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IN VITRO SCREENING OF EXTRACTS FROM SELECTED MEDICINAL PLANTS IN GHANA FOR ANTI-LEISHMANIA PROPERTIES

By

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DECLARATION

I hereby declare that this submission is my own work towards an MPhil and that, to the best of my knowledge, it contains no materials previously published by another person nor material which has been accepted for the award of any other degree of the University except where due acknowledgement has been made in the text.

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DEDICATION

In Loving Memory of Madam Patience Quaye, always in my heart Grand ma.

Mum and Dad, Christy and Kwasi, I am blessed to have you as parents.

Nana and Paapa, Proof that there is a God : if you need a reason Celyna,

Love.

Mitsuko, my definition of kindness and selflessness.

This dedication represents the past, present and future, life; my life.

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LIST OF ABBREVIATIONS

FACS	Fluorescent Activated Cell Sorting
Ms	Micro seconds
µg	Microgram
Ml	Milliliter
IC_{50}	Concentration of extract that inhibits parasite proliferation by 50%
DAPI	4,6-Diamidino-2-Phenyl indole
PBST	Triton X 100 in PBS
PBS	Phosphate Buffered Saline

ABSTRACT

Leishmaniasis is an infectious disease caused by protozoans of the *Leishmania spp*. It is a chronic infection that is associated with global morbidity and mortality. The main form of control, chemotherapy, is hampered by high toxicity of available drugs and emergence of resistant parasite strains. This study was aimed at screening some Ghanaian medicinal plants for anti-*Leishmania* activity. Plant extracts were prepared with ethanol and their anti-*Leishmania* activity tested at a concentration range of 1-100 µg/ml using the alamar Blue® assay (Invitrogen, USA). Out of 96 ethanolic plant extracts screened, 32 were found to have varying degrees of activity. The eight (8) extracts with the highest activity obtained from seven (7) plants, (*Cassia alata* [045L], *Zanthoxylum xanthoxyloides* [064R], *Cola cordifolia* [032L], *Annona senegalensis* [035L], *Clausena anisata* [036R], *Bridellia ferruginea* [038L] and *Parkia clappertoniana* [050SBL and 050L]) were selected and tested for their apoptosis inducing properties and effect on parasite morphology using FACS analysis and immunohistochemistry. None of the eight (8) extracts had induced apoptosis in *Leishmania* parasites but had varying effects on the parasites' morphology. Extracts 035L caused kinetoplast disintegration while 032L, 038L, 050SBL and 050L caused nuclear fragmentation. Extract 045L, on the other hand, did not affect nuclear or kinetoplast division but rather inhibited cytokinesis. Five out of 7 plants whose extracts showed high activity were tested for the first time in this study. Data obtained from this study are useful as an initial database for Ghanaian medicinal plants with anti*Leishmania* activity. This study recommends further analysis on the eight active extracts, for their potential as anti-*Leishmania* drug candidates.

CHAPTER ONE

1.0 Introduction

Leishmaniasis is a parasitic infection caused by parasites of the genus *Leishmania*. It is characterized by painless but disfiguring lesions and is a major cause of morbidity and mortality. It is transmitted by the sand fly of the genus *Phlebotomus.* The geographical distribution of Leishmaniasis is restricted to regions where the sand fly vector is endemic which puts over 350 million people at risk of infection worldwide. In 2006, it was reported to have affected 14 million people in Africa, Asia, Europe and the Americas causing a morbidity of 2.4 million disability adjusted life years and about 70,000 deaths annually (Kimutai, *et al*., 2009).

There are three main forms of Leishmaniasis; cutaneous, muco-cutaneous and visceral. Cutaneous Leishmaniasis is the most common form of the disease and is caused by over twenty (20) different species of *Leishmania*. There are about 1.5 million cases of cutaneous Leishmaniasis every year, representing about 75% of Leishmaniasis worldwide (Sundar & Rai, 2002). Cutaneous Leishmaniasis is characterized by one or several ulcer(s) or nodule(s) in the skin caused by the invasion of macrophages in the dermis (Chappuis *et al*., 2007). Although it is self-limiting, it results in the formation of disfiguring scars. The wound healing is very slow in individuals with a compromised immune system. Muco-cutaneous Leishmaniasis, which is also caused by over twenty (20) different species of *Leishmania*, is characterized by a progressive destruction of the mucosa by ulcers that extend from the nose and mouth to the pharynx and larynx. The ulcers are not self-limiting and are usually seen several months and, in some cases, years after the first episode of cutaneous Leishmaniasis (Chappuis *et al*., 2007).

The visceral form of the disease is a systemic infection characterized by prolonged fever, anemia, hepatosplenomegaly and weight loss as a result of the multiplication of the parasite in the reticulo-endothelial system (Boelaert & Bhattacharya, 2007). If left untreated, visceral Leishmaniasis can lead to death. *L. donovani*, *L. chagasi* and *L.infantum* are the species of *Leishmania* known to cause visceral Leishmaniasis.

Incidences of visceral Leishmaniasis co-infection with HIV have been reported in 25%70% of patients in Southern Europe (Sundar & Rai, 2002).

