine for the treatment of diseases and infections is as old as mankind (Surendra & Talele, 2011). The World Health Organization supports the use of traditional medicine provided they are proven to be efficacious and safe (Government of India, 2001). In the developing

countries, vast numbers of people live in extreme poverty who suffer and die, for want of medicine among others, and lack alternative for primary health care (GOI, 2001). Therefore, the need to use medicinal plants as alternatives to orthodox medicines in the provision of primary health care cannot be over-looked. Herbal medicines now seem to be the remedy for both traditional and modern medicine (Zerehsaz et al., 1999). Additionally, herbal medicines have received much attention as sources of lead candidate compounds since they are considered as time tested and relatively safe for both human use and environmental friendliness (Fazly-Bazzaz, Khajehkaramadin, & Shokooheizadeh, 2005), including easily available and affordable. There is therefore, the need to look inwards to search for herbal medicinal plants with the aim of validating the ethno medicinal use and subsequently an isolation and characterization of compounds which will be added to the potential lists of drugs.

Modern synthetic drugs for leishmaniasis are simply not available or the few available ones are expensive while some come with adverse side effects. To obtain herbal medicine or an isolated active compound, different research strategies can be employed, among them are; the investigation of the traditional use, the chemical composition, the toxicity of the plants, or the combination of several criteria (Rates, 2001). For purification and isolation, the active extracts of the plant are sequentially fractionated, and each fraction and/or pure compound can be evaluated for biological activity and toxicity.

Aim

To screen *E. ivorense, O. ahia, A. aubryanum* and *C. oxycarpum* for their *in vitro* anti-leishmanial activities

Objectives

1. To determine the efficacy of extracts from four native plants identified for leishmaniasis treatment
2. To identify various phytochemicals found in the selected plant extracts
3. To isolate and determine the activity of active compound (s) found in the most active crude extract (s).

Statement of the problem

Leishmaniasis presents as the world's ninth infectious disease and it is a globalburden in over 98 countries (Rodrigues et al., 2014), with a mortality rate of 60,000 (Mohapatra, 2014; Salehabadi, Karamian, Farzad, & Namaei**,** 2014). It is as well the most common neglected tropical disease (NTD) in countries with poor socioeconomic conditions (Neris et al., 2014) in Africa, Asia, Southern Europe and Latin America (Daneshbod et al., 2011; de Medeiros et al., 2011; Monzote, 2011). At least, 350 million people world wide are estimated to be infected, with 12 million new cases reported each year (Kumar & Kumar, 2013).

No form of leishmaniasis had been reported in Ghana until 1999 when there were reported cases of chronic skin lesions in health centres in a district of Ho in the Volta region of Ghana (Raczniak et al., 2008). In a follow up in 2002, 12.2 to 32.3 % of local school children were identified with the suspected lesions which tested positive to histopathological examination confirming the initial lesions as indeed cutaneous leishmaniasis. In 2003 alone, the Ghana Health Service Annual Report also presented a retrospective data of almost 9,000 cases in the districts of Ho, Hohoe and Kpando. In the reported outbreak, Ho recorded the highest victims of 8553, followed by

Hohoe and then Kpando with 176 and 167 respectively. Due to the lack of treatment available for leishmanisasis in Ghana, case detection by health workers and self-reporting of cases has dwindled over the years to zero as at 2008. The Ministry of Health, has mentioned leishmaniasis among diseases that are classified to almost exclusively affect the poor (Ministry of Health, 2007) in Ghana. This notwithstanding, the disease was not listed among the 5 neglected tropical diseases operated under the Disease Control Unit of the Public Health Division of the Ghana Health Service (GHS),whose main strategy of intervention is annual and/or bi-annual mass drug administration (MDA), (Ghana Health Service, 2014).

Meanwhile report by the GHS to the WHO in 2010 stated that there is no treatment available for cutaneous leishmaniasis (CL) because no drugs have been registered in Ghana and that self-medication was frequently observed during outbreaks in Ho (GHS, 2010). Aside this, many of the medicines required to treat leishmaniasis and other neglected tropical diseases (NTDs) are not produced in West Africa and provision of NTD medicines is needful on sources such as the global fund (TGF) (Gyansa-Lutterodt, 2007). Leishmaniasis is not being talked about not because the impact is low or negligible, it is because people have no idea that the disease exists. The use of chemical agents has in times past been the only effective way to treat all forms of the disease. An effective vaccine against leishmaniasis is however not available. The current therapy is not only toxic but also expensive and hence not likely affordable to the victims who are predominantly poor. Since almost all victims of leishmaniasis are generally poor and as such, the lengthy treatment using expensive drugs with related costs is far beyond the means of

such families (Oryan, Alidadi, & Akbari, 2014). Many patients in turn seek for alternative therapy such as plant medicine which is cheaper and readily available. Unfortunately, most of the herbs traditionally used in treatment have still not been evaluated scientifically.

Significance of the study

Given the aforementioned reasons, development of new, less toxic and more cost-effective drugs with better efficacy as well as accessible to low-income populations to treat the disease is needed (Lage et al., 2013). The treatment of leishmaniasis with available chemotherapy has been associated with systemic toxic effects, economic cost, and poor treatment compliance, necessating most research in the past decade and even now to focus on the advancement of substitute dosage schedules, approaches of delivery or management (Croft & Yardley, 2002). Almost all victims of leishmaniasis are poor and as such the lengthy treatment using expensive drugs with related costs is far beyond the means of such families (Oryan et al., 2014). Therefore, many patients seek for alternative plant medicine which is cheaper and readily available, yet, most of the herbs traditionally used in treatment have still not been evaluated scientifically. Natural products have long been providing important drug leads for infectious diseases and while vaccination for leishmaniasis is not yet available, and conventional treatments are arguably unsatisfactory, the need for potential anti-leishmanial agents from natural products that have lower side effects are urgently needed. This research seeks to provide affordable, accessible and quality but less toxic drugs against *L. donovani* and *L. gh.*

Hypothesis

Erythrophleum ivorense, Omphalocarpum ahia, Anthostema aubryanum and *Coelocaryon oxycarpum* extracts are effective against the promastigotes of both *L. gh* and *L. donovani*

Limitations to the study

1. The absence of readily available reagents and equipment leading to the exclusion of some vital components of the research such as testing of extracts and isolated compounds on clinically important amastigote form of the parasite. A selectivity index test could have been performed on the plants and isolates whose toxicity is not known provided there were available macrophage cell lines and its accompanying reagents.

2. Lack of adequate storage devices and unreliable source of power which has contributed immensely to shortcomings in this research. Parasites were lost in culture such that it was impossible to perform the antileishmnaial activity of the isolated compounds on *L. gh* promastigotes.

3. Comparing the activities of the fractions, it was seen that methanol fraction gave the best activity and hence should have been used to isolate the compounds. However, ethyl acetate was rather used because of the absence of readily available reagents/solvents and expertise due to the high polarity of methanol.

Organisation of the study

The study is organised into six chapters. Chapter one is the introduction and comprises background to the study, aim and objectives, statement of the problem, significance of the study, hypothesis, limitations to the study and Organisation of the study. Chapter two is made up of literature

review associated to the study. Chapter three is materials and methods that describe the various experimental procedures used in the course of the study. Chapter four is made of results whereas chapter five is the discussion. The final chapter is chapter six, comprising summary of findings, conclusion and some recommendations.

CHAPTER TWO

LITERATURE REVIEW

Leishmaniasis is a parasitic disease considered to be a growing public health concern for several countries. Based on the World Health Assembly (WHA), Resolution 2007/60.13, the WHO convened the Expert Committee on Leishmaniasis in March 2010, which subsequently issued the first updated technical report on leishmaniasis in over 20 years (WHO, 2007, 2010), has emphasised on the public health concern of the disease. Over 12 million individuals are estimated to be infected with leishmaniasis and 350 million are at risk of being infected (WHO, 2010), however, this is probably an underestimation, as many cases are not diagnosed, not diagnosed or not reported (Collin, Coleman, & Davidson, 2006).

Globally, the disease is endemic in 98 countries and territories and is the cause of 2.35 million disability-adjusted life years (DALYs) lost (PAHO, 2014). The DALY measurement was first promoted in the 1993 and its accuracy depends on the incidence, duration of incidence, severity and mortality data and also the basic asumptions used for the calculation (King, Dickman, & Tisch, 2005). Leishmaniasis is endemic in many countries of the world including Central and South America, the Mediterranean, Asia, Africa, the Middle East, China, India and the Caribbean (Centres for Disease Controls, 2012, Centre for Food Security and Public Health, 2009), thus the disease has been reported in all continents except for Antarctica. Among the

countries however, the tropical and sub-tropical communities are the worst hit, but creeping recently across the Mediterranean as well as in Germany, Turkey and Italy (Bodgan, 2001; Dujardin, 2008; Maroli et al., 2008). The indigenous cases in the United States are rare. It is normally reported in individuals returning from countries that have the disease and sand flies, as a result of military intervention or tourism (CDC, 2012). Nevertheless, sand flies capable of spreading the disease have been found in southern Texas and leishmaniasis has been reported in 21 states in the US and Canada (CDC, 2012; CFSPH, 2009).

History of Leishmaniasis

Leishmaniasis has been known for several hundreds of years, with one of the leading clinical descriptions made in 1756 by Alexander Russell (Arfan, 2006). He examined a Turkish patient and thereafter named it Aleppo boil in terms which are quite relevant: "after it is cicatrized, it leaves an ugly scar, persistent through life, and has existed with people in Aleppo and Baghdad by the 18th century AD. The inhabitants, as a matter of fact, had no idea about the causal agent (Dedet & Pratlong, 2003; Herwaldt, 1999). The Old World cutaneous leishmaniasis which is known also as oriental sore is an ancient disease with its history traced centuries ago. There exist records of what seems to be cutaneous leishmaniasis at least as far back as 650 BC, and possibly much earlier in the Tigris/Euphrates basin (Arfan, 2006). Arab physicians who identified oriental sores comprising Avicenna, described what was named and is currently called Balkh sore from northern Afghanistan, with later records from places such as Baghdad and Jericho in the Middle East as far back as the 10^{th} century (Bern, Maguire, & Alvar, 2008).

Texts from the 15th and 16th centuries, and then during the Spanish colonization, indicate the risk run by seasonal agricultural workers who returned from the Andes with skin ulcers which, in those times were attributed to "valley sickness" or "Andean sickness" (Arfan, 2006). Later on, cases of disfigurements of the nose and mouth were known as "white leprosy" due to their strong similarity to lesions caused by leprosy. In the Old World,
Indian physicians used the Sanskrit term kala azar for an ancient disease which later was defined as visceral leishmaniasis.

The discovery of *Leishmania* species in the old world owes its attribute to military men (Arfan, 2006). It was first described in 1885 by sergeant major Cunningham of the Indian medical service in Calcutta from a tissue taken from a sore called the Delhi boil (Cunningham, 1885). Cunningham (1885), found nucleoid bodies of equal sizes clustered in masses and thought they were spores and thus postulated that the Delhi boil had a fungal origin. Three years on, Russian military sergeant D.F. Borovsky also made a report from the Tashkent military hospital that bacterial agents described in Start sores were artifactual and that the actual causative organism was protozoan and described the anatomy of the organism and pointed out the kinetoplast (Arfan, 2006). The cause remained unknown, and several eminent clinicians, including Ronald Ross, were convinced that kala azar was a virulent form of malaria (Arfan, 2006). It was not until 1900, when Scottish army doctor, William Leishman (Leishman, 1903) and the Professor of Physiology, Charles Donovan (Donovan, 1903) independently discovered the parasite in the spleens of patients with kala azar and attributed the etiology of this life-threatening Indian disease, now called visceral leishmaniasis.

Etiology of Leishmaniasis

Leishmaniasis results from infection by various species of *Leishmania*, a protozoan parasite of the family Trypanosomatidae (order Kinetoplastida). Nearly, 30 species of the parasites have been described, with at least 20 of these organisms being pathogenic to mammals (Gramiccia & Gradoni, 2005) including humans. By geographical location, the organisms fall within two main groups which are the old world species occurring in Europe, Africa and Asia, and the new world species which occur in Americas (CFSPH, 2009).

The genus *Leishmania* encompasses two subgenera, *Leishmania* and *Viannia*, differentiated by the site of multiplication inside the gut of the insect vector (CFSPH, 2009). The classification of *Leishmania* is quite multifaceted which in some cases are debateable. In some instances, more than one species name may be used for an organism while some names may be nullified ultimately. Take for example that two different names were used for this organism, *L. infantum* in the "Old World" and *L. chagasi* in the "New World" since these two organisms had been assumed to belong to different species. They have however been reclassified into one species, *L. infantum*, with the help of a series of genetic studies. Despite this insight, *L. chagasi* is still used in South America. Primarily, visceral leishmaniasis is caused by *L. donovani* and either *L. infantum* or *L. chagasi* (Desjeux, 2004). While *L. donovani* is anthroponotic and is mostly transmitted amid individuals who act as the reservoir hosts, *L. infantum* is zoonotic. Once in a while, other species such as *L. tropica* and *L. amazonensis*, which would normally have caused cutaneous leishmaniasis, are able to cause visceral leishmaniasis.

In the New World, the species that are capable of causing cutaneous leishmaniasis include the members of the *L. braziliensis* complex (*L. braziliensis, L. panamensis/ L. guyanensis, L. shawi* and *L. peruviana,*) and the *L. mexicana* complex (*L. mexicana, L. amazonensis, L. venezuelensis*), as well as *L. lainsoni, L. naiffi* and *L. lindenbergi* (CFSPH, 2009). Species in the Old World that cause cutaneous leishmaniasis include *L. tropica, L. major* and *L. aethiopica*, which are all members of the *L. tropica* complex. In addition, some strains of *L. infantum* are capable of causing cutaneous leishmaniasis and not affect internal organs. All the organisms mentioned are zoonotic except for *L. tropica*, which is anthroponotic (CFSPH, 2009).

Transmission of *Leishmania*

The species of *Leishmania* are in most cases transmitted indirectly between hosts by invertebrate vector hosts which are small insects of the order diptera, belonging to the *Phlebotominae* subfamily and only two of the six genera described are of medical importance: *Phlebotomus* of the "Old World"; Africa, Asia, and Europe and *Lutzomyia* of the "New World"; the Americas (Killick-Kendrick, 1990, 1999). Each species of *Leishmania* is adapted to transmission in certain species of sand flies (Mutinga, Basimike, Kamau, & Mutero, 1990). Like mosquitoes, only the female sand fly feeds on blood and are usually most active at dawn, dusk and during the night, but they will bite if they are disturbed in their hiding places in animal burrows, holes in trees, caves, houses and other relatively cool, humid areas during the day (Mutinga et al., 1990). The flies are attracted to light and as such may enter buildings at night. Other arthropods including ticks (*Dermacentor variabilis* and *Rhipicephalus sanguineus*) and canine fleas may also act as mechanical

vectors (Mutinga et al., 1990). Where sand flies transmit *Leishmania* spp., ticks and fleas are probably unimportant in the epidemiology of the disease; however, they might be involved in rare cases of dog-to-dog transmission in other locations (Dantas-Torres, 2007).

The dimorphic nature of the parasite specifically makes its life cycle quite complex. There is an extracellular stage within the phlebotomine host and one intracellular stage within a vertebrate host, of which the two morphological forms are respectively promastigotes and amastigotes (Koutis, 2007). The disease is transmitted to its vertebrate host by the female infected sand fly which needs a blood meal for maturation of its eggs (Zavitsanou, Koutis, & Babatsikou, 2008).

Infection occurs when an infected sand fly regurgitates infective promastigotes from its proboscis into the blood while feeding on human/vertebrate host (CDC, 2013). The promastigotes that reach the wounds created are phagocytised by macrophages and transform into tissue stage amastigotes which then multiply by binary fission inside the macrophages, often provoking a cutaneous ulcer and lesion at site of bite (CDC, 2013). The released amastigotes through multiplication in the macrophages proceed to infect other neighbouring mononuclear phagocytic cells. The cycle is continued when the female sand fly takes a blood meal from an infected person and in the process ingests the amastigote-filled macrophages.

In the sand fly, amastigotes transform into promastigotes, develop in either the gut or midgut depending on the species of *Leishmania* before final migration to the proboscis (CDC, 2013). Parasite, host, geographic region and other factors affect whether the infection becomes symptomatic or not. The

interplay of such same conditions determines the type of disease manifested in an individual (CDC, 2013). It is worth noting that recent evidence indicates that day-biting midges are responsible for transmitting leishmaniasis to kangaroos in Australia (Dougall, 2011). By this, it could be inferred that midges may be involved in transmission of the Ghanaian parasites (unpublished data) despite the presence of candidate sand flies which research has failed to isolate the parasites (Boakye, Wilson, & Kweku, 2005). Moreover only 0.4 % of the flies caught by Raczniak et al. (2008) were Phlebotomus flies whereas the rest of the 99.6 % were all *Sergentomya* species. A pictorial summary of the lifecycle is shown below in figure 1.

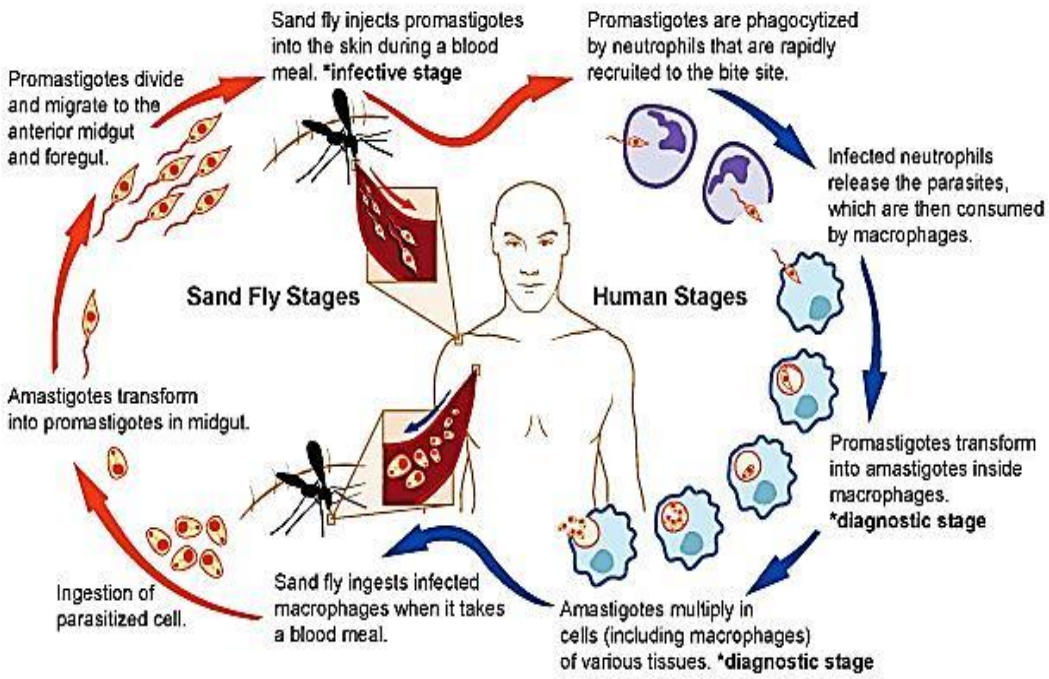

Figure 1: Life cycle of *Leishmania* species; Source; WHO, 2013

Pathology of leishmaniasis

Successful infection of *Leishmania* is achieved by alteration of signaling events in the host cell, leading to enhanced production of the autoinhibitory molecules like TGF-beta and decreased induction of cytokines such as interleukins (IL) 12 for protective immunity (Murray & Delph-Etienne, 2000). Nitric oxide production is also inhibited. In addition, defective expression of major histocompatibility complex (MHC) genes silences subsequent T cell activation mediated by macrophages, resulting in abnormal immune responses (WHO, 2010). Generally, a number of diseases are prompted by different *Leishmania* species, and individual species are unique in pathogenicity in different human populations.