There is currently no vaccine against Leishmaniasis and the only preventive measure is the reduction of human-vector contact. Chemotherapy is known to be the main form of disease treatment. The first line treatment drugs for Leishmaniasis are pentavalent antimony based drugs such as sodium stibogluconate and meglumine antimoniate. The downside to this treatment is that, it requires long courses, parenteral administration and is generally expensive. There have also been reports of resistance to both sodium stibogluconate and meglumine antimoniate in parts of India where incidence of the disease is highest (Guerin *et al*., 2002). The second line drugs currently used, pentamidine and amphotericin B, are characterized by toxicity, are expensive and not easily accessible (Guerin *et al*., 2002). In 2002, miltefosine was disseminated as the first oral treatment for Leishmaniasis. Unfortunately, this drug is also characterized by high toxicity and is contraindicated in women of child-bearing age mainly because it is teratogenic and inhibits development of fetus embryo (Pink, Hudson, Mouriès, & Bendig, 2005). Due to the high toxicity and cost of available drugs, they are usually not prescribed unless the parasites are seen in a stained tissue smear.

Microscopy is the gold standard for diagnosing *Leishmania* infection. This technique however requires a lot of time and skill in both sample collection and observation. It is also not effective in detecting low concentrations of parasitemia (Bensoussan & Nasereddin, 2006). Other techniques that are currently used in the diagnoses of Leishmaniasis are antigen/antibody-detection based rapid test kits, ELISA and Polymerase Chain Reaction (PCR) (Boelaert & Bhattacharya, 2007). All these new techniques come with some limitations. A major limitation is their efficiency in distinguishing past infections from current ones (Bensoussan & Nasereddin, 2006; Chappuis *et al*., 2007).

The use of traditional medicine is a very common occurrence in Africa. The World Health Organization (WHO) estimates that 80 % of the population living in developing countries use traditional medicine and there is a gradual increase in the use of traditional medicine in the developed world (Darko, 2012). In some parts of Africa, including Ghana, traditional medicine is the first line of therapy against varying diseases. Although there are some reports on the anti-parasitic qualities of some of these medicinal plants, there is still a lack of scientific evidence to back most of these claims. There have been some reports on the *in vitro* anti-*Leishmania* activity of some medicinal plants across the world. Most of these plant extracts were tested on the promastigote form of the *Leishmania* parasite (the form found in the sandfly) but no mechanistic studies were performed on the extracts found to have anti-*Leishmania* activity (de Medeiros *et al*., 2011; Peraza-Sánchez *et al*., 2007).

1.1 Rationale

Leishmaniasis is fast becoming a major health problem across the world. In Ghana, cutaneous Leishmaniasis is endemic in the Volta Region which is outside the

Leishmaniasis belt. Reports of the co-infection of visceral Leishmaniasis and HIV in southern Europe is an even bigger concern as it highlights the opportunistic nature of *Leishmania* infections (Sundar & Rai, 2002). In Ghana, there has been a single report on cutaneous Leishmaniasis and HIV co-infection (Lartey, *et al*.,, 2006). The opportunistic nature suggests the presence of asymptomatic human reservoirs of the *Leishmania* parasite as well as those who may be showing symptoms but have undetectable levels of the parasite. Available drugs used in the treatment of Leishmaniasis cannot be used for mass drug administration due mainly to their high toxicity. This toxicity also makes the treatment of identified cases very difficult and uncomfortable for the patient. This has given rise to the need to develop an alternate drug therapy for the treatment of identified cases and possibly the elimination of reservoirs.

In Africa, there have been reports of plants that have anti-*Leishmania* properties, but the mode of action is not known. This study therefore seeks to screen some selected Ghanaian medicinal plants for anti-*Leishmania* properties and their possible mechanisms of action.

1.2 AIM

To screen selected Ghanaian medicinal plants for anti-*Leishmania* activity

1.3 SPECIFIC OBJECTIVES

- To establish an *in vitro* anti-*Leishmania* screening system in the laboratory
- To screen 96 crude plant extracts on promastigote *Leishmania* parasites
- To determine the mechanism of action of active plant extracts

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 LEISHMANIASIS

Leishmaniasis is a disease that is fast gaining recognition as a serious health problem. It is still, however, regarded as a neglected tropical disease. In 2012, it was estimated to cause the ninth largest disease burden among infectious diseases (Alvar *et al*., 2012). Leishmaniasis is known to affect both humans and animals. It is caused by a kinetoplastid of the genus *Leishmania* which is transmitted by the female sand fly. The geographical distribution of the sand fly places about 350 million people at risk of infection worldwide, with 14 million people in Asia, Africa, Europe and the Americas directly affected by the

disease (Kimutai *et al*., 2009). The overall burden of Leishmaniasis has remained quite stable over the years with a morbidity and mortality rate of approximately 2.4 million disability adjustable life years (DALYs), which is a measure of overall disease burden, expressed as the amount of years lost due to illhealth, disability or early death (Kimutai *et al*., 2009). Leishmaniasis has been estimated to cause about 70,000 deaths a year placing it among the high ranking communicable diseases (Kimutai *et al*., 2009). The number of deaths caused by Leishmaniasis must always be approximated bearing in mind that it is usually obtained by hospital deaths and may not include all the cases that failed to reach the hospital (Alvar *et al*., 2012).