Generally, it has been agreed that the control of *Leishmania* within the host is mediated by both innate and adaptive immune responses. The interaction between *Leishmania* and human host response is not only manifested in terms of the clinical or subclinical outcome of the disease but also on the rate of spontaneous healing and recurrent disease (Jeronimo et al., 2000). At the site of inoculation, neutrophils are the first cells to confront *Leishmania*. Cells of the innate immune system, including natural killer cells, have been implicated to impact the course of infection and disease (Belkaid, Hoffmann, & Mendez, 2001).

Experimental evidence has indicated the pathogenesis of some species of *Leishmania*, such as *L. major*, is improved by neutrophil intermediation of infection, where as in others such as *L. donovani* and *L. amazonensis*, it is the neutrophils that contribute to protection. Whether a person's immune system is under compromised or over competent can lead to chronic, therapeutically

challenging disease presentations. The absence of *Leishmania* specific cell-mediated responsiveness characterizes non ulcerating diffuse cutaneous leishmaniasis; infection-mediated immunosuppression in the course of visceral leishmaniasis leaving the host frail against a huge parasite burden and heightened cell-mediated immune hypersensitivity that produces disfiguring chronic mucosal and cutaneous disease.

Apart from evidence based on clinical trials, the defining role of the immune response was clearly well-known by capsizing susceptible and resistant phenotypes in genetically defined experimental models (Kaye, Curry, & Blackwell, 1991). Selective deletion and replacement of immunocompetent cell populations and, in recent times, targeted deletion of the genes coding cell products involved in the immune response have been used to dichotomise the immunopathogenic and curative responses to experimental infection. It should be noted however, the strict Th1 and Th2 dichotomy in many experimental animal models does not necessarily reflect human disease (Muraille, De, & Brait, 2003).

In visceral leishmaniasis (VL) infection that is caused by *L. donovani,* reticuloendothelial hyperplasia occurs while in the case of *L. infantum,* the spleen, liver, mucosa of the small intestine, the bone marrow, the lymph nodes and the other lymphoid tissues are affected (Mohapatra, 2014). Many of these cells are heavily parasitized, rendering lymphocytic infiltration scanty atrophy of paracortical may be observed in the spleen and other lymphoid organs. The lifespan of white blood cells and red blood cells is reduced, causing granulocytopenia and which results in anaemia. Moreover the function of the liver is likely to be altered and then later, prothrombin production declines

(WHO, 2010). Together with thrombocytopenia, the prothrombin depletion may result in severe mucosal haemorrhage. Hypoalbuminaemia is associated with oedema and other features of malnutrition. Diarrhoea may occur as a result of intestinal parasitization and ulceration or secondary enteritis. In the advanced stage of the disease intervening infections are recurrent, especially pneumonia, dysentery and tuberculosis and these are common causes of death.

Hyperglobulinaemia (mainly polyclonal immunoglobulin G) and polyclonal B cell activation is common in visceral leishmaniasis, but its pathological role is not known. VL causing amastigote forms can be found within bone marrow macrophages and occasionally in neutrophil and eosinophil granulocytes. Human visceral leishmaniasis is associated with a mixture of both Th1 and Th2 responses (Muraille et al., 2003). Cure following treatment is accompanied by increased interferon-γ and interleukin (IL)-12 and decreased IL-10 and transforming growth factor-β. The number of $CD4^+$ $CD25^+$ T cells are reported to be increased during active visceral leishmaniasis and to decrease at cure (Belkaid et al., 2001). These regulatory T cells may contribute to the state of immunosuppression characteristic of visceral leishmaniasis. In the case of Post Kala azar Dermal Leishmaniasis, the disease is triggered immunologically following treatment of VL. The cells of inflammation that are in play are predominantly $CD3^+$, IL-10 which is prominent in the lesions, interferon-γ which is found uniformly, and IL-4 which is present in varying amounts. Diminished expression of interferon- γ receptor 1 and tumour necrosing factor (TNF)-R1 and -R2 receptors during PKDL (Ghalib, Piuvezam, & Skeily, 1993), may interfere with an effective host response. IL-10-expressing $CD3^+CD8^+$ lymphocytes are prominent, and

their level decreases with treatment. Patients with PKDL usually have raised levels of immunoglobulins G3 and G1 and increased serum levels of IL-10 (Belkaid et al., 2001). High serum concentrations of IL-10 during visceral leishmaniasis correlate with subsequent development of PKDL.

Immune system regulation

The uptake of promastigotes by macrophages is a receptor-mediated process that involves the the use of energy by the macrophage and not by the parasite (Mosser & Rosenthal, 1993). The obligate intracellular behavior of the *Leishmania* species allow them to express several different ligands on their surface and are able to interact with various macrophage receptors,therby ensuring their uptake by phagocytic cells (Mosser & Rosenthal, 1993). Some of these include receptors for complement (Kane & Mosser, 2000), fibronectin as well as mannose-fucose receptor (Chang, Chaudhuri, & Fong, 1990). The receptors are able to bind parasites with avidities that are different. For example, low affinity receptors have the ability to significantly contribute to parasite internalization without making an obvious contribution to parasite adhesion. This is seen in fibronectin receptor (FnR), which binds to the parasite surface molecule gp63 with very low low affinity.

In *in vitro* phagocytosis assays, FnR does not play much role but it is seen that cells lacking FnR however, or parasites that have undergone any forms of mutation in the fibronectin recognition domain on gp63, show significant delays in parasite uptake (Chang et al., 1990). This is an indication that FnR plays an important role in uptake. Certain ligands that have also been implicated in parasite update include lipophosphoglycan (LPG) and gp63, and

other phosphoglycans in the parasite glycocalxy (Palatnik, Previato, & Mendonca-Previato, 1990).

Even the most important ligands needed for parasites uptake are not parasite-encoded at all and because of this promastigotes rely exclusively on host-derived opsonins to achieve optimal uptake by phagocytic cells. The complement system also represents an important mediator of promastigote adhesions to phagocytic cells (Mosser & Edelson, 1987). The third component of complement together with its the receptor Type 3, CR3, Mac-1, CD11b/CD18, is probably the most important of the macrophage complement receptors for parasite phagocytosis due to the abundance of CR3 expression on macrophages and to the very transient nature of the C3b molecule, whose half-life on opsonized particles is measured in minutes (Da Silva, Hall, & Joiner, 1989). Promastigotes make their way into macrophage phagosomes, which acidify and fuse with lysosomes. In these acidified medium of phagolysosomes, the parasites are able to replicate. The proof that these phagolysosomes are fully competent is the fact that debris and dead organisms are degraded in the same. Phagolysosomes that house viable amastigotes are the same that contain the deabris and degraded dead organisms which certify that phagolysosomes are fully competent (Cunningham, 2002).

Some research evidence by Swanson and Fernandez-Moreira (2002) has indicacted a delay in the maturation of promastigote phagosomes such that this “pregnant pause”may give the promastigote enough time to transform into amastigotes and upregulate the genes necessary for intracellular survival (Desjardins & Descoteaux, 1997). On the surface promastigotes is LPG which may contribute to the delay in maturation.

It is now believed that changess in lipid content of cellular organelles influence the fusagenic competence of vesicles with each other (Duclos & Desjardins, 2000). Just like promastigotes, amastigote forms of the parasite are also taken up by more extraordinarily efficient receptor-mediated phagocytotic process. At its resting phase, macrophage can internalize at least a dozen amastigotesin just about 30 minutes. Heparin binding activities are possessed by amastigotes and that allows them to adhere to cellular proteoglycans. This adhesion in turn surges the efficacy of receptor-mediated phagocytosis. Receptors such as the mannose receptor have been implicated in amastigote phagocytosis (Peters, Aebischer, & Stierhof, 1995). A study has shown that amastigotes may mimic apoptotic cells and bind to phosphatidylserine receptors on macrophages (Wanderley, Moreira, & Benjamin, 2006) resulting in the failure of the parasites to activate inflammatory cytokine production from macrophages.

Forms of leishmaniasis

Depending on the species of the parasite, leishmaniasis is manifested in three main clinical forms, which are cutaneous, mucocutaneous and visceral leishmaniasis (Goto & Lindoso, 2010).

Visceral leishmaniasis

Human visceral leishmaniasis (VL), more popularly known as kala-azar, is primarily caused by *L. donovani* in Africa and the Indian subcontinent, *L. infantum* in the Mediterranean and *L. chagasi* in Latin America. *L. donovani* is anthroponotic, transmitted mainly between people, who act as the reservoir hosts. The term kala-azar which means black (kala) in Hindi as a result of skin pigmentation that can occur as a symptom is regularly reserved

for severe cases of VL, although the terms kala-azar and visceral leishmaniasis sometimes are used interchangeably (Hashim et al., 1994). It is the most severe form of the disease in which the amastigotes migrate to vital organs such as the spleen and liver. It is the reason why splenomegaly is a common clinical feature of one with VL, since generally only about 5 % of cases present without splenomegaly (Hashim et al., 1994).

When VL is left untreated, it can result in 100 % mortality of infected patients (DeWitt, Girma, & Simenew, 2013). The mortality may occur either directly from the disease or indirectly from other complications, such as secondary bacterial infection or haemorrhage (DeWitt et al., 2013). The impact of VL is rigorous and unbearable, often characterized by other symptoms such as prolonged fever, and pancytopenia (Berman, 1997). The conventional manifestations of clinically manifest visceral infection among hepatosplenomegaly (usually, the spleen is more prominent than the liver) and fever, include anemia, leukopenia, and thrombocytopenia, a high total protein level and a low albumin level, with hypergammaglobulinemia (Berman, 1997). Lymphadenopathy may be noted, particularly in some geographic regions, such as Sudan (Berman, 1997). Some of these symptoms such as the long-lasting fever, has sometimes led to the misdiagnosis of the disease and often mistaken to be malaria, because of prolonged fever (Alvar, Canavate, Molina, & Moreno, 2004). There are two forms of VL, which are different in their transmission features. Zoonotic transmission of VL is from animal to vector to human while anthroponotic transmission of VL is from human to vector to human (Chappuis, Sundar, Hailu, Ghalib, & Rijal, 2007). In the former, humans are intermittent hosts and animals, largely dogs, are the

reservoir of the parasite (Alvar et al., 2004). Anthroponotic VL is found in areas of *L. donovani* transmission whiles transmission of zoonotic VL is prominent in areas where *L. infantum* is common (Chappuis et al., 2007).

Although VL is highly prevalent mostly in the Indian subcontinent as indicated in figure 2, large numbers of cases of VL have been reported in Africa and in Southern America causing important medical problems (Berman, 2006). It has been reported in Sudan as far as 1904 (de Beer et al., 1991), Ethiopia and Kenya (Berman, 2006). Every year in Africa, health facilities report a number of cases with deaths. In epidemic years, the toll of the disease can be much alarming while the epidemics of VL during the 1980s and 90s killed 100,000 people in Sudan alone (Chappuis et al., 2007). With routine surveillance in the region mostly limited to passive case detection at a few health facilities equipped to diagnose and treat the disease, the current morbidity and mortality figures are likely to underestimate the regional leishmaniasis burden. Despite the wide spread of the disease all over the world, six countries account for more than 90 % of the global incidence of VL; India, Nepal, Bangladesh, Sudan, Ethiopia, and Brazil (Chappuis et al., 2007). Out of these six countries three are African, a rather fascinating situation since the continent has the least surveillance data on the disease; Sudan, South Sudan and Ethiopia. The other three countries are India, Bangladesh, and Brazil (Chappuis et al., 2007).

After recovery from VL, patients have high chance of developing a chronic cutaneous form called post-kala-azar dermal leishmaniasis (PKDL), which usually appears nearly two years after apparent clinical cure of the infection (Salotra & Singh, 2006**)**. PKDL is a syndrome characterized by skin

lesions that can be of various types and initially are most prominent on the facial. Some of the skin lesions include erythematous or hypopigmented macules, papules, nodules, and patches which are typically first noticed which develops at variable intervals during or after treatment of VL (WHO, 2010). PKDL have been described in cases of *L. donovani* infection in South Asia and East Africa. In general, PKDL is more frequent, develops earlier, and is less chronic in patients in East Africa (Chappuis et al., 2007). In Sudan for example, PKDL is noted in up to 60 % of patients, typically from 0 to 6 months after VL therapy, and often heals spontaneously (Chappuis et al., 2007). In contrast, in South Asia, PKDL is noted in approximately 5 to 15 % of patients several years after initial therapy which usually require further treatment (Gonzalez, Pinart, Reveiz, & Alvar, 2008). Patients with chronic PKDL can serve as important reservoir hosts of infection (Chappuis et al., 2007). It should be noted that PKDL is a relapse from VL; proper treatment of VL will automatically eliminate PKDL.

VL is the fifth leading opportunistic infection occurring as an opportunistic infection in HIV-infected patients (Bal, 2005; DeWitt et al., 2013) and is presented in figure 2 below. It is estimated that almost 30 % of VL patients have HIV, with Ethiopia having the highest VL/HIV co-infection in the world (DeWitt et al., 2013). Coinfection of VL with human immunodeficiency virus (HIV) was first reported in European Mediterranean countries in the mid 1980s and extended progressively to other regions (WHO, 2010). Visceral leishmaniasis presented atypical clinical features as an opportunistic infection in HIV adults, with high rates of relapse and mortality. Visceral leishmaniasis is more likely to develop in HIV-infected patients and

impairs their response to antiretroviral treatment. Lartey et al. (2006) described the association between the two diseases as a vicious cycle of mutual reinforcement because both diseases attack the cell-mediated immune system. The WHO in 2010 stated that AIDS increases the risk of VL by 100-1000 times in endemic areas. In the revised WHO Clinical Staging for HIV for the African region, VL is assigned to WHO stage 4 which implies severe immune suppression (WHO, 2010). As figure 2 portrays, in south-western Europe *Leishmania*/HIV co-infection has been reported highest among male adults with more than 55 % of the cases that occur in this area have been reported to occur in the age group from 31 to 40 years and over 70 % of the cases of *Leishmania*/HIV co-infection reported from south-western Europe among intravenous drug users.

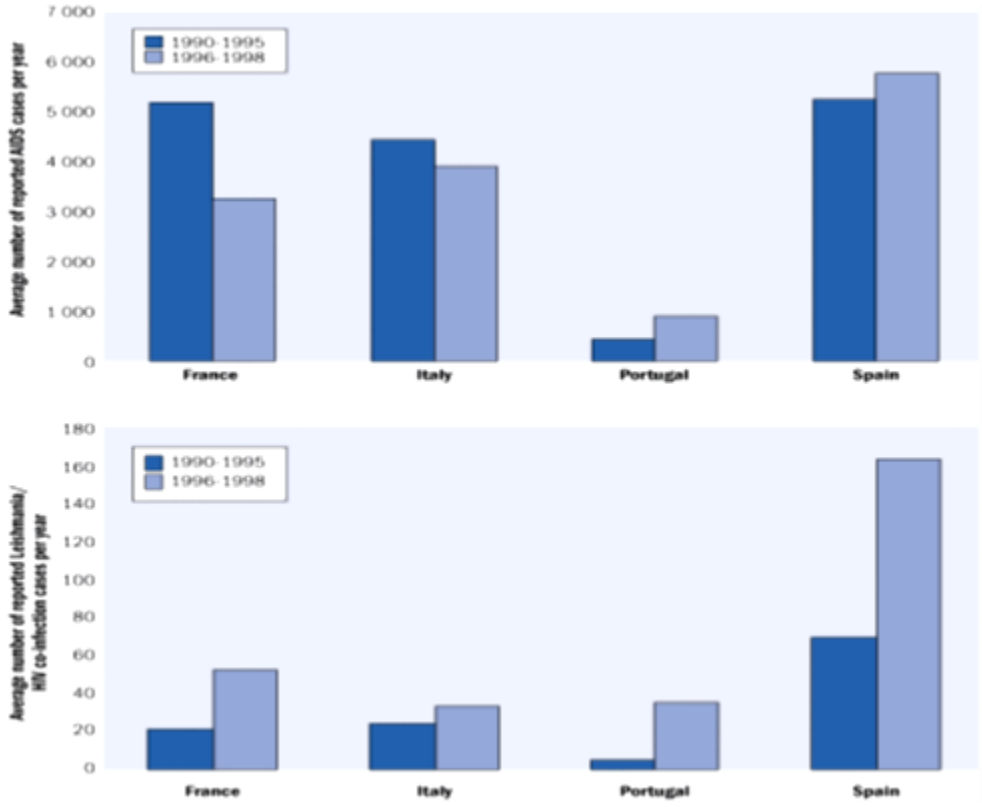

Figure 2: Average number of AIDS cases and *Leishmania* /HIV co-infection cases per year, reported to WHO, South-western Europe, Source; WHO, 1999

Due to deficient diagnostic capacities and surveillance, the burden of VL-HIV-coinfection in Africa remains grossly unidentified; however, HIV-coinfection is emerging in this continent (DeWitt et al, 2013).

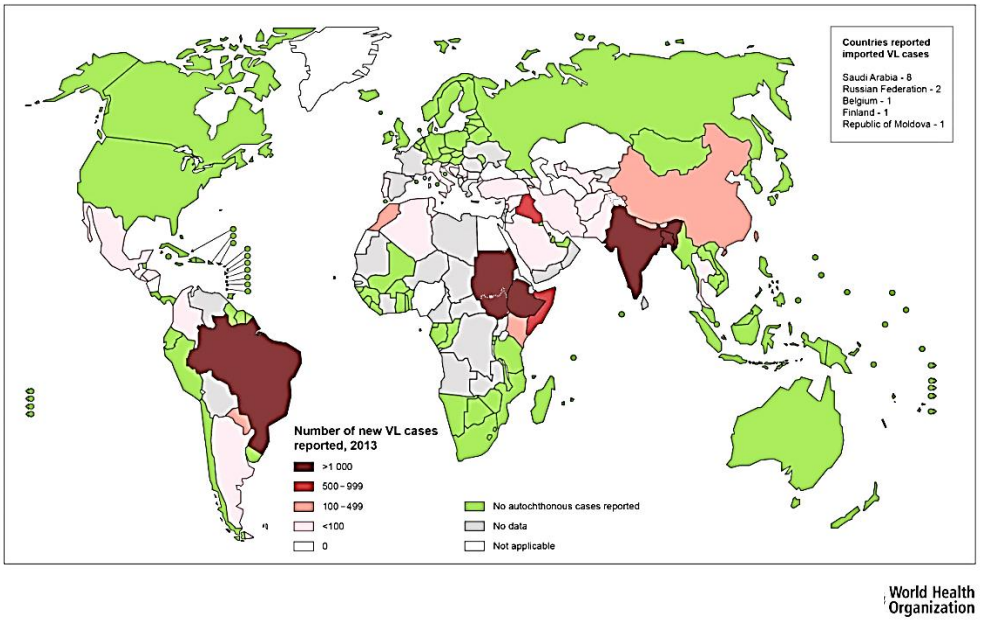

Figure 3: Worldwide endemic status of visceral leishmaniasis; Source; WHO, 2013

Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) is an international public health concern and a social predicament in various developing countries. It is the most common form of the disease and can impinge on the skin and mucous membranes. It is caused by dissimilar *Leishmania* species widespread in many countries in both New and Old World (Alvar et al., 2012). The majority of *Leishmania* species cause CL in 1,500,000 individuals (Assimina, Charilaos, & Fotoula 2008). CL causes skin lesions that, depending on the species, can be localized on a specific place on the body (mostly on the limbs or the face) or spread. The skin lesions usually develop within few weeks after being

infected. In specific varieties of cutaneous leishmaniasis these lesions can cause massive tissue damage, leaving the patient disfigured and the subject of social prejudice and stigma. Wounds from cutaneous infections are sometimes self-healing due to the development of acquired immunity through cellular and humoral responses (Peters & Sacks, 2006). Spontaneous healing frequently results in lifetime protection from disease, which may or may not be restricted to the same *Leishmania spp*. (Reithinger et al., 2007). Resolution of disease results in a lifelong cutaneous scar, which depending on its size and location, may cause extensive trauma in affected individuals (Yanik et al., 2004). Whiles other forms of CL stay nodular, others can progress to form diffuse cutaneous leishmaniasis. In the classic course of the cutaneous disease, lesions first appear as papules, progress to ulcers, and then instinctively heal with scarring over months to years (Berman, 2006). Treatment is however highly recommended as sores can spread, take months or even years to heal or even cause death following secondary infections (CFSPH, 2009). CL occurs in 82 tropical, subtropical and temperate countries worldwide with an estimation of 1.2 million new cases of occurring each year (Alvar et al., 2012). Surveillance data has indicated an increase in the global number of CL cases during the past decade, as documented in Afghanistan (Assimina et al., 2008), Bolivia (Davies et al., 2000), Brazil (Brandao-Filho, Campbell-Lendrum, Brito, Shaw, & Davies, 1999), Colombia (Davies et al., 2000), Syria (Tayeh, Jalouk, & Cairncross, 1997), and Turkey (Svobodova et al., 2009).

In West Africa, several cases of the CL have been reported in Niger (Stevene, 1911), Mali (Lefrou, 1948), Nigeria (Dyce, 1924), Senegal (Riou & Advier, 1933), Cameroon, Burkina Faso, Mauritania, Gambia and Guinea

(Boakye et al., 2005) and Ghana (Kwakye-Nuako et al., 2015). Ten countries have been reported to have the highest estimated CL case counts in the world comprising Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru which together account for not less than 70 % of global estimated CL incidence as shown in figure 3 (Desjeux, 2004; WHO, 2013). Algeria reportedly accounts for 90 % all CL cases in the world wide (Desjeux, 2004). Clinically, CL may also be subdivided into localised cutaneous and diffused cutaneous leishmaniasis. Despite its increasing worldwide incidence, but because it is hardly ever fatal, cutaneous leishmaniasis has become one of the so-called neglected diseases, with little interest by financial donors, public-health authorities, and professionals to implement activities in the areas of research, prevention, or control.

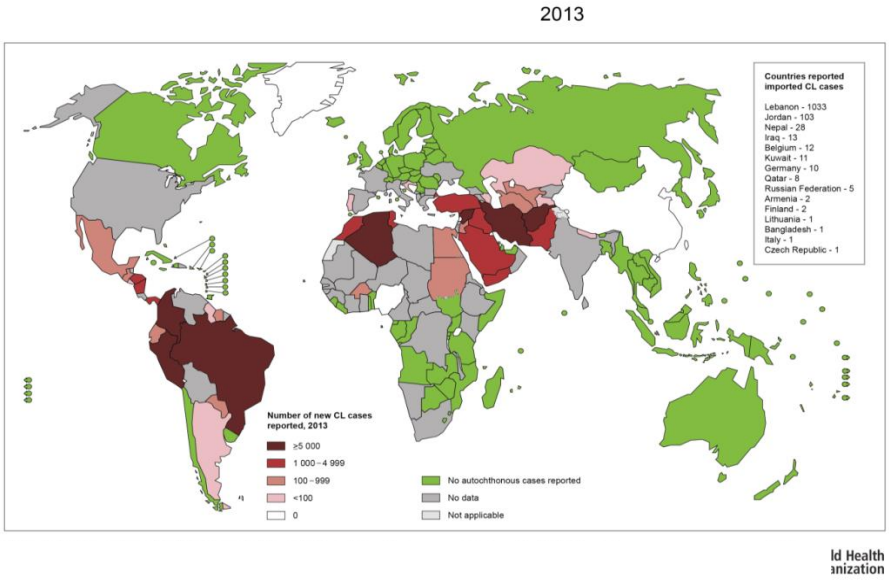

Figure 4: Worldwide distribution of cutaneous leishmaniasis; Source; WHO, 2013

Mucosal cutaneous leishmaniasis (espundia)

Mucosal cutaneous leishmaniasis (MCL) conventionally refers to a metastatic consequence of New World cutaneous infection, which results from

circulation of *Leishmania* parasites from the skin to the naso-oropharyngeal mucosa (Desta, Shiferaw, Kassa, Shimelis, & Dires, 2005). The parasite and host factors influence the determinants and magnitudes of the mucosal dissemination and mucosal disease vary among geographic regions. The disease usually becomes clinically evident after 1 to 5 years of the original cutaneous lesions (DeWitt et al., 2013).

However, mucosal and skin lesions may be noted concurrently resulting in mucocutaneous leishmaniasis. The initial manifestations of mucosal leishmaniasis usually are persistent, unusual nasal symptoms (such as stuffiness or bleeding), although oral or pharyngeal symptoms sometimes are noticed first (Acha & Szyfres, 2003). If untreated, the disease can progress to ulcerative destruction of the naso-oropharyngeal mucosa (such as perforation of the nasal septum). MCL also results in extensive disfiguring lesions of the nose, mouth and throat mucous membranes and in the process rendering its victims disfigured for life (DeWitt et al., 2013). It is characterised by the ability of the parasite to metastasise to mucous tissues by either lymphatic or haematogenous distribution. Classically, MCL begins with nasal inflammation and stuffiness, followed by ulceration of the nasal mucosa and perforation of the septum. In some cases, the lips, cheeks, soft palate, pharynx, or larynx are also involved. Mucosal leishmaniasis never heals spontaneously, is very difficult to treat, with secondary bacterial infections common, and is potentially fatal (Marsden, 1986). The destructive and disfiguring lesions of the face, resulting from MCL is most often caused by *Leishmania* (*Viannia*) *braziliensis*, but cases caused by *L. aethiopica* have also been rarely described (Kimutai, Ngure, Tonui, Gicheru, & Nyamwamu, 2009). MCL is often

referred to as a group of diseases because of the varied spectrum of clinical manifestations, which ranges from small cutaneous nodules to gross mucosal tissue destruction (Reithinger et al., 2007).

A particular species of *Leishmania* are more likely than others to progress to MCL; for example, it is estimated that 3 % of patients with *L. braziliensis* develop mucosal disease. Additionally, 90 % of all cases of MCL occur in Bolivia, Brazil and Peru. The time delay between first signs of the cutaneous ulcer to the noticeable involvement of mucosal membranes of nose and mouth is estimated to be from one month to 24 years (Kimutai et al., 2009).

Diagnostic devices in leishmaniasis

A number of procedures are available to diagnose leishmaniasis, some of which include conventional parasite detection techniques, immunological tests, antigen detection tests and molecular methods among others. Cutaneous leishmaniasis can be diagnosed by direct observation of the parasites in skin scrapings, impression smears or skin biopsies stained with Giemsa, Leishman's, Wright's or other stains (Anjili et al., 1998). Amastigotes are easiest to find in recent lesions. In areas where polymerase chain reaction assays (PCR) are available, PCR are used for diagnosis (CFSPH, 2004). *Leishmania* species can also be cultured. However, each species will grow only in certain media, and some species can be difficult to isolate. Novy-MacNeil-Nicole (NMN) medium, brain-heart infusion (BHI) medium, Evan's modified Tobie's medium (EMTM), Grace's medium and Schneider's Drosophila medium might be used initially (Ayllon et al., 2008).

Animal inoculation into hamsters may also be valuable, especially with contaminated material. Diagnosing leishmaniasis by *in vitro* culture requires between 5 and 30 days, while animal inoculation can take several weeks or months. The species, subspecies and/or strain can be identified by PCR, DNA hybridization, kinetoplast DNA restriction endonuclease analysis, isoenzyme analysis, or immunological techniques that use monoclonal antibodies (Barnes, Stanley, & Craig, 1993). A delayed hypersensitivity test, the leishmanin skin test (Montenegro skin test), is useful in the diagnosis of cutaneous and mucocutaneous leishmaniasis, but it is usually negative in the diffuse cutaneous form (Anjili et al., 1998). Antibodies are often slow to develop and of low titer.

Visceral leishmaniasis can be diagnosed using some of the same techniques, including direct observation of the parasites (Banuls, Hide & Prugnolle, 2007). Amastigotes may be found in peripheral blood, or more often, in aspirates or biopsy smears from the spleen, bone marrow or lymph nodes. PCR, culture or animal (hamster) inoculation may be particularly useful early, when parasite numbers are low. Serology can also be helpful in this form of leishmaniasis. Common serological tests used in humans include the immunofluorescent antibody test (IFA), direct agglutination, enzyme-linked immunosorbent assay (ELISA), fast agglutination-screening test (FAST), and a rapid immunochromatographic assay (K39 dipstick or striptest (Beck et al., 2008). Other assays including gel diffusion, complement fixation, indirect hemagglutination and countercurrent electrophoresis have also been used (CFSPH, 2004). Cross-reactions can occur in some serological tests with leprosy, Chagas disease, malaria and schistosomiasis (Baneth, Koutinas,

Solano-Gallego, Bourdeau & Ferrer, 2008). The leishmanin skin test/ Montenegro skin test is usually negative in cases of visceral leishmaniasis, but reactions can be seen once the disease is cured.

Leishmaniasis in Ghana

Three types of ecological zones are distinct in Ghana. These are the arid northern savanna, forest middle belt and coastal savanna zone. Epidemiological data as shown in figure 4 has suggested that the northern zone of Ghana is contained in the leishmaniasis belt in Africa (Fryauff, Hanafi, Klena, Hoel, & Appawu, 2006). No case has however been found to confirm this suggestion in this area. Reported cases are however from a focal outbreak in a moist semi-deciduous forest area, outside the so called leishmaniasis belt (Fryauff et al., 2006).

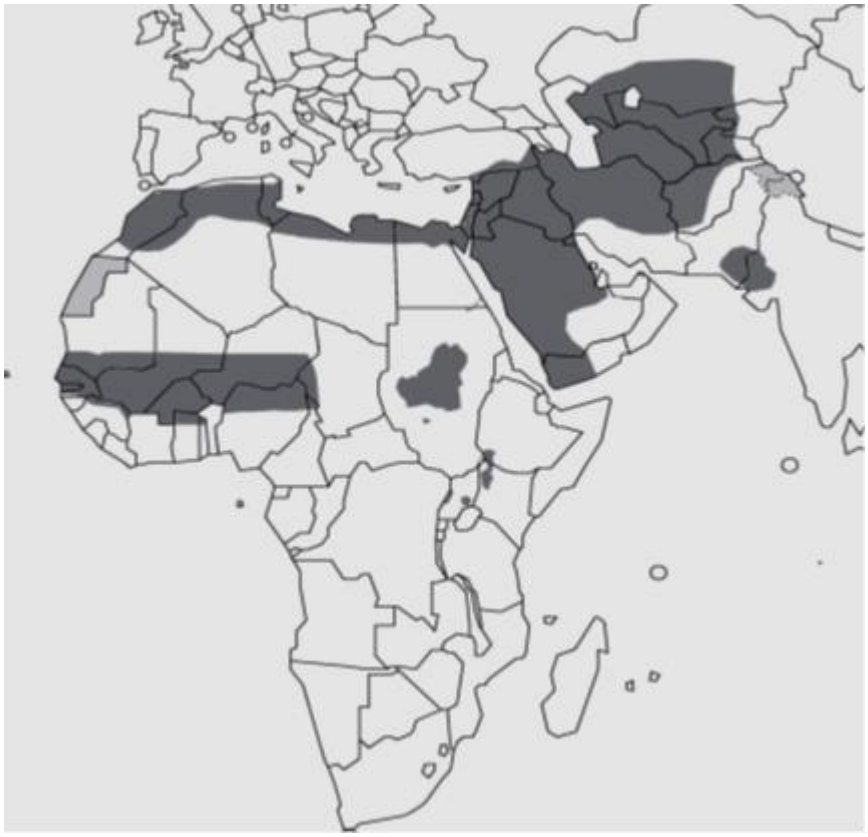

Figure 5: Geographical distribution of Old World cutaneous leishmaniasis due to *L. major*, Source; WHO, 2010

In Ghana, the first cases of CL were reported in the Ho district between 1999 and 2001, when health centres in two sub districts recorded cases of chronic skin ulcers (Fryauff et al., 2006). All through 2002, surveys conducted in towns in the Ho municipality, identified suspected lesions in 12.2-32.3 % of local school children (Fryauff et al., 2006). Being new to the sudden and quite strange ulcers, inhabitants named the disease in the area as 'Agbemekanu', (Kwakye-Nuako et al., 2015) literally connoting 'a gift from a visitor or somebody who had travelled and just came back'. This refers to the local belief that the disease has been brought in from neighbouring Togo, since travel across the border between the Volta region and Togo is quite frequent. Whether this implied importation of CL is true remains uncertain.

Other than young children, the disease is found in older children and adults who often are newcomers to the area. Thus the current pattern of infection more likely reflects an exposure of naïve individuals to what has become an established endemic focus in Ghana. Even though no official reported cases in neighbouring Togo, there were frequent reported cases of CL in Burkina Faso during that same period of emergence of CL in Ghana (Boakye et al., 2005). While an active case search found 2,348 infected individuals in the Ho district, 2 in Hohoe and 76 in Kpando in 2002, an outbreak of over 6,000 cases was reported in Ho again in 2003 alone, affecting 116 villages (Fryauff et al., 2006). These figures are quite alarming considering a more than 100 percent increase in prevalence within the scope of a year. This shocking case load decreased drastically to 105 cases in 2004 and to only 14 cases in 2005. Reported cases remained incredibly low and dwindled to zero in 2008 and 2009. Opinions subscribed to the decline in

number include the lack of the management of the disease in Ghana and self-reporting by victims. On the other hand, the decline could mean an actual clearing of the disease with individuals treating themselves during the outbreak. The insecticide spraying campaign that took place after the outbreak (Boakye et al., 2005) is yet a laudable reason for the low incidence of the disease between these years.

Leishmaniasis had been in existence since the 1930s despite recently reported in Ghana (Fryauff et al., 2006). It is possible that the disease existed in the country but had not yet been reported. The period between 1930 and 1999 may be responsible for the large numbers found during the outbreak years, with the lack of proper diagnosis serving as the main challenge since the disease shares similar clinical symptoms with vast numbers of microbial infections. Moreover, the self-healing nature of the disease cannot be under estimated, to have played a role in the reduction of the reported cases over the years.

There is high probability that the burden of leishmaniasis will amplify due to increasing resettlement, regional climate change, and weakened immunity, resulting from poor nutrition and/or HIV. Regardless of this, efforts to control the disease are mostly non-existent or not prioritised. It is worth noting that Kwakye-Nuako et al. (2015) have for the first time been able to isolate, culture and identify the species of *Leishmania* responsible for the lesions in Ghana, which are rather related to one of the several species grouped within the *L. enrietti* complex. These species are perhaps the first new human-infective *Leishmania spp*. to be isolated in Africa for over 40 years (Kwakye-Nuako et al., 2015). Based on current evidences reported by

Kwakye-Nuako et al. (2015) one can now say confidently that the suspected lesions in Ho, are actually due to leishmaniasis. Interestingly, the complex of *L. enrietti* has been mostly associated with zoonotic infections only (Lainson, 1997).

Treatment of leishmaniasis

Although treatment of leishmaniasis may not necessarily remove the parasite, an improved clinical outcome can be produced. Current treatment options including pentavalent antimonials (sodium stibogluconate (SSG) and meglumine antimoniate), paromomycin, miltefosine, the amphotericin Bs; deoxycholate and liposomal amphotericin B (L-AmB) have all been reported to suffer from limitations of cost, specific toxicities or parenteral administration (Croft, Sundar, & Fairlamb, 2006). Combination treatments with existing drugs have been developed to optimize the efficacy and safety of treatment and reduce costs and hospitalization.

The advantage of the new formulations is that they are less toxic than amphotericin B; therefore, the total doses can be administered over a brief interval of days. For situations in which toxicity and duration of therapy are the major considerations, the new formulations will be attractive. For situations in which cost is a major concern, amphotericin B may be preferred to the new and relatively expensive formulations. The non-existent comparative studies have made it somewhat difficult to determine which formulation should be used when the decision is made to use lipid-associated amphotericin B (Berman, 1997). On the basis of infusion-related side effects, ABCD seems to be the most toxic formulation, and L-AmB seems to be the least toxic formulation (Berman, 1997). Findings on comparison of the

minimum effective doses of L-AmB in Europe and in India suggest that efficacy data from one geographic area may not pertain to another geographic area and as such the relative efficacies of the three formulations can be determined only from studies performed in the exact geographic area (Berman, 1997).

Secondary regimens such as pentamidine monotherapy, paromomycin monotherapy, and interferon-γ combined with antimony are all mostly likely to be less effective than amphotericin B containing formulations (Berman, 1997). Although use of the amphotericin B formulations is associated with a higher initial cure rate (100 %) than is use of antimony (50 %) (Altos, Salas, & Riera, 1991; Rosenthal Marty, & Poizot-Martin, 1995), relapses typically occur, even when liposomal amphotericin B is used (Davidson, Di Martino, & Gradoni, 1994). Since it is unlikely that initial therapy will eliminate all organisms, maintenance regimens are needed to prevent relapses. Antimony and amphotericin B are generally used and should probably be reserved for initial and subsequent treatment courses. Therefore, the secondary parenteral agents; pentamidine and paromomycin, alone or perhaps combined with putatively effective drugs such as interferon, ketoconazole/fluconazole, or allopurinol, are the choices for maintenance therapy.

Pentavalent antimonials

Pentavalent antimonials which are made of meglumine antimoniate and sodium stibogluconate (SSG) have been the standard first-line treatment of VL for the last seven decades (DeWitt et al., 2013). In 2007, Richard et al., stated in their report that the pentavalent antimonials were the recommended drugs used for the treatment of both CL and VL similar to the claim of den

Boer, Argaw, Jannin & Alvar (2011). Immediately after the parasite was discovered in 1904, the first recognition of organic antimonials drugs for leishmaniasis was also discovered in 1912 (Yardley & Croft, 2000). In as much as both meglumine antimoniate and SSG are chemically similar, they differ in the dose administered; 8.1 % Stibogluconate (Sb) 5+ (81 mg/mL) versus 10 % Sb 5+ (100 mg/mL) respectively. As a result of this difference, the two forms cannot easily be substituted within control programmes. As leishmaniases became more extensively treated and more carefully studied, treatment failures with Sb and clinically resistant isolates became recognized. Alternatives to Sb have recently been found for some syndromes. Nevertheless, the mainstay of chemotherapy is still the pentavalent antimonials. The primacy of pentavalent antimonial therapy has been maintained by increasing the dosage for syndromes for which the cure rate has been found to be low. When available, pentavalent antimonials are often used for treatment and in the US, these drugs are provided through the Centers for Disease Control and Prevention (CDC, 2013). When used alone, SSG require 30 days of painful daily intramuscular injections. This drug also has serious (cumulative) toxic side effects and is dangerous in HIV co-infected patients, with mortality being five to ten times higher than in non HIV-infected. Although the pentavalent antimony was first recognized as clinically effective in 1947 (Berman, 1988), stibogluconate and meglumine antimoniate are still the mainstay of treatment for all the leishmaniases. Although stibogluconate produced in India is widely used in that country, there have been no formal comparisons of Indian stibogluconate with Pentostam or with Glucantime.

A review of the data in 1992 by Herwaldt and & Berman, showed that for Kenyan (Anabwani, Ngira, Dimiti, & Bryceson, 1983) and Indian (Thakur Kumar, Kumar, Mishra, & Pandey, 1988), kala-azar and for Panamanian cutaneous disease (Ballou, McClain, & Gordon, 1987), Sb at a dosage of 20 mg/(kg/d) was more effective at a cure rate greater than 90 % compared to Sb at a dosage of 10 mg/(kg /d), and it was recommended that the daily dose be 20 mg/kg. It is worth mentioning that systemic antimony is used for severe and complex lesions. Even though the exact mechanism of the antimonials is not clear, Lindoso, Costa, Queiroz and Goto (2012), have stipulated that the drug inhibits the activity of the glycolytic and oxidative pathways of the fatty acids for the reduction of ATP in the amastigotes. Also, the pentavalent form has been stated to utilize thiols from the parasite and the cell surface of the host and is reduced into the trivalent which, a more active and toxic form inside the macrophages (Alviano et al., 2012; Lindoso et al., 2012; Mohapatra, 2014). The metabolism of thiols with its high intracellular levels plays prominent roles in developing antimonial resistance. Thiol molecule increases the oxidative stress within the macrophages preventing antioxidant formation and reduction of pentavalent antimonials to trivalent form (Jain & Jain, 2013; Mohapatra, 2014). Inhibition of fatty acid oxidation and initiation of apoptosis and also induction of the DNA topoisomerase enzyme are other mentioned mechanisms that have been described (Alviano et al., 2012).

Table 1: Efficacy of pentavalent antimonial regimens for the treatment of leishmaniasis

Type of disease/ Study site	drug, dosage	no. of patients cured/ total no. of patients
Visceral		
Kenya	Sb, 20 mg/ (kg/d) for ≤ 28 d	21/21(100)
	Sb, 10 mg/ (kg/d) for ≤28 d	12/20(60)
India	Sb, 20 mg/ (kg/d) × 40 d	62/64(9
	Sb, 20 mg/ (kg/d) ×20d	51/63(81)
	Sb, 20 mg/ (kg/d) ×40d	45/61(74)
	Sb, 10 mg/(kg/d) ×20d	33/58(57)
Sudan	Sb, 20 mg/ (kg/d) × 30d	
	2562/3076(83)	
Cutaneous		
Panama	Sb, 20 mg/(kg/d) × 20d	19/19(100)
	Sb, 10 mg/(kg/d) ×20d	16/21(76)
Guatemala	Sb, 20 mg/(kg/d) ×20d	24/25(96)
Guatemala	MA, 20 mg/(kg/d) ×10d	19/21(91)
Mucosal		
Panama	Sb, 20 mg/(kg/d)×28	10/16 (63) *
Peru	Sb, 20 mg/(kg/d) ×28d	6/8(75)*
		2/21(10)$^{\Psi}$

Source; WHO, 2013, Key; MA= Meglumine antimoniate, stib=stibogluconate, Mild disease*, Severe disease $^{\Psi}$

Paromomycin (PM)

Paromomycin, is an amino glycoside antibiotic originally identified as an antileishmanial drug in the 1960s, which acts synergistically with antimonials *in vitro* (Berman, 2006). Like other amino glycosides, PM acts by impairing the macromolecular synthesis and alters the membrane properties of *Leishmania* (Sunder et al., 2007). The drug is licensed in Europe for the parenteral treatment of bacterial diseases for which aminoglycosides are typically used (Berman, 2006), with recommended dose is 15 mg/(kg × d) for 10 days.

Although PM differs from neomycin B only in the substitution of CH_2OH for CH_2NH_2 on one of the three sugar groups of neomycin B, PM has broad antiparasitic activity not shared by the neomycins or other aminoglycosides (Berman, 2006). As injectable, PM has been used as monotherapy for VL, of which attempts have been made to boost the efficacy of PM by administering it in combination with Sb. It is a cheap anti-leishmanial drug that needs to be administered in combination with another drug to achieve optimal efficacy (Berman, 2006).

Africans countries are in the process of switching from 30 days SSG to the WHO-recommended combination regimen of 17 days SSG-PM in those without HIV (MSF, 2012). The combination of PM plus pentostam, given for 20 days, cured 82 % of Indian VL patients (Thakur & Gothoskar, 1992), a cure rate comparable to that observed when pentostam is administered alone for twice as long as 40 days (MSF, 2012). Similarly, for VL in Sudan, PM and pentostam, administered at full daily doses for approximately half the usual duration of pentostam therapy, were equal in efficacy to pentostam

administered for the full time (Seaman, 1993). The use of half as much PM or Sb in combination resulted in less efficacy that was less than 70 % **(**Thakur, Bhowmick, & Dolfi, 1995). Injectable PM has been used less successfully for the treatment of cutaneous leishmaniasis. Even a high daily dose (22.5 mg/kg), given for 14 days, it cured only 50 % of patients in Colombia (Soto, Buffet, Grogl, & Berman, 1994) and similar results were seen in Belize (Hepburn, Tidman, & Hunter, 1994).

The combination of PM and Sb has also been used as therapy for more serious syndrome of diffuse cutaneous disease (Teklemariam et al., 1994). Although effective for visceral disease, Berman (1997) has established that PM monotherapy is probably not as effective as antimonial therapy in regions where *Leishmania* are susceptible to antimony. By its aminoglycoside nature, PM has the potential for renal toxicity and eighth cranial nerve toxicity (Berman, 1997). Although these side effects are rarely seen if PM is used as monotherapy at the recommended dosage, longer administration of PM or in combination with other agents may result in these side effects (Scott et al., 1992). It is registered in India, which was expected to be registration in Bangladesh and Nepal in 2011 and African countries in between 2011 and 2012 even though it has been suggested that no additional registrations are foreseen to be necessary in the near future (den Boer et al., 2011).

Table 2: Efficacy of parenteral paromomycin regimens for the treatment of visceral or cutaneous leishmaniasis

Type of disease/ Study site	Regimen	no. of patients cured/ total no. of patients
Visceral		
Kenya	15 mg/(kg/d)19d	15/19(79)
	15 mg/(kg/d)×14d+Sb(20 mg/kg//d)×19d*	20/23(87)
India	17 mg/(kg/d) × 20 d+Sb(20mg/(kg/d))×20d	18/22(82)
	Sb(20mg/(kg/d))×30d*	62/67(93)
Cutaneous		
Colombia	22.5 mg/(kg/d)×14d	15/30(50)
Belize	14 mg/(kg/d)×20d	10/17(59)
	Sb (20 mg/((kg/d)×20d*	15/17(88)

*** Comparison regimen, Source; Berman, 1997**

Amphotericin B and lipid associated amphotericin B

Amphotericin B is a pollen antibiotic that was commended as first line drug in India by National Expert Committee for Sb^V refractory regions of VL (Mishra, Kale, Prasad, Tiwari, & Singh, 2011). It is extracted from the filamentous bacteria, *Streptomyces nodusus*. It is the second line treatment drug and is capable of acting on both amastigotes and also promastigotes (Jain & Jain, 2013; Lindoso et al., 2012). The drug can perturb both parasitic and mammalian cells, but the selective lethality of Amphotericin B (Amp B) for parasitic cells is the result of its great affinity towards substituted sterols, such as ergesterol and cholesterol the major cell membrane sterols (Thakur et al.,

1988). It binds to cholesterol and forms pores in the cell membrane of the parasite leading to cell death as results of increased permeability and the leakage of cellular content (Oryan et al., 2014). Consequently, it may lead to generation of oxygen free radicals causing damage to cell, followed by cell death (Jain & Jain, 2013; Mohapatra, 2014).

Liposomal amphotericin B has been described as the safest and most effective drug because of its excellent therapeutic index and long half-life by far, allowing for ultra-short regimens (Chakravarty & Sundar, 2010; Bern, 2006). A single dose of 10 mg/kg AmBisome was recommended by the WHO as the preferred first-line treatment option for the Indian subcontinent (Matlashewski, Arana, & Kroeger, 2011; WHO, 2010). This regimen is ultra-rapid, extremely safe and highly efficacious and opened a new dimension for large-scale control programmes, of which Bangladesh, Nepal and India had agreed on a concerted effort to eliminate VL by 2015 (WHO, 2005). L-AmB is administered intravenously and must be stored and transported in a manner that ensures the vial is not exposed to temperatures over 25 °C (MSF, 2012). Its current cost remains an important barrier to treatment, nevertheless, there are many reasons to believe that L-AmB could soon become the mainstay of first-line treatment for all patients, either single dose or in combination with an oral drug (MSF, 2012). In India and Bangladesh, Médecins Sans Frontières, (MSF) has used short-course regimens of L-AmB (15-20 mg/kg total dose) with an initial cure rate of over 98 % with a very good safety profile (MSF, 2012). In 2010, WHO Expert Committee recommended L-AmB in a single dose or in short-course regimen as first-line therapy in South Asia (MSF, 2012). MSF is now working together with DNDi and other partners in a

clinical study evaluating effectiveness and feasibility in the field of single dose L-AmB (10 mg/kg) and combination regimens (L-AmB-miltefosine and miltefosine-paromomycin) (MSF, 2012). The results of this study will help countries in South Asia to update their treatment recommendations.

Table 3: Efficacy of regimens of amphotericin B (AmB) and of lipid-associated Amb for visceral leishmaniasis

Study site (Leishmanial resistance)	**drug, regimen**	**No. of patients cured /total no. of patients**
India (Sb and pentamidine)	AmB,1 mg/kg20 injections	298/300(99)
India	AmB, 1 mg/(kg/d)×20d	40/40(100)
India	AmB, 0.5 mg/kg× 14 injections;	40/40(100)
	Sb, 20 mg/ (kg/d) ×40d*	25/40(62)
India (Sb)	AmB, 0.5 mg/kg×14 injections;	59/60(98)
	Pentamidine, 4 mg/kg ×20 injections*	46/60(77)
Europe$^{\psi}$	L-AmB, 1 mg/(kg/d) ×21d$^{\lambda}$	10/10(100)
Europe	L-AmB, 1 mg/(kg/d) ×21d; 3 mg/kg ×10d	13/13(100)
Europe	3 mg/kg on d 1-4, 10	41/42(98)
	L-AmB, 3-5 mg/kg on d 1, 4, 11;	
Sudan	3-5 mg/kg on d 1, 4, 7, 9, 11, 14	29/32(91)
	L-AmB, 1.5 mg (kg/ d) ×21d	8/16(50)

Table 3 cont.

Europe	ABCD, 2 mg/(kg/d) ×10d;	14/16(88)
Brazil	2 mg/(kg/d) ×7d	3/11(27)
	2 mg/(kg/d) ×5d	10/10(100)
	ABLC, 3 mg/kg ×5 injections	9/9(100)
India (Sb)	3 mg/(kg/d) ×5d	9/10(100)
		21/21(100)
		4/4(100)

Key; ABCD = amphotericin B colloidal dispersion; ABLC = amphotericin B lipid complex; L-AmB = liposomal amphotericin B; AmB = *amphotericin B; NA = not available; Sb = antimony; * Comparison regimen; One patient was cured λ; Study of HIV infected patients ψ· Source; Berman, 1997

Miltefosine

Miltefosine was initially developed as an anticancer drug and is the first effective oral treatment for VL and the latest antileishmanial drug to enter the market (Croft & Coombs, 2003). It blocks *Leishmania* proliferation, alters phospholipid and sterol composition and activates cellular immunity. However, due to high cost and adverse side effects, medical advisors generally avoid miltefosine in their prescriptions (Sundar et al., 2003). It was developed by Zentaris (Frankfurt, Germany), in close cooperation with WHO/Special Programme for Research & Training in Tropical Diseases (TDR). Currently, it is manufactured by Paladin (Quebec, Canada), after the rights of production and marketing were obtained from Zentaris. Miltefosine is produced under Good Manufacturing Practices (GMP) standards that are equivalent to WHO GMP (MSF, 2012).

As the current price is relatively high compared with other treatments (Table 1), negotiations for new and reduced preferential prices are needed (MSF, 2012). The WHO will aim for a price per treatment similar to a treatment with generic SSG and Glucantime (around US$ 50/treatment), valid for all endemic low-income and middle-income endemic countries, and in an agreement to maintain the production for as long as is necessary (MSF, 2012).

Miltefosine is registered in India, Bangladesh, Nepal and Pakistan with agreements in place for registration in African countries (MSF, 2012). Despite the convenience of using miltefosine as an oral treatment, MSF in their 2012 report stated the drug is not the ideal. The drug has been noted as a teratogen and as such contra-indicated in pregnancy making it unusable in women of child bearing age without strict contraception and also requires up to 28 days of treatment (MSF, 2012).

Pentamidine

Pentamidine is the drug of choice for CL except for *L. mexicana* and can also be used as an alternative treatment for VL. Pentamidine hampers replication and transcription at the mitochondrial level in pathogen, and is the first drug used for the treatment of patient refractory to Sb^V (Jha, 1983). However, the efficacy of pentamidine has gradually declined over the years, which now cures only 70 % of patients producing serious adverse effects like shock, hypoglycemia and death in significant proportion (Thakur, Kumar, & Pandey, 1991). To circumvent the problem of clinical resistance to Sb in India, pentamidine has also been tried for the treatment of visceral disease (Berman, 1997).

The pentamidine regimen consisted of 4 mg/kg given three times per week until initial parasitological cure was achieved (Berman, 1997). Seventy-seven percent of patients were cured after 15 injections had been administered (5 weeks), and 94 % were cured after a total of 27 injections had been administered (Thakur et al., 1991), however, 21 % of these patients relapsed. Since a 99 % cure rate was seen in this region of endemicity in the early 1980s after 5-week courses of injections were administered (Jha, 1983), clinical resistance to pentamidine had been engendered by 1990s (Berman, 1997).

The rationale for using pentamidine to treat cutaneous disease was to try to avoid the toxicity and the duration of treatment associated with the use of Sb. For a regimen of pentamidine to be superior to a regimen of Sb, it would have to be less toxic, involve fewer injections, and be equally effective. The repeated administration of 4 mg/kg would be unattractive; therefore, the administration of 2 mg of pentamidine isethionate/kg every other day for 7 days was studied for the treatment of cutaneous disease in Colombia. To further decrease dosage, the same dose of pentamidine (2 mg/kg) was administered every other day for only 4 days, but the cure rate was 84 %, however, a higher dose (3 mg/kg).

Table 4: Price per leishmaniasis treatment (January 2010)

Compound	treatment regimen in days	drug cost in US$[a]
Visceral		
L-Amb 10mg/kg	1	126
L-Amb 20mg/kg	2-4	252
Amphotericin B deoxycholate	30	20
	1 mg/kg (alternating days)	
MF 100 mg/kg /day	28	65-150
PM 15 mg/kg /day	21	15
SSG 20 mg/kg/day	30	55.8
MA 20 mg/kg/day	30	59.3
L-Amb 5 mg/kg+MF100 mg/day	8	88.2-109.5
L-Amb 5 mg/kg+ PM 15 mg/kg/day	11	79
MF 100 mg/day+ PM 15 mg/kg/day	10	30.2-60.7
(SSG 20 mg +PM 15 mg)/kg/day	17	44
Cutaneous		
SSG systemic 20 mg/kg/day	20	37.2
SSG intralesional [b]	until lesion is healed	12
MA systemic 20 mg/kg/day	20	39.5
MA intralesional [b]	until lesion is healed	13.2
Pentamidine	up to 4 months	free
		(donation programme)

Source; Berman, 1997, Key; [a] for a patient weighing 35 kg, [b] intralesional treatment is commonly estimated at a third of the cost of systemic treatment, L-Amb= Liposomal amphotericin B, MF= Miltefosine, PM= Paromomycin, SSG= Sodium stibogluconate, MA= Meglumine antimoniate

Medicinal plants and leishmaniasis

Medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents (Nostro, Germanò, D'angelo, Marino, & Cannatelli, 2000). Phytochemicals naturally occur in medicinal plants in parts such as leaves, fruits and roots that have defence mechanism and protect the plant from various diseases. Phytochemicals comprise both primary and secondary compounds such as terpenoids, alkaloids and phenolic compounds (Krishnaiah, Sarbatly, & Bono, 2007). Terpenoids display a number of essential pharmacological activities that include anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities (Mahato & Sen, 1997), whereas alkaloids have been used as anaesthetic agents (Kappers, Aharoni, van Herpen, Luckerhoff, & Dicke, 2005). Table 5 compares some leishmanial activities of some plants and their fractions that have been reported some 10 years ago (Tiuman, Santos, Ueda-Nakamura, Filho, & Nakamura, 2011).

Table 5: **Plant extracts, fractions and isolated compounds evaluated against *Leishmania***

Plant species	Extracts/compounds	*Leishmania sp.*	IC_{50} (µg/mL)
Aloe nyeriensis	Methanolic	*L. major*	68
	Aqueous	*L. major*	53.3
Annona coriacea	Total alkaloids	*L. chagasi*	41.6
Annona crassiflora	Total alkaloids	*L. chagasi*	24.9
Annona muricata	Ethyl acetate	*L. amazonensis*	25
Guatteria australis	Total alkaloids	*L. chagasi*	37.9

Table 5 cont.

Polyalthia suaveolens	Methanolic	*L. infantum*	1.8
Rollinia exsucca	Hexane	*L. amazonensis*	20.8
Rollinia pittieri	Hexane	*L. amazonensis*	12.6
Xylopia aromatica	Methanolic	*L. amazonensis*	20.8
Acacia tortilis	Aqueous	*L. major*	52.9
Albizia coriaria	Aqueous	*L. major*	66.7
Laetia procera	Casearlucine A	*L. amazonensis*	11.1
Scaevola balansae	Dichloromethane	*L. amazonensis*	8.7
Premna serratifolia	Dichloromethane	*L. amazonensis*	4.4
Asparagus racemosus	Methanolic	*L. major*	58.8

Source: Tiuman et al., 2011

Utility of natural products in drug discovery and development is not surprising as many of medicinal plants such as barks of *Cinchona calisaya*, *Strychnos pseudoquina* and *Remijia ferruginea* and roots and leaves of *Deianira erubescens* which have been historically used against different parasitic diseases (Mishra, Singh, Srivastava, Tripathi, & Tiwari, 2009). Ancient records as well as recent literature reports have established the effectiveness of natural products as potentially rich sources of new and selective agents for the treatment of important tropical diseases caused by protozoans and other parasites (Wright & Phillipson, 1990).

In 1970s, artemisinin, an important antimalarial drug was known from traditional Chinese medicine *Artemisia annua* and since then many artemisinin derivatives have been prepared and evaluated in various pre-clinical and clinical trials to use for the treatment of malaria (Mishra et al., 2011). The

interest in plants products, specifically in medicinal plants or their extracts, surfaced all over the world due to the belief that many herbal extracts have been extensively used by native populations to treat leishmaniasis and scientific reports have demonstrated their potential (Chan-Bacab et al.,2003; Kvist, 2006). Natural products literature provides a growing research on plant derived antileishmanial agents and several natural products have thus been discovered with excellent activity against *Leishmania* parasites, however, not many of them have been clinically evaluated in studies or projected to reach the clinical applications in near future.

A focus to cover the entire formal and constant research on leishmanicidal natural products from the mid-1980 to 2010 with special attention on structure-activity relationship (SAR) based activity and mechanism of action is being considered (Mishra et al., 2009). Betulinic acid, (3β-hydroxylup-20(29)-en-28-oic acid) is a naturally occurring pentacyclic lupine type triterpenoid which exhibits a variety of biological properties highly disseminated across the plant kingdom (Cichewicz & Kouzi, 2004). Some of the known biological activities include inhibition of human immunodeficiency virus (HIV) (Fujioka et al., 1994), and anti-bacterial (Chandramu et al., 2003; Fujioka et al., 1994). Betunilic acid also acts as anti-malarial (Bringmann et al., 1997), anti-inflammatory (Alakurtti, Mäkelä, Koskimies, & Kauhaluoma, 2006; Bernard, Scior, Didier, Hibert, & Berthon, 2001; Huguet, Recio, Manez, Giner, & Rios, 2000; Mukherjee, Saha, Das, Pal & Saha, 1997), anthelmintic (Enwerem, Okogun, Wambebe, Okorie & Akah., 2001), antinociceptive (Kinoshita et al., 1998), anti-HSV-1 (Ryu et al., 1993, 1992), and anti- cancer activities (Fulda & Debatin, 2000; Fulda, Jeremias, Steiner, Pietsch, &

Debatin, 1999; Gambacorti-Passerini & Formelli, 2002). The *Betula* spp., *Ziziphus* spp., *Syzygium* spp., (Chang et al., 1999), *Diospyros* spp., (Singh & Sharma, 1997), *Paeonia* spp. (Lin, Ding, & Wu, 1998) and *E. ivorense* (Armah et al., 2015) are a few of the plants species from which betulinic acid has been isolated from, in good measure. Indole alkaloids such as dihydrocorynantheine**,** corynantheine and corynantheidine isolated from the bark of *Corynanthe pachyceras*, are the respiratory chain inhibitors exhibiting IC_{50} of 3 µM against *L. major* whereas pleiocarpine isolated from stem bark of *Kopsia griffithii*, shows *in vitro* antileishmanial activity with an IC_{50} < 25 µg/mL against *L. donovani* promastigotes. Xylopine, an aporphine alkaloid isolated from *Guatteria amplifolia* has shown activity against promastigotes of *L. mexicana* with an IC_{50} of 3 µM and *L. panamensis* with IC_{50} of 6 µM (Correa et al., 2006). Unonopsine, a dimeric aporphine alkaloid isolated from the *Unonopsis buchtienii* displays antileishmanial activity (IC_{100} value 25 µg/mL) against *L. donovani* promastigotes (Waechter, Hocquemiller, Bories, Munoz, & Fournet, 1999). Others such as *Albertisia papuana, Pseudoxandra sclerocarpa, Gyrocarpus americanus* and *Caryomene olivasans*, have been reported to display activity against *L. donovani, L. braziliensis* and *L. amazonensis* with IC_{100} nearly 50 µg/mL (Mishra et al., 2009).

Erythrophleum ivorense

E. ivorense belongs to the family fabaceae. Members of this pan tropical woody genus are represented from North-East Asia (*E. fordii, E. succirubrum, and E. densiflorum*), Australia (*E. chlorostachys*), Madagascar (*E. couminga*) and Africa (*E. ivorense, E. suaveolens, and E. africanum*) (Duminil et al., 2013). This species is found in evergreen forests and requiring

high rainfall. It is a large tree found growing in tropical regions in Africa including Ghana, Cote d'Ivoire and Liberia. It is also described as the 'ugly' plant growing up to 40 m tall, usually bole cylindrical, but it may occasionally be fluted at the base, with or without buttresses at old age (Adu-Amoah et al., 2014). It is called "potrodom" among the Akans in Ghana (Armah et al., 2015), "Epo-obo" among Yoruba people in Nigeria and referred by several other names in West Africa countries such as 'forest ordeal tree', 'red water tree' and 'sasswood tree' (Wakeel et al., 2014). *E. ivorense* is among plants that have been used to treat brain disorders such as epilepsy (Schachter, 2009). The stem bark has also traditionally been used in the treatment of convulsive disorder, emesis, pain, swelling, smallpox and as anthelminthic and laxative (Oliver-Bever, 1986). Ethanol extracts of the stem bark of *E. ivorense* as demonstrated by Adu-Amoah et al. (2014) in mice models showed that it has anticonvulsant and sedative properties while the methanol extract has been found to possess anti-microbial and cytotoxic effects (Wakeel et al., 2014). Aqueous extracts of other members in the family that include *Acacia tortilis* and *Albizia coriaria* as indicated in table 5 have shown good activity on *L major* promastigotes. The stem-bark of *E. ivorense* is widely known to contain diterpene-alkaloids, cassaine, cassaidine, cassamidine coumidine, erythropillamine and erythrophleguin all of which are important compounds with several potencies against disorders, emesis, pain, edema, smallpox, and laxative and as anti-helminthic (Oliver-Bever, 1986).

Omphalocarpum ahia

The members of genera *Omphalocarpum* are classified commonly as navel fruit and belong to the family sapotaceae. It is endemic to tropical Africa

and comprises nearly 7 species closely related to the genus *Tridesmostemon* from central Africa. It is a tall tropical African tree present in Ghana, Sierra Leone, Gabon, Nigeria, Cameroon, Liberia and Angola. In Africa, plants of the genus are prepared for various purposes and have been used in traditional medicine the treatment of headaches constipation, fever, rheumatism elephantiasis and wounds from skin diseases (Betti, 2004). A decoction of the bark of *O. ahia* is used in the treatment of malaria while the mixture of leaves and barks are used for treating yaws. Also the bark of the stem is used as antihelminths. A closely related plant, *O. elatum* is used to treat yaws, coughs, and constipations and also used as a purgative. Phytochemical investigations by Baliga, Pai, Bhat, Palatty and Boloor (2011), revealed the presence of alkaloids, flavonoids, saponins and triterpenoids from *O. procerum*. The activity of crude extracts from *O. procerum* against protozoans such *T. cruzi*, *L. donovani* and *P. falciparum* had been discussed previously by Orhan in 2010. In a more recent development, six pure compounds have been isolated from the fruit pericarp of *O. procerum* and include one new fatty acid triterpnoids, procerenone (Ngamgwe, et al., 2014). It is interesting to know that this class of secondary metabolite has been isolated from other members of the genera of sapotaceae and could be considered as a chemotaxonomic marker (Wandji et al., 2002). The six compounds were tested for their biological activity against *T. cruzi, L. donovani, P. falciparum* and *T. brucei rhodesiense* which showed weak to moderate activity against the tested protozoans with IC_{50}s that ranged between 9 and 80 µg/mL (Ngamgwe, et al., 2014).

Anthostema aubryanum

A. aubryanum is a monoecious shrub that comprises 3 species, 2 in mainland Africa and 1 in Madagascar, closely related to dichostemma. The plant occurs in evergreen forests, wooded savannah and equally widespread in its distribution. A latex present in the bark, young shoots, leaves, flowers and fruit is very toxic, bitter and is able to cause blisters on the skin. It is harmful to mucous membranes and there by capable of causing blindness. The macerated bark is used in the treatment of all acute illnesses and also to expel intestinal parasites, kidney problems, oedema, impotence and mental illness while crude water extracts of the stem bark of a *A. senegalense* has shown strong *in vitro* antihelminthic activity against *Haemonchus contortus* (Grewal, 2000). In Sierra Leone, the young leaves are ground with flour and the dried paste taken as laxative. A crude stem bark extract has exhibited significant activity against *L. donovani* as well as moderate antibacterial and antifungal activities (Abreu et al., 1999). Not much is known about the active properties of *Anthostema* species, yet it is known that phorbol esters with phytochemical activity have been shown in *A. aubryanum*

Coelocaryon oxycarpum

C. oxycarpum is a member of the myristicaceae family, with nut meg as the most popular member of the family. The family is tropical with appreciable representative in central Africa and the Americas. Members of the family are reported to possess antifungal and antimicrobial action against *Streptococcus mutans*. Other members of the genera include *C. botryoides, C. preussii* and *C. sphaerocarpum* although information regarding any of these species is undeniably scanty. The sap of the plant is used for laxative purposes

by local folks and a polyketide derivative has been isolated from a member of the family and found to show moderate cytotoxicity against Myco*bacterium tuberculosis* and *Plasmodium falciparum* (Rangkaew, Suttisri, Moriyasu, & Kawanishi, 2009).

Extraction methods

The development of phytomedicine requires some basic considerations such as the choice of extraction method which would undoubtedly have an effect on the final product. Since the end product should necessarily be pharmacologically viable, care should be taken such that the end products have properties such as potency, selectivity, duration of action, safety/toxicology assessments and pharmacological properties, good aqueous solubility, and good stability. Methods such as maceration, ultrasound assisted extraction(UAE), microwave assisted extraction (MAE) and accelerated solvent extraction (ASE) all employ the use of solvents in their procedures and are as such very much affected by the type of solvent used in the process. It has been however noted by Trusheva, Trunkova and Bankova, (2007) that maceration, UAE and MAE methods are not affected by the volume of solvent. Thus, the biologically active compounds in in the poplar type propolis at ratio (1:10w/v) suggesting that the use of solvents at large volumes are not significant. Maceration is thought of as a more convenient applicable and less costly (Vongsak, Sithisarn, Mangmool, Thongpraditchote & Wongkrajang, 2013). Chemical waste is however a challenge with maceration in comparison with MAE and UAE which have been recently referred to as 'green method' (Dhanani, Shah, Gajbhiye & Kumar, 2013).

Maceration method

This type of plant extraction involves soaking either powder or coarse plant materials into a solvent in a covered container and allowed to stand in room temperature for a period of time and shaking it once in a while (Handa, Khanuja, Longo & Rakesh, 2008) to soften and break the plant's cell walls so that soluble phytochemicals are released. The mixture is then strained by filtration. Heat is then applied by convection and conduction and as such, the choice of solvent used in this method will determine the kind of compounds extracted from the plant materials (Azwanida, 2015).

Soxhlet or hot continuous extraction

Finely grounded plant materials are put into spongy/permeable bags usually made from strong filter papers or rather cellulose and placed in the spongy bags in the soxhlet apparatus. The solvent to be used for the extraction process is placed at the bottom chamber of the soxhlet apparatus which vaporizes into the sample sponge, condenses in a condenser and drip back. Upon reaching the siphon arm the liquid content is emptied into a bottom flask while the process repeats itself.

Ultrasound assisted extraction

This method of extraction is also known as sonication and involves the usage of ultrasounds ranging from 20-2000 khz (Handaet al., 2008). The effect of sound cavitation from the ultrasound rises to the surface contact between solvents and plant materials and the permeability of the plant cell walls. The plant cell walls are which are subjected to ultrasound are therefore disrupted by both physical and chemical properties and in that sense facilitate the release of compounds (Dhanani et al., 2013). This method of extraction is ideal for

use in both small and commercial scale extraction of phytochemicals (Azwanida, 2015).

Accelerated solvent extraction

Compared to maceration and soxhlet, this method is one of the most efficient forms of solvent extraction in that, it consumes very little solvent in its process. In ASE, the plant materials are packed with inert material to prevent the samples from clustering together to block the system tubing (Rahmalia, Fabre, & Mouloungui, 2015). ASE packed cells includes layers of sand-plant materials mixture in between cellulose filter paper and sand layers. ASE also uses automated technology to control temperature and pressure for every plant sample requiring barely an hour to complete each extraction process. The outcome of a product in ASE is solvent dependent.

Superficial fluid extraction

In 2010, Patil & Shettigar, described the (SFE) method as the most technologically advanced system. It is also in other texts as dense-gas sharing physical properties of both gas and liquid at its critical point. Temperature and pressure are some factors that are able to determine and push substances into their critical points. Even though the system behaves more like a gas, it does have salvating characteristics of liquids. The advantages of SFE are, the versatility it offers in handpicking the constituents you desire to extract from a given material and the fact that your end product has virtually no solvent residues since all the CO_2 evaporates completely (Azwandi, 2015). There are many other gases and liquids that are highly efficient as extraction solvents when put under pressure (Patil & Shettigar, 2010).

CHAPTER THREE

MATERIALS AND METHODS

Plant materials

Identification, collection and authentication of plant materials

Samples of *O. ahia* (Sapotaceae), *E. ivorense* (Fabaceae), *A. aubryanum* (Euphorbiaceae) and *C. oxycarpum* (Myristicaceae) were collected from Cape Coast in the Central Region of Ghana between the months of April 2012 and August 2013. The plants were authenticated by Botanists in the School of Biological Sciences, University of Cape Coast and the Department of Pharmacognosy, Kwame Nkrumah University of Science and Technology. Voucher specimen numbers have been assigned to each plant and deposited at the herbarium of School of Biological Sciences for future reference; *O. ahia*, BHM/Omph/018A/2014, *E. ivorense*, BHM/Eryth/017R/2014, *A. aubryanum*, BHM/Anth/019A/2014 and *C. oxycarpum*, BHM/Coel/O16O/2014.

Preparation of crude extracts

The procedure for the preparation of crude extracts was adopted from the work of Wadood et al. *(*2013). The root barks of *E. ivorense* and *C. oxycarpum*, leaves of O. *ahia* and stem bark of *A. aubryanum* collected were carefully separated from other morphological parts of the plants and washed clean over running water, to remove dust particles and other water soluble impurities settled on them. The barks of roots and stems of *E. ivorense* and *A. aubryanum* respectively, were further cut into smaller pieces. The plant

samples were then air dried for three weeks and afterwards pulverized into fine powder using a miller at the Chemistry Department of University of Cape Coast and stored afterwards in polythene bags for later use. The powdered extracts before use were weighed, keeping record of the weight of each powdered extract and macerated in 70 % ethanol for three days in round bottom flasks. Afterwards, the ethanol was decanted and filtered using Whattman filter papers. The filtering was done to further remove any debris suspended in the extracts. The filtrates were concentrated to a semi solid paste with a rotary evaporator regulated at 60 °C. The pastes were transferred into crucibles and kept in desicators which had been activated by heating silica gels into them to completely dry the extracts. The now dried extracts were finally weighed and recorded. Four fractions of *E. ivorense* were obtained using three different solvents for extraction; petroleum ether, methanol and ethyl acetate.

Table 6: Plants analysed for their antileishmanial activities

Scientific name	Local Akan name	Parts used	Family
E. ivorense	Potrodom	Root bark	Fabaceae
O. ahia	Duapompo	Leaves	Sapotaceae
C. oxycarpum	Abruma	Leaves	Myristicaceae
A. aubryanum	Kyirikusa	Stembark	Euphorbiaceae

Source: Fieldwork, Anning (2016).

Phytochemical tests

The phytochemical analysis for the various plant parts were performed according to Wadood, et al. (2013) and Nizam, Sajid, Sajid, & Yasser, (2013).

Test for Saponin

Half a gram of the crude extracts were diluted in 2 mL of distilled water and shaken rigorously for three minutes in a graduated cylinder. The formation of a stable layer of foam that is about 1 cm confirmed the presence of saponins.

Test for Alkaloids (Wagner test)

Half a gram of the crude extracts were dissolved in 5 mL of distilled water followed by filtration. The filtrate was then treated with Wagner's reagent and the formation of a red brown precipitate was looked out for to indicate the presence of alkaloids.

Test for flavonoids

An amount of 5 mL of dilute ammonia solution was added to a portion of the aqueous filtrate of extract followed by addition of concentrated Sulphuric acid. Appearance of yellow coloration indicated the presence of flavonoids.

Test for tannins

To half a gram of the extracts, 1 mL of 5 % ferric chloride was added. Formation of greenish black color indicated the presence of tannins.

Test for glycosides

To 1 mL of extracts, 1.5 mL of chloroform and 10 % ammonia solution was added. Pink colour formation indicated the presence of glycosides.

Test for triterpenoids

To 1.5 mL of the extracts, 1 mL of Libemann–Buchard Reagent (acetic anhydride + concentrated sulphuric acid) was added. Formation of blue green color indicated the presence of triterpenoids.

Test for anthraquinons

A 0.5 g of each extract was boiled with 10 mL sulphuric acid and filtered while it was still hot. The filtrate was shaken with 5 mL of chloroform. The chloroform layer was then pipetted into another test tube and 1 ml of dilute ammonia was added. A colour change was observed in the resulting solution.

Test for phytosterols

To 0.5 mL of the extracts, 2 mL of cold acetic acid and few drops of 5 % ferric chloride added. This was under layered with 1 mL of concentrated sulphuric acid. Brown ring formation at the interface indicated the presence of steroids.

Anti-leishmanial assay

Field collection of *Leishmania* parasites

The parasites were collected from Ho, in patients who had the infection with visible sores on the scalp and around the arms by Dr. Kwakye-Nuako of the Department of Biomedical Sciences, University of Cape Coast. The clinical aspect of the study has had an approval with protocol ID MS-Et/M.6.1-P.3/2006-07. The sites of infection were thoroughly cleansed using gauze soaked in 70 % alcohol, making sure that the alcohol did not enter the open sores. The parasites were aspirated from the swollen edges of the lesions using 1.5 mL syringes fitted with short syringe that contained 0.5 mL sterile

normal saline. The sterile saline water was pushed in and out of the swollen edges several times, aspirating as much liquid as possible and the content emptied into a flask that contained Novy-McNeal-Nicolle (NNN) transport media. Culture flasks were labelled with codes and dates.

Using strict aseptic conditions in the laboratory, the aspirates suspected to contain the parasites were emptied into M199 in as many culture flasks as were available making sure to use only a small volume of media in each flask. This was done to increase the chances of growth of the parasites. The flasks were tightly closed and incubated between 22 and 25 °C. The cultures were subsequently examined regularly in aseptic conditions in at least every two days. The primary cultures that remained negative after two weeks were sub passaged into fresh M199 and incubated for a week after which those that still remained negative were discarded. Cultures that were positive showed active growth of promastigotes by multiplying in number and were as such cryopreserved in -86 °C freezer for later use. The parasites isolated from the Ho were identified by Kwakye-Nuako et al., (2015) and designated as *L. gh. L. donovani* was a gift from the Department of Parasitology at the Noguchi Memorial Institute of Medical Research (NMIMR).

Preparation of Culture Medium 199 Media

To every 500 mL of M199 media, 10 % (50 mL) of foetal bovine serum (FBS), 1 % (5 mL), basal medium eagle (BME) vitamin and 0.25 % (1.25 mL) gentamicin were added. This was supplemented with 5 mL of urine and the whole solution filtered using a suction pump in an aseptic environment. The prepared media was labelled with all the constituent

ingredients including the date of preparation and stored in a fridge at 4 °C for later use.

Cryomedia and cryopreservation

Leishmania promastigotes (*L. gh* and *L. donovani*) actively dividing in culture were harvested for cryopreservation. Late log phase growing promastigotes are preferred since they survive freezing and thawing better. The minimum concentration of parasites was $1x10^6$ as concentration lower than this would take longer times to re-establish in culture. To prepare a 20 mL volume of cryomedia, 15 % of glycerol of the total volume of 20 mL was used. Thus 3 mL of glycerol is gently poured into 17 mL of M199 and mixed thoroughly. The glycerol serves as cryoprotectant in the extreme cold in which the promastigotes will be stored. To cryopreserve *Leishmania* promastigotes, the 20 mL of cryomedia was added drop wise into the 20 mL of parasites in the culture flask. This was done so that the parasites could adjust to the new environment being exposed to and not die out. The cryoprotected samples were then dispensed into 2 mL NUNC cryo tubes making sure not to overfill them. The tubes were then labelled, sealed and stored in -86 °C freezer for later use.

Anti leishmanial activity of treatment regimens

The stabilates were thawed by rapidly rubbing NUNC cryo tubes in gloved hands to provide some warmth. The thawed parasites were transferred aseptically into fresh M199 supplemented with 10 % FBS and then inspected under the microscope with X40 power looking out for motility of the promastigotes which serves as a good indicator for viability. The cultures were checked daily for parasite viability and those that were not showing motility

after 72 hours were sub cultured again into new M199 media to eliminate any possible glycerol residues.

A two fold serial dilution of the treatment regimen was performed to achieve concentrations of 500, 250, 125, 62.5, 31.3 and 15.6 µg/mL. These concentrations were achievd after a series of trials at higher concentrations. A 0.001 g of crude ethanol extract of *E. ivorense* was weighed into 1.5 mL Eppendorf tube and dissolved completely in 20 µL (1 %) of dimethyl sulfoxide (DMSO) and consequently topped up with 980 µL of M199 supplemented with 10 % FBS. Using a micropipette, 1 mL of M199 diluent was dispensed to all the wells on the first column of the microwell plate. The micropipette was then used to transfer 1 mL of the extract solution to the first well. The micropipette was used to mix the diluent and extract solution by drawing up the solution and expelling again for three times. Afterwards, 1 mL of the extract solution and M199 was dispensed to the second well with the same tip to carry out the first two-fold serial dilution. A second two fold serial dilution was carried out and the series of two-fold dilutions carried out until the last well. In the last well 1 mL of the solution is discarded so that the final volume in each microwell plate was 1 mL. Finally, 117,000 promastigotes of *L. gh* were dispensed into each microwell plate and then incubated at 25 °C. Each dilution process was carried out in triplicates. The procedure was repeated for all the plant extracts. Parasite growth and/or inhibition were observed by counting routinely on 8, 12, 24, 48 and 72 hours after incubation.

Two negative controls were set up for the experiment. In one well, 1mL of M199 diluent was dispensed after which 117,000 promastigotes were added and mixed gently in the well. For the second negative co control, 20 µL

of DMSO was mixed thoroughly in 980 µL of M199. The mixed solution was then dispensed into a well prefilled with 1 mL M199 diluent. The new solution was then mixed evenly eventually discarding 1 mL of the solution. To this solution also, 117,000 promastigotes were added and mixed.

For a positive test control, amphotericin B was used as the standard antileishmanial drug. A 0.001 g of amphotericin B was weighed into a 1.5 mL Eppendorf tube and dissolved in 20 µL DMSO and 980 µL of M199. The 1 mL of the extract solution was then dispensed into a microwell plate prefilled with 1 mL of M199 diluent and then mixed thoroughly discarding 1 mL out of the well. An amount of 117,000 promastigotes were dispensed into the solution finally. The experiment was repeated using the treatment against *L. donovani* as described above.

Counting *Leishmania* promastigotes

The haemocytometer was used for all cell counting. Before cell counting, the haemocytometer was prepared and made ready for the counting by wiping its entire surface clean using 70 % ethanol. The shoulders of the haemocytometer were moistened and a cover slip was fixed using gentle pressure. The parasite suspension to be counted was mixed by gently swirling the culturing flasks and 10 µL of the parasites immediately is taken using a pipette and dispensed into an Eppendorf tube. This was then mixed evenly and gently with 10 µL of 4 % formalin. Again, 10 µL of this new parasite/formalin solution was drawn using a pipette and carefully filled the haemocytometer. By capillary action the sample filled the edges of the haemocytometer. The grid lines of the haemocytometer were focused using the X10 objective of the microscope. Finally the parasites were counted using objective lens.

Isolation of compounds from *E. ivorense*

The bioactive compounds present in the crude extract of *E. ivorense* were isolated from the Department of Pharmacognosy, KNUST. This was done after cold macerating 1.2 kg of the powdered air-dried root bark of *E. ivorense* with 70 % ethanol for 72 hours. The resulting extract was then filtered and concentrated under reduced pressure at 40 °C to give the crude extract in a yield of 8.7 % (w/w). A 100 g of this extract was partitioned with 5 L each of petroleum ether, ethyl acetate, and methanol, to yield 15, 36.3 and 41.2 g fractions respectively. The 25 g ethyl acetate fraction was further subjected to column chromatography via silica gel (70-230 mesh) and eluding with a gradient of petroleum ether-EtOAc that yielded five major fractions, which are pet-ether/EtOAc 9:1; 0.72 g, pet-ether/EtOAc 4:1; 0.26 g, pet-ether/EtOAc 7:3; 4.2 g, pet-ether/EtOAc 3:2, 5.9 g and pet-ether/EtOAc 1:4; 10.8 g. The fractions referred to as fractions I, II, III, IV and V respectively. Both fractions I and II were combined on the basis of their TLC profile and repeated with much smaller silica gel column chromatography as previously mentioned to yield 400 mg of **compound 1**. Compound 1 was further recrystallized from acetone to give colourless needle crystals. **Fraction III** gave an oily yellow mass which was dried and washed several times with petroleum ether to produce 200 mg of **compound 2**, an amorphous powder. **Fraction IV** was also column chromatographed over silica gel as mentioned, and eluted with pet-ether and EtOAc mixtures of 7:3, 1:1 and 2:3. The eluted fractions were combined with pet-ether/EtOAc 7:3 and concentrated to give **compound 3** in a yield of 300 mg. X-ray analysis, revealed the colourless needle **fraction 1** (mp 187-189 °C) as Erythroivorensin, **fraction 2** as

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid and **fraction 3** as Eriodictyol ((2S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydro-4H-chromen-4-one,3).

Data analyses

All statistical analyses were performed using GraphPad Prism version 5 for Windows and Microsoft Excel 2010 worksheet. The experimental set ups were all carried out in triplicates and as such the mean inhibitory concentrations to the parasites were obtained from average of the triplicates. The results were expressed as mean±standard error of mean. The activity of the treatments and both positive and negative controls at various concentrations were compared using one way ANOVA with Tukey's post-test, setting P values of <0.05. Concentrations for 50 % of the maximal effect (IC_{50}) for each drug were determined by using an iterative computer least squares method, following non-linear regression (3-parameter logistic) equation. The active extracts that showed IC_{50}s that were less than 50 µg/mL were selected as active against promastigotes form as expressed by Garcıa, Monzote, Scull, and Herrera (2012).

CHAPTER FOUR

RESULTS

Phytochemical analysis

The phytochemical analyses of crude extracts from four different plants revealed the presence of active curative biological constituents. Important biological phytochemicals that include saponins, tannins, steroids, anthraquinons, phenols, alkaloids, flavonoids, triterpenoids and glycosides were present in either one or all of the samples. *E. ivorense* had the most biological constituents tested for; only glycosides and triterpenoids were absent while *O. ahia* had the least biological constituents with only saponins and tannins present. *A. aubryanum* followed next in active constituents lacking anthraquinons, glycosides and triterpenoids. Present in *C. oxycarpum* were saponins, tannins, steroids and anthraquinons. The results of the phytochemical tests have been summarized in Table 7.

Table 7: Phytochemical analyses of *Erythrophleum ivorense, Anthostema aubryanum, Coelocaryon oxycarpum* and *Omphalocarpum ahia*

Chemical Constituents	Inference			
	C. oxycarpum	*E. ivorense*	*A. aubrynum*	*O. ahia*
Alkaloids	-	+	+	-
Flavonoids	-	+	+	-
Saponins	+	+	+	+
Tannins	+	+	+	+
Steroids	+	+	+	-
Anthraquinone s	+	+	-	-
Glycosides	+	-	-	-
Triterpenoids	-	-	-	-

Key: (+) Present, (-) Absent

Source: Fieldwork, Anning (2016).

Anti-leishmanial activities

Field collection of Leishmania parasites

Promastigotes of *Leishmania* were observed in M199 culture after several days of incubation at 20 °C. These promastigotes were further sub cultured for and used for the *in vitro* biological activities. The observations were made under the inverted microscopes through the culture flasks.

***In vitro* antileishmanial activities of treatment regimens**

The growth inhibitions of all crude extracts were assessed on the promastigotes of both *L. donovani* and *L. gh*. Percentage inhibitions at the highest concentration of 500 µg/mL were recorded between the values 90 and

100 % and 70 and 80 % respectively for *L. donovani* and *L.gh*. The most inhibition came from the activity of *E. ivorense* on the promastigotes of *L. gh* with a growth inhibition of 67 % even at the least concentration of 15.6 µg/mL of the treatment while a complete growth inhibition of 100 % at 500 µg/mL was observed as presented in Table 12. *O. ahia* had the least inhibition on either of the promastigotes that is 2 and 71 % at the least and highest concentrations respectively. However, no complete inhibition at any concentrations was observed in any other crude extracts aside *E. ivorense*.

The inhibitions of the treatments on *L. gh* was similar to the treatment on *L. donovani*, however, crude *E. ivorense* gave a rather high inhibition among all the crudes and the fractions at the lowest concentration, with an inhibition of 67 % but the inhibition of all other crudes and fractions were less than 40 %. The promastigotes of *L.gh* were very sensitive to the crude extracts and the fractions at the highest concentration just as promastigotes of *L. donovani*. Crude extracts against *L. gh* and *L. donovani* gave inhibitions greater than 70 %. This was shown by the large percentages of the inhibitions. The crude extracts of leaves *E. ivorense* and *C. oxycarpum*, root and stem barks of *O. ahia* and *A. aubryanum* respectively, demonstrated different *in vitro* activities against the promastigotes of *L.gh* with IC_{50}s between 6 and > 100 µg/mL respectively. The % inhibitions of all the crude extracts on the promastigotes of *L. gh* have been enumerated in Tables 8-15.

Table 8: The growth inhibitions of the crude extracts of the leaves of *E. ivorense* on the promastigotes of *L. donovani* after 72 hours

E. ivorense (µg/mL)	% inhibition
15.6	17
31.2	21
62.3	23
125	52
250	67
500	88

Source: Fieldwork, Anning (2016).

Table 9: The growth inhibitions of the crude extracts of the leaves of *O. ahia* on the promastigotes of *L. gh* after 72 hours

O. ahia (µg/mL)	% inhibition
15.6	12
31.2	12
62.3	15
125	21
250	45
500	82

Source: Fieldwork, Anning (2016).

Table 10: The growth inhibitions of the crude extracts of the leaves of *A. aubryanum* on the promastigotes of *L. donovani* after 72 hours

A. aubryanum (µg/mL)	% inhibition
15.6	1.9
31.2	8
62.3	10
125	27
250	55
500	73.4

Source: Fieldwork, Anning (2016).

Table 11: The growth inhibitions of the crude extracts of the leaves of *C. oxycarpum* on the promastigotes of *L. donovani* after 72 hours

C. oxycarpum (µg/mL)	% inhibition
15.6	2
31.2	6
62.3	8
125	29
250	45
500	70

Source: Fieldwork, Anning (2016).

Table 12: The growth inhibitions of the crude extracts of the leaves of *E. ivorense* on the promastigotes of *L. gh* after 72 hours

E. ivorense (µg/mL)	% inhibition
15.6	67
31.2	78
62.5	88
125	90
250	92
500	100

Source: Fieldwork, Anning (2016).

Table 13: The growth inhibitions of the crude extracts of the leaves of *O. ahia* on the promastigotes of *L. gh* after 72 hours

O. ahia (µg/mL)	% inhibition
15.6	2
32.1	4
62.5	17
125	20
250	29
500	71

Source: Fieldwork, Anning (2016).

Table 14: The percentage inhibitions of the crude extracts of the leaves of *C. oxycarpum* on the promastigotes of *L. gh* after 72 hours

C. oxycarpum (µg/mL)	% inhibition
15.6	10
31.2	50
62.5	70
125	73
250	95
500	96

Source: Fieldwork, Anning (2016).

Table 15: The growth inhibitions of the crude extracts of the leaves of *A. aubryanum* on the promastigotes of *L. gh* after 72 hours

A. aubryanum (µg/mL)	% inhibition
15.6	4
32.1	35
62.5	57
125	71
250	82
500	90

Source: Fieldwork, Anning (2016).

Fractionation and isolation of compounds from *E. ivorense*

E. ivorense was the most effective anti-leishmanial agent among the crude extracts tested. This necessitated the fractionation and isolation of compounds from the plant extract. The fractions obtained were methanol, ethyl acetate and petroleum ether fractions. The methanol and ethyl acetate

fractions exhibited significant (P<0.001) anti-leishmanial activity which were greater than the other extracts as shown in figure 6. The activity of the methanol fraction was comparable to the crude *E. ivorense* extract. The ethanol fraction was the most active fraction. The ethyl acetate fraction was also more active against the *Leishmania* promastigotes than the petroleum ether fraction. Subsequently, three pure compounds isolated from the ethyl acetate fraction were eriodictyol, betulinic acid and erythroivorensin.

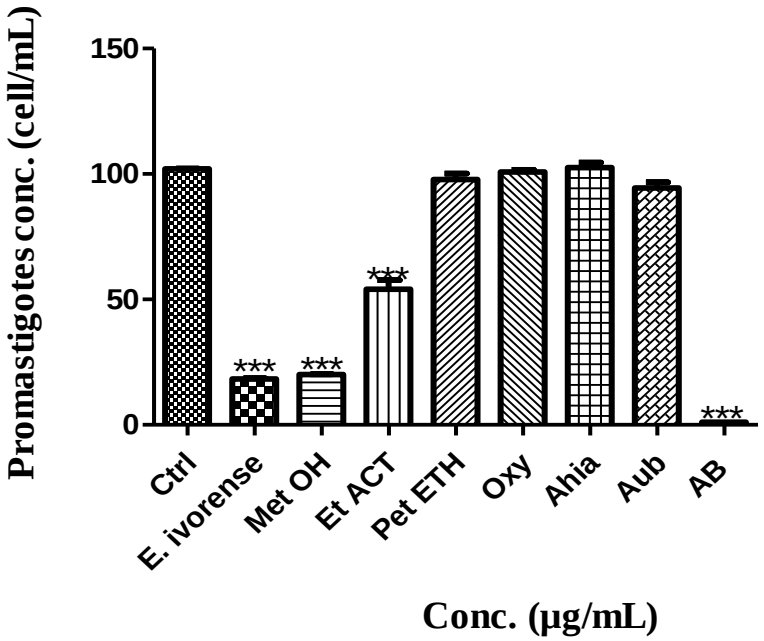

Figure 6: The activity of all the crude extracts in comparison with the fractions from *E. ivorense* at the least concentration of 15.6 µg/mL on the promastigotes of *L. gh*. Data is presented as Mean ± SEM, One-way ANOVA when compared with control (*p<0.001) followed by Tukey's post-test.**

Source: Fieldwork, Anning (2016).

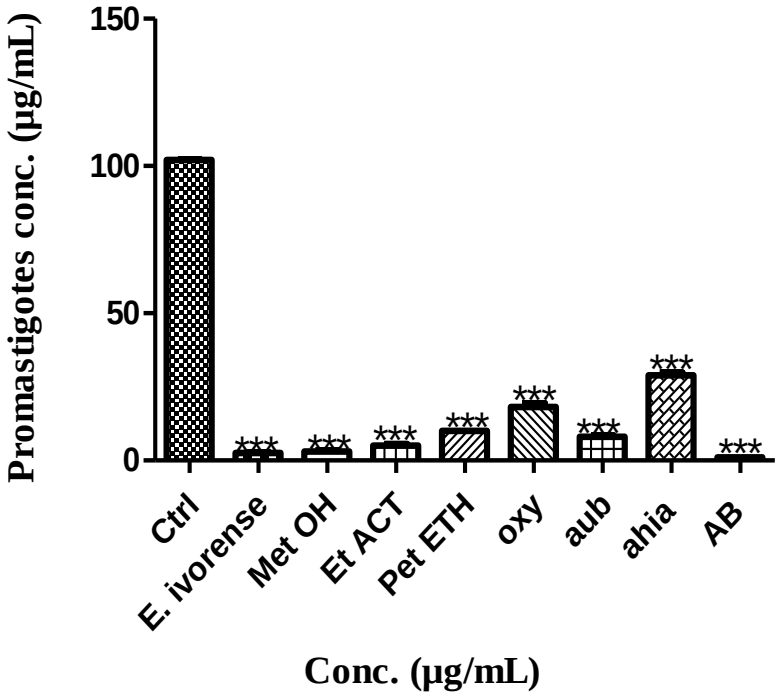

Figure 7: The activity of all the crude extracts in comparison with the fractions from E. ivorense at the highest concentration of 500 µg/mL on the promastigotes of *L. gh*. Data is presented as Mean ± SEM, One-way ANOVA when compared with control (*p<0.001) followed by Tukey's post-test.**

Source: Fieldwork, Anning (2016).

Estimation of IC_{50}

All the estimated IC_{50}s were calculated after 72 hours of administering treatment and was obtained by non-linear regression of plots. The IC_{50} of *E. ivorense* was 6.3 and 123 µg/mL respectively for *L. gh* and *L. donovani*. The IC_{50} of the methanol, ethyl acetate and petroleum ether fractions were 26.7, 57.6 and 150.2 µg/mL respectively on *L. gh* species. Similarly, the IC_{50} of methanol, ethyl acetate and petroleum ether were 1.6, 43.8 and 31.2 µg/mL respectively on promastigotes of *L. donovani*. Also, the IC_{50} of eriodictyol and erythroivorensin were 61.80 and 61.82 µg/mL respectively on the

promastigotes of *L. donovani.* All treatment regimens were classified according to their antileishmanial activity using a scale by Robledo et al., 2015 as follows, active: IC_{50}< 20 µg/mL; moderately active: IC_{50}> 20 and <50 µg/mL; or potentially nonactive; IC_{50} > 50 µg/mL. The crude extracts of the leaves of *E. ivorense* exhibited the most activity of all the crude extracts with an IC_{50} of 6.3 µg/mL while *O. ahia* was the weakest with an IC_{50} of 336 µg/mL after 72 hours. The IC_{50}s of *C. oxycarpum* and *A. aubryanum* were 39.9 and 59.7 µg/mL respectively after 72 hours. The same activities were also measured on the promastigotes of *L. donovani.* Due to the high activity presented by the crude *E. ivorense*.

Figures 6 to 8 present details of the IC_{50}s that were recorded of the fractions of *E. ivorense*. The IC_{50} of amphotericin B was 2.5 µg/mL.

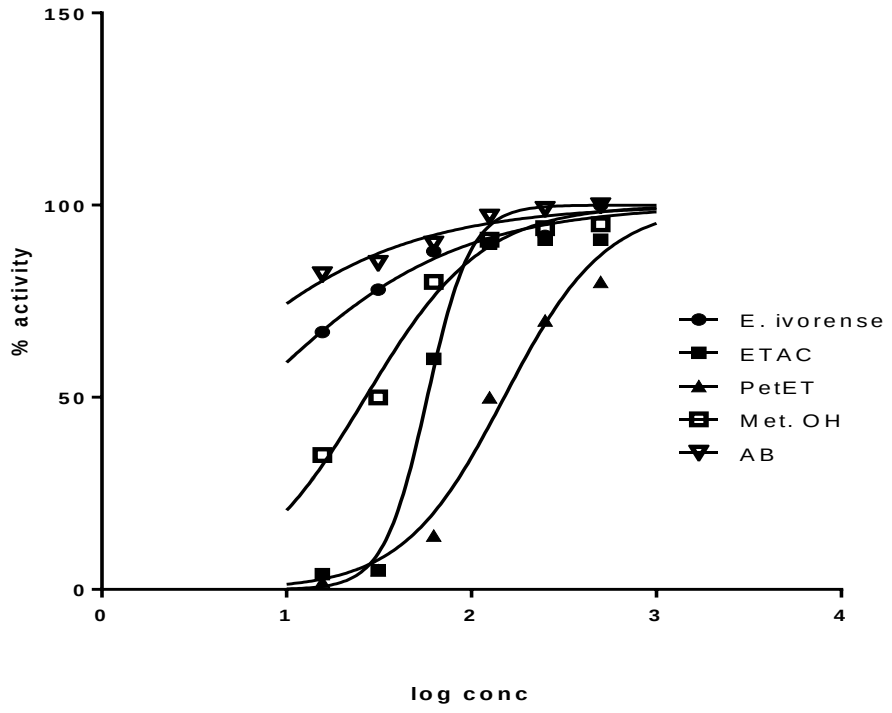

Figure 8: The IC_{50}s of the fractions that were derived from the crude extract of *E. ivorense* after 72 hours of treatment on *L. gh* species

Source: Fieldwork, Anning (2016).

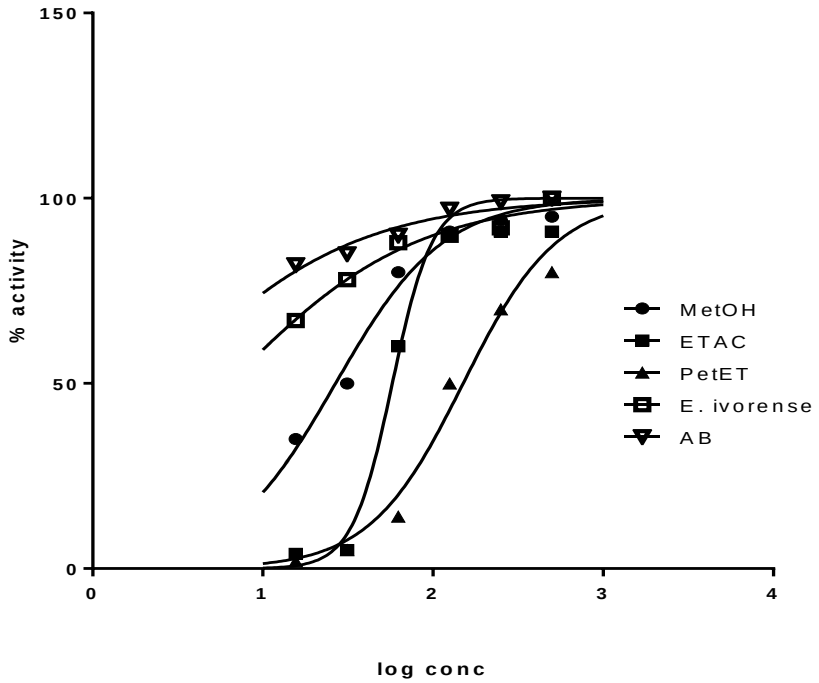

Figure 9: A comparisons of the IC_{50}s of fractions of *E. ivorense* on the promastigotes of *L. donovani* after 72 hours

Source: Fieldwork, Anning (2016).

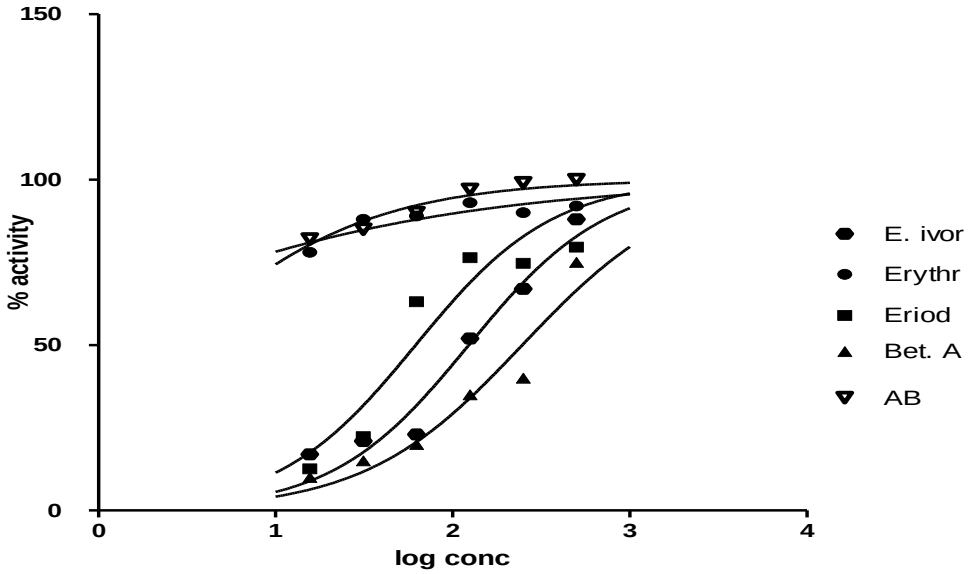

Figure 10: IC_{50}s of the isolated compounds on the promastigotes of *L. donovani* after 72 hours

Source: Fieldwork, Anning (2016).

CHAPTER FIVE

DISCUSSION

The practice of herbal treatment is well established in Ghana like most other developing countries of the world. Large numbers of people are involved in this practice especially in the rural areas of the country where access to medical care is a huge cost (Smolinski, Hamburg, & Lederberg, 2003). The dynamic features of local system of treatment are their safety, affordability and availability to large population. Traditional health care systems using medicinal plants can be recognized and used as a starting point for the development of novelties in drugs (Khan et al., 2012). Natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoan and other parasites. In this sense, the potential of plant products as a source of antileishmanial drugs has been demonstrated and considered as a promising approach. Artemether for example, is a derivative of artemisinin which is extracted from the plant *Artemisia annua* and is a new anti-malarial drug used for treatment against the erythrocytic stages of chloroquine-resistant *Plasmodium falciparum* and for cerebral malaria.

Several Ghanaian medicinal plants and their products have been screened for their biological activities as elaborated by Boampong, Karikari, & Ameyaw (2015), Boampong et al., 2013 and Ameyaw et al., 2014. While several studies about screening of plants extracts against *Leishmania* have been reported, no report on the screening of the plant extracts used in this

study has yet been reported yet on their activity against any form of *Leishmania*. This current study was thus designed to explore the antileishmanial activity of various parts of different plants using established *in vitro* protocols.

Phytochemical screening of the crude extracts of all the plants revealed the presence of phytochemicals similar to the report of Ayoola et al. (2008). Natural products such as alkaloids and terpenes, have shown potent growth inhibition of *L. brasiliensis* (Wright & Phillipson, 1990). The major phytochemicals tested for included saponins, tannins, flavonoids, steroids, glycosides, triterpenoids, alkaloids and anthraquinons. Each plant extract revealed the presence of at least three phytochemicals. Of the eight phytochemicals, only saponins and tannins were present in all the extracts while glycosides and triterpenoids were present only in *C. oxycarpum* and *O. ahia*. Glycosides, alkaloids and steroids were present in the crude extracts of the leaves of *E. ivorense*. This is similar to previous reports of the methanolic leaf extracts recounted by Adu-Amoah et al., (2014). While *E. ivorense* had the highest number of phytochemicals present, *O. ahia* lagged as the lowest having found only saponins, tannins and triterpenoids present. Thus all phytochemicals except triterpenoids and glycosides were present in *E. ivorense*. Among the phytochemicals tested for, *A. aubryanum* and *C. oxycarpum* contained five but different constituents. While *A. aubryanum* revealed saponins, tannins, flavonoids, alkaloids and steroids, *C. oxycarpum* presented steroids, saponins, tannins, anthraquinons and glycosides. Results from phytochemical assay have been presented in Table 1. Natural products

such as alkaloids and terpenes have shown potent growth inhibition of *L. brasiliensis* (Wright & Phillipson, 1990).

Approaches such as promastigote, intracellular amastigote, or axenic amastigote forms of the parasites are among the different approaches used to evaluate drug models. Garcıa et al. (2012) have discussed that the most important of these approaches is the counting of intracellular amastigotes which are the clinical significant stage of the parasite in its mammalian host. In spite of this, the promastigote form has been used in several screening studies despite the fact that it is a less relevant clinical stage. Moreover, tests involving promastigotes have been described as easy and highly reproducible method (Estevez, Castillo, & Pisango, 2007; Garcıa, et al., 2012) and as such preliminary tests for the screening of plant extracts are widely demonstrated by the use of promastigotes (Gachet et al., 2010; Peraza-Sanchez, Cen-Pacheco & Noh-Chimal, 2007; Tempone, Sartorelli & Teixeira, 2008), even when it is time consuming.

All the plants showed promising activities at different concentrations against the promastigotes and the biological activities measured as percentage inhibition. The inhibition is the ability of the various extracts to reduce the viability of the growth or proliferation of the promastigotes. It is evident from the study that inhibition of promastigote growth was time and concentration dependent. At the lowest concentration of 15.6 µg/mL, all the crude extracts gave inhibitions lesser than 20 % whereas the fractions and pure compounds gave inhibitions greater than 50 %. This may be due to the low concentration of the pure compounds or phytochemicals present in the extracts at the lower concentrations. At a concentration of 500 µg/mL, all the treatments gave

inhibitions greater that 70 % except for betunilic acid isolate which gave 25 % inhibition. This is a clear indication that the inhibition activity of betulinic acid against *L. donovani* amastigotes is weak, confirming previous studiy reported by Moghaddam, Ahmad, and Samzadeh-Kermani (2012).

Betulinic acid has been isolated from the crude extract of the leaves of *Pentalinon andrieuxii* by Domínguez-Carmona et al. (2010), and was tested for its antiprotozoal activity against *L. amazonensis, L. braziliensis, Trypanosoma cruzi tulahuen* and *Plasmodium falciparum*. The betulinic acid revealed moderate trypanocidal activity against *T. cruzi* with IC_{50} of 50 µM and a good antiplasmodial activity which also recorded an IC_{50} of 22.5 µM against *P. falciparum*. There was however no leishmanicidal activity noticed for betulinic acid against *L. amazonensis* and *L. braziliensis* confirming the weak activity of betulinic acid observed in this present study against *L. donovani* promastigotes. Aside this, betulinic acid has been shown to exhibit incredible activity such as anti-cancer (Kumar, Mallick, Vedasiromoni, & Pal, 2010), anti HIV (Fujioka et al., 1994), anti-bacterial (Woldemichael, Singh, Maiese & Timmermann, 2003) and anti-malarial (Steele, Warhurst, Kirby, & Simmonds, 1999). Further *in vitro* screening of betulinic acid for inhibitory activity against *T. brucei* revealed a glycolytic enzyme GAPDH inhibition and was a good reversible inhibitor of this enzyme with respect to its cofactor NAD^+ (Moghaddam et al., 2012). Meanwhile, a DNA polymerase β inhibition has also been noticed in the presence and absence of bovine serum albumin which could be a possible route of mechanism for the acid in cases where it is found active (Ma, Starck, & Hecht, 1999). Another known compound that was isolated from the root-bark of *E. ivorense* is eriodictyol, a common flavonoid

known to possess anti-inflammatory properties in a number of test models. Research has demonstrated that eriodictyol is able to suppress nitric oxide (NO) production and also capable of expressing pro-inflammatory cytokines and inducible NO synthase (Lee et al., 2013). Eriodictyol is also known for its antioxidant and polyphenolic properties and thereby possessing numerous pharmacological actions in a number of models (Habtemariam & Dagne, 2010) which could be the explanation for its activity.

Eriodictyol is a flavonoid and occurs naturally in plant materials. It is a known component of the group of related flavonoid substances collectively designated as vitamins P which are concerned with the maintenance of normal conditions in the walls of the small blood vessels. It has previously been reported that eriodictyol is present in citrus fruits and in many other plants including *Ericdictyon californicum*, *Lespedeza crytobotrya*, *Prunus campanulata, P. serrulata, Helitropium stenophyllum* and *H. sinuatum* (Torres et al., 1996; Wollenweber, Wehde, Dör, & Stevens, 2002). Eriodictyol in ethanol extract of *Vernonanthura tweedieana* leaves revealed weak trypanocidal activity with no significant leishmanicidal activity (da Silva et al., 2015). In this current research however, eriodictyle showed a moderately potent activity against the promastigotes of *L. donovani* as opposed to the result found by da Silva et al. (2015). The difference in results observed is not out of place since different species of the parasites were used for both studies. Again the isolate, eriodictyl, was isolated from different plant materials each existing in a completely different geographical location; *E. ivorense* for the current research and *V. tweedieana* for the reported research.

The mechanism of action of the novel erythroivorensin rests with further research but it is postulated to have similar mechanism of action as the betulinic acid (Armah et al., 2015).

Amphotericin B was used as the reference drug which gave the best activity on both *L. donovani* and *L. gh* after 72 hours with an IC_{50} of 2.4 µg/mL and 99 % inhibition at 15.6 µg/mL. This result is similar to the report of Luize et al., (2005), in which Amphotericin B was used as positive control against *L. amazonensis* and indicated 90 % of growth inhibition at concentration of 0.116 µg/mL. The DMSO and M199 culture controls that were set were all found to be inactive in all of the experiments. It can thus be said that the amount of DMSO that was used in each of the experiments to dissolve the samples had no active effect on the growth or inhibition of the parasites which was the same as reports by Jaafari, Hooshmand, Samiei, & Hossainzadeh (2007), Luize et al. (2005) and Yong et al. (2000). Meanwhile, reports on phytochemical screening of aerial parts of *P. abrotanoides* for example, used for medicinal purposes, have shown the presence of high content monoterpenes, diterpenoids and sesquiterpenes such as myrcene, pinene, camphor, caryophyllene, humulene, camphene and bisabolol (Morteza-Semnani, 2004; Sajjadi, Mehregan, Khatamsaz, & Asghari, 2005) while the root extracts have been reported by Tan et al. (2002), to be involved in some level of leishmanicidal activity. Anti-leishmanial activities of terpenoides on *L. major* promastigotes in a dose-dependent mode have again been reported by Tan et al. (2002). Therefore, *P. abrotanoides* root extract could be suitable topical treatment candidate for the treatment of cutaneous leishmaniasis similar to results obtained from this study and even though no

anti-inflammatory tests were performed, it could be deduced due to similar advances between literature and this study that extracts are probable candidates for treatment for *Leishmania*.

Even though the exact mechanisms underlying the anti-leishmanial activity of the plant extracts and their compounds are yet to be fully understood, possible involvement of antimicrobial mechanisms by permeating cell membranes and disrupting the structure of the different layers of membrane polysaccharides, fatty acids, and phospholipids, leading to serious cell damage have been suggested by Bakkali, Averbeck, and Averbeck (2008). Evidence from photomicrographs of *L. chagasi* treated with essential oil from *C. cajucara* displayed different degrees of cell damage that was time dependent (Rodrigues et al., 2013). In another research, there were increase in mitochondrial volume, loss of mitochondrial cristae and presence of vacuoles in the flagella were seen within the first 5 minutes of incubation. After mitochondrion is damaged, kDNA which make up 5-10 % of total DNA (Saraiva et al., 2005**)** is fragmented followed and then finally condensation of nuclear chromatin, although no changes were observed in the shape of the nucleus. After 40 minutes of treatment, the cells had showed thorough disorganization of the cytoplasmic organelles with dilated mitochondria, flagella pocket with intense release of vesicles, and numerous vesicles in the cytoplasm were noted. In Rodrigues's (2013) report, these similar features were however seen after 40 minutes of extract administration. In this present study, there was similar increase in cell volume such that the usual parasite long spindle-shaped had been lost; looking short and bulging toward the mid portions for all crude extracts at concentration of 500 µg/mL. This was

followed by membrane disruption and then loss or break-off of flagella in some cases limiting the motility of the parasites. These characteristics were seen using an inverted light microscope as has been reported by Rodrigues et al. (2013). Ultimately, there is lysis of cells resulting in parasite death. A lot of mechanisms of action of phytochemicals have been suggested. Some phytochemicals may inhibit microorganisms, interfere with some metabolic processes or even modulate gene expression and signal transduction pathways (Kris-Etherton et al., 2002; Manson, 2003; Surh, 2003) and are either used as chemotherapeutic or chemo preventive agents.

Polyphenols such as flavonoids and tannins are known to induce nitric oxide (NO) production which in turn enhances the extract and/or compound killing potental (Cheon et al., 2000; Ishii, Horie, Shibano, Kitanaka, & Amano, 1999). For this study, tannins were present in all four plant extracts whiles flavonoids were present only in *E. ivorense* and *A. aubryanum*. In Lemesre et al. (1997) study, it has been reported that the effects of authentic NO gas, mimics macrophage-mediated cytotoxicity, on the *in vitro* viability and proliferation of axenically cultured amastigote and promastigote forms of *L. mexicana, L. amazonensis,* and *L. chagasi*. Moreover, a number of papers have reported beneficial effects of polyphenols on infectious diseases that may be due to immunomodulatory activities, though the mechanism of action remains to be clarified (Kolodziej & Kiderlen, 2005). In that study, the authors demonstrated that NO action led to lethal metabolic inhibition in both parasite developmental stages by, at least in part, triggering iron loss from enzyme(s) with iron–sulfur prosthetic groups, particularly aconitase. Kolodziej and Kiderlen (2005) again observed the correlation between association index, NO

production and parasite elimination, suggesting that NO could exert an important role in the clearance of infection. In that report, the infection of macrophages 24 hours prior to treatment with crude extract reduced the association index by 71 %, with a concomitant increase of 90.4 % in NO production suggesting that the aqueous extract used in that study possibly induced the synthesis of NO by murine macrophages, which potentially heightens the killing mechanisms of these cells. Still, literature strongly supports the beneficial effects of polyphenols on infectious diseases that may be due to immunomodulatory activities, though the mechanism of action remains to be clarified (Kolodziej & Kiderlen, 2005). The leishmanicidal activities of phenolic compounds against *Leishmania* promastigotes and amastigotes have been described in several works (Kolodziej, Radtke, & Kiderlen, 2008; Kolodziej & Kiderlen, 2005; Mendonca-Filho et al., 2004). Plant extracts, secondary metabolites or biomolecules are capable of exerting immunostimulatory properties (Chouhan, Islamuddin, Sahal, & Afrin, 2014). The tritepene fraction containing oleanolic and ursolic acids can modulate immune cells to produce and increase levels of IL-12 and IFN-γ (Yamamoto et al., 2014). Tannins and related compounds kill *Leishmania* via a NO-mediated mechanism (Chouhan et al., 2014). Increase in cytokines related to Th1 lymphocytes has been reported in *Berberine chloride*, by Saha et al. (2011). A decrease in tcytokines associated with Th2 cells have been reported in *Asparagus racemosus* by Sachdeva et al., 2014b and have been considered to be involved in mechanisms just as high IgG2/IgG1 ratio levels in mixture of *Tridax procumbens* and *Allium sativum*, (Gamboa-Leon et al., 2014). These mixtures are therefore being prospectively considered as new mechanisms for

anti-leishmanial, leishmanicidal or immunomodulatory activities of plant-derived formulations (Chouhan et al., 2014). Leishmanicidal activity of *Allium sativum* (garlic) has been demonstrated against infection with *L. major* and *L. maxicana*, so that it induces a Th1-type response and stimulates INF-γ and NO production in macrophage, and thus prevents the progression of the infection (Gamboa-Leon et al., 2014 & Gharavi et al., 2011).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Summary

Four plants have been identified to be used for medicinal purposes. The plants include *E. ivorense, O. ahia, C. oxycarpum* and *A. aubryanum* and have been noted for their use in the treatment of various parasitic infections including *Leishmaniasis.* Secondary metabolites such as flavonoids and tannins were found present in all the plant extracts while the other constituents were found in either one or more of the plants.

The assessment of the efficacy of the plants was described as plant activity on the promastigotes of *L. donovani* and *L gh*. The plant extracts inhibited the promastigotes of *L. gh* remarkably. The percentage inhibitions of *E. ivorense* at the least concentration of 15.6 µg/mL was 67 % and 100 % for the highest concentration of 500 µg/mL whereas *C. oxycarpum* followed closely at inhibitions of 10 and 96 % respectively for lowest and highest concentrations of plants. *O. ahia* recorded the least activity with 2 and 71 % at least and highest concentrations respectively. The lowest IC_{50} was 6.3 µg/ mL for *E. ivorense* and 336 µg/mL for *O. ahia* confirming their inhibitory activities recorded earlier. The IC_{50}s for both *A. aubryanum* and *C. oxycarpum* were 60 and 40 µg/mL respectively.

Even though the percentage activity of the plants on *L. donovani* were somewhat different, it was still obvious of the high activity of *E. ivorense* on

the promastigotes of *L. donovani*. The highest activity again was recorded from *E. ivorense*, but this time with lower values of 88 and 17 % for highest and lowest inhibitions respectively. The IC_{50}s for the highest and lowest inhibitory plants were 123 and 274 µg/mL respectively on the promastigotes of *L. donovani*.

Conclusion

Three unique compounds were isolated from the ethyl acetate fraction of *E. ivorense* which were labelled as **compounds 1-3**. **Compound 1** was erythroivorensin, **compound 2**, eriodictyol and **compound 3** as betulinic acid with IC_{50}s of 0.5, 61.8 and 247 µg/mL respectively on the promastigotes of *L. donovani*. The IC_{50} of the standard drug, amphotericine B, used as positive control was 2.5 µg/mL on both *L. donovani* and *L. gh* promastigotes. Due to the active nature of these compounds against the parasites, it is a possibility that they could be used as markers for the standardization of treatments with *E. ivorense* or its compounds bearing in mind its reported toxicity.

Recommendations

- There is an urgent need for cytotoxicity assay on all treatment regimens to ascertain how selective the treatments are using macrophage cell lines.
- The mechanisms of action of the treatment regimens, especially *E. ivorense* and its compounds, need to be urgrntly researched due to its high activity on the parasites.

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APPENDICES

APPENDIX A

1. Crystal data and structure refinement for Erythroivorensin

Empirical formula	$C_{20}H_{30}O_2$
Formula weight	604.88
Temperature/K	150 (1)
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a/Å	11.99271 (7)
b/Å	14.66394 (7)
c/Å	19.91449 (10)
α/°	90
β/°	90
γ/°	90
Volume/Å3	3502.17 (3)
Z	8
P_{calc} g/cm^3	1.147
μ/mm^{-1}	0.554
F(000)	1328.0
Crystal size/mm3	0.28 × 0.1 × 0.08
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.486 to 147.294
Index ranges	$-14 \leq h \leq 14$, $-18 \leq k \leq 18$, $-24 \leq l \leq 24$
Reflections collected	59,230
Independent reflections	7031 [R_{int} = 0.0305, R_{sigma} = 0.0141]

Data/restraints/parameters	7031/0/405
Goodness-of-fit on F^2	1.021
Final R indexes [$I \geq 2\sigma$ (I)]	R_1 = 0.0309, wR2 = 0.0843
Final R indexes [all data]	R_1 = 0.0317, wR2 = 0.0853
Largest diff. peak/hole/e $Å^{-3}$	0.23/−0.15
Flack parameter	−0.12 (4)

Source; Armah et al., 2015

The details of the structural analysis are described in the Supporting information along with the CIF file, which has been deposited at the Cambridge Crystallographic Data Centre (CCDC No. 1051612).

2. NMR spectroscopic data (500 MHz, CDCl3) for erythroivorensin

1	38.7, CH_2	1.75 dd (12.6) 0.91 m	C2, C5
2	31.9, CH_2	2.01 dd (12.2, 2.2)	
		1.12 m	
3	42.2, CH	1.43 m	
		1.38 m	
4	33.2, C	–	
5	55.4, CH	0.92 m	C4, C9, C20, C18, C19
6	21.8, CH_2	1.68 dd (13.3, 3.0)	C8
7	18.9, CH_2	1.61 dt (12.9, 3.4)	
		1.43 m	
8	37.4, CH	2.48 m	C14
9	52.9, CH	0.97 m	C7, C20
10	36.8, C	–	
11	20.6, CH_2	1.86 dd (12.6, 5.9) 1.15 m	C8, C9, C12, C13

		1.15 m	
12	26.1, CH_2	2.46 m 2.18 m	C13, C13, C14, C13, C13, C14
13	136.5,C	–	
14	134.5, C	–	
15	134.9, CH	6.84 dd (17.2, 10.9)	C12, C13, C16
16	115.6, CH_2	16a 5.16 d (10,9)	C13, C15
	174.3, C	16b 5.35 d (17.2)	C13, C15
17	174.3, C	–	
18	33.4, CH_3	0.85 s	C3, C4, C5, C19
20	14.2, CH_3	0.87 s	C1, C5, C9, C10

Source: Armah et al., 2015

APPENDIX B

1. Extraction from plant material

Plate1

Plate2

Plate 1: **The plant materials soaked in 70 % ethanol in round bottom flask for three days and then filtered using a filter paper into a measuring cylinder.**

Plate 2: **Resultant extract in a crucible for drying.**

PLATE 3

Plate 3: **A rotary evaporator set up containing the macerated samples. This was done at the Department of Chemistry, University of Cape Coast.**

2. Leishmanial assay

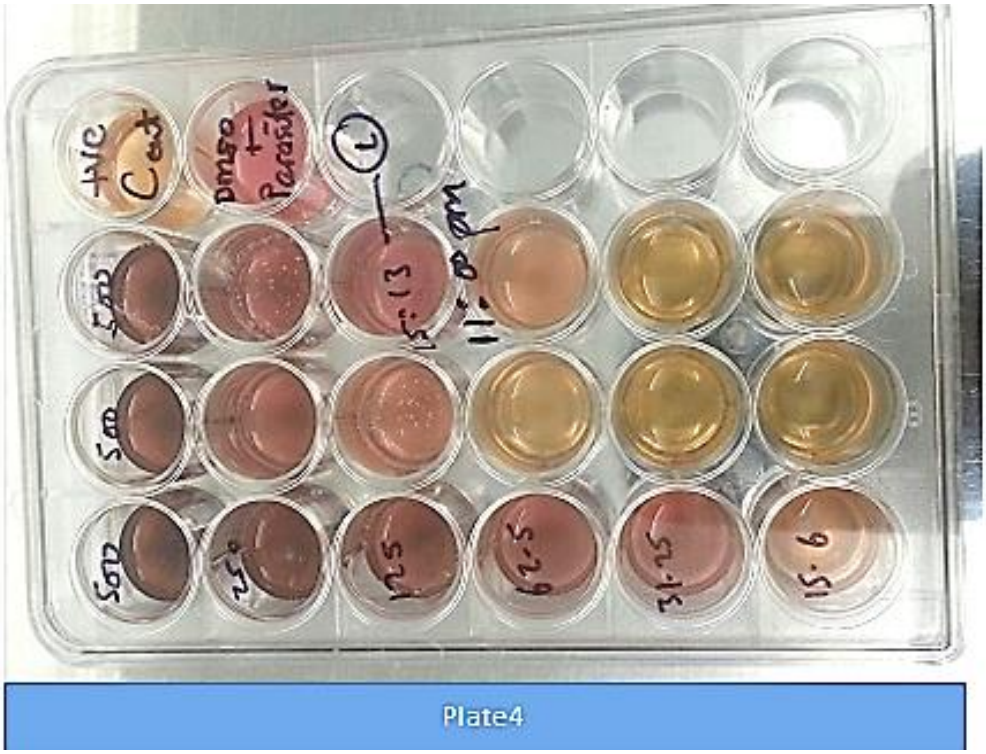

Plate 4: A 24 well plate containing extracts and parasites ready to be incubated and tested for their antileishmanial activity. Each row contained the same concentration of extract and parasites. DMSO was set as a negative control while a positive control was made of only parasites and M199.

Plate 5: A girl with multiple lesions of suspected CL on the scalp. The lesions are being dressed for treatment and for samples. The amastigotes are found around the raised edges of the lesions

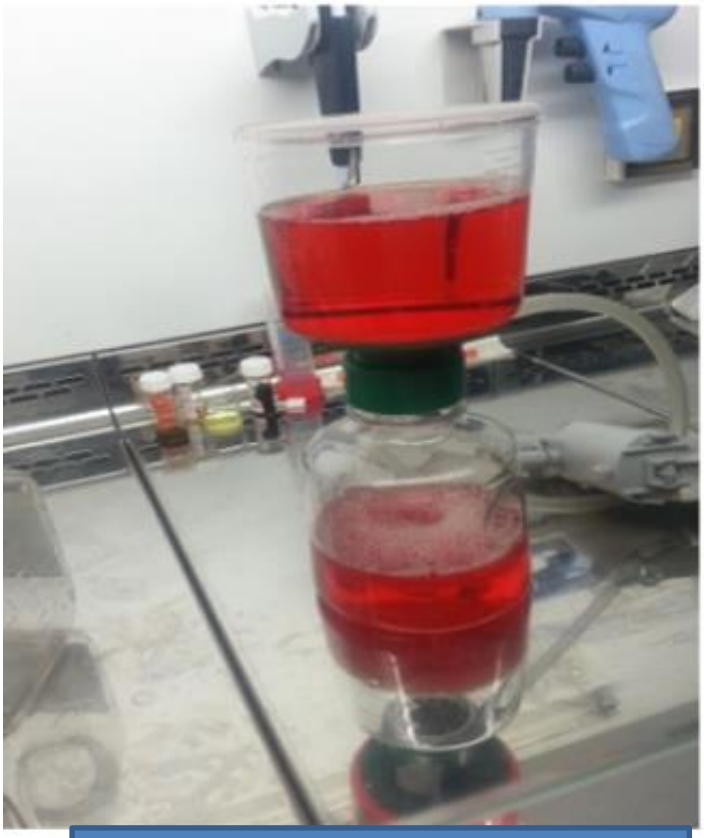

Plate 6

Plate 6: Preparation of M199 in a class II cabinet using a suction pump.

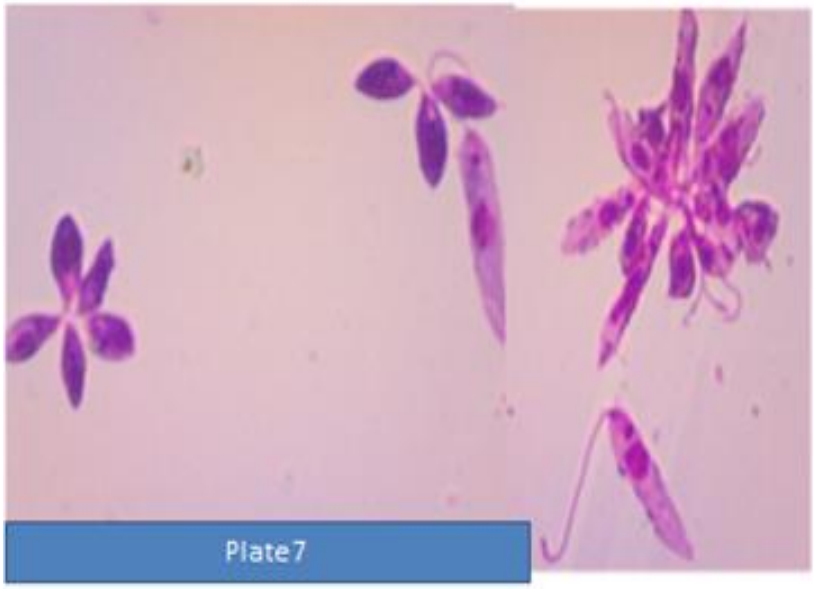

Plate 7: Giemsa stained promastigotes of *L. gh*. that were isolated in patients from Ho.