2.1.1 Forms of Leishmaniasis and Global Distribution

There are several forms of human Leishmaniasis. Leishmaniasis can broadly be divided into symptomatic and asymptomatic infections. The three main forms of symptomatic infections are cutaneous, muco-cutaneous and visceral with increasing degrees of severity (Figure 1). This means that Leishmaniasis ranges from asymptomatic infection through sores on the skin to a more systemic infection.

Among the three main forms of the disease, cutaneous and muco-cutaneous are the most widely distributed. They are endemic in over 70 countries with 1.5 to 2 million new cases reported each year (Kobets, Grekov, & Lipoldova, 2012). The Americas, the Mediterranean basin and western Asia (which is from the Middle East to central Asia) are the most affected regions. Of all the countries in these regions, ten (10) countries are reported to have the highest incidences of cutaneous Leishmaniasis recording over 7075% of cases. These are Afghanistan, Algeria, Columbia, Brazil, Iran, Syria, Ethiopia,

North Sudan, Costa Rica and Peru (Alvar *et al*., 2012). On the other hand visceral Leishmaniasis is reported to record 0.2 to 0.4 million cases every year. It is not as widespread as cutaneous Leishmaniasis with 90% of visceral Leishmaniasis occurring in six countries which include India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar *et al*., 2012). In West Africa, visceral Leishmaniasis has been reported in Niger, Mali, Nigeria, Senegal, Cameroon, Burkina Faso, Mauritania, Gambia and Guinea. In West Africa, cutaneous Leishmaniasis is transmitted by *L. major*, while visceral Leishmaniasis is transmitted by *L. donovani* (Kimutai *et al*., 2009).

2.1.2 Leishmaniasis in Ghana

In Ghana, one of the three ecological zones, arid Northern savanna, epidemiologically lies within the Leishmaniasis belt in Africa. There have however been no reports of Leishmaniasis in this region of the country. Most reported cases of Leishmaniasis in Ghana have come from the Ho district of the Volta Region which is the moist semideciduous forest region of Ghana (Figure 2) (Landau, 2008). Cutaneous Leishmaniasis (which may include muco-cutaneous, because of problems with diagnosis) is the only kind of Leishmaniasis reported in this region. There have been no reports of visceral Leishmaniasis in Ghana but a single case of cutaneous Leishmaniasis/HIV co-infection (Kimutai *et al*., 2009). The disease was first reported between the years 1999 and 2001 when health workers reported Leishmaniasis-like lesions. This led to an intensive case detection research that saw an increase in recorded cases from 2,348 to 6000 between 2002 and 2003 (Landau, 2008). The disease became known by the local folk as "Agbamekanu", which means "a gift from a visitor", or "a native who just returned from travelling". Although Ghana is endemic for Leishmaniasis, the expertise for clinical diagnoses of

Leishmaniasis by microscopy is lacking in most health centers and hospitals (Landau, 2008). The lack of clinical diagnoses of Leishmaniasis in Ghana has led to a decrease in recorded cases (in health facilities) since year 2003. The decrease in recorded cases in health facilities have also been attributed to people suffering from Leishmaniasis opting for herbal or local medicine since there is no treatment available in health centers or hospitals (Landau, 2008). Cutaneous Leishmaniasis being self-limiting as well reduces the need for individuals to seek health care (Landau, 2008). A less convincing argument may be that the mass spraying of insecticides upon detection of the disease in Ghana may have played a key role in the decrease in cases.

2.1.3. Factors that Affect the Incidence of Leishmaniasis

Leishmaniasis over the last decade has spread into more communities and countries across the world due to risk factors which include climate change, population movement, tourism and trade. Climate changes are considered the main cause of spread of the sand fly into new habitats or areas and with it Leishmaniasis. These climate changes are due mainly to urbanization, deforestation and global warming. Cutaneous Leishmaniasis is considered one of the top ten skin diseases that affect tourists returning from tropical regions. On a less frequent but very serious level, Leishmaniasis has been found to affect individuals through organ transplant (Kobets *et al*., 2012).

A major complication with Leishmaniasis infection is the emergence of Leishmaniasis and Human Immunodeficiency Virus (HIV). This has led to advocacies that Leishmaniasis be considered as an Acquired Immuno-Deficiency Syndrome defining disease (Kimutai *et al*., 2009). In Africa, specifically Ethiopia and Tunisia, 70% of people with visceral Leishmaniasis are also HIV positive.

Leishmaniasis is classified amongst the diseases of poverty. This is because the cost of treatment is so much so that it is either not affordable to most infected people or imposes significant financial burden on them. In extreme cases, it leads to the complete loss of wages either through treatment or inability of the infected to work actively. Until a suitable vaccine is found, the main form of control remains chemotherapy and vector control. Available drugs are however limited by toxicity and emerging resistance.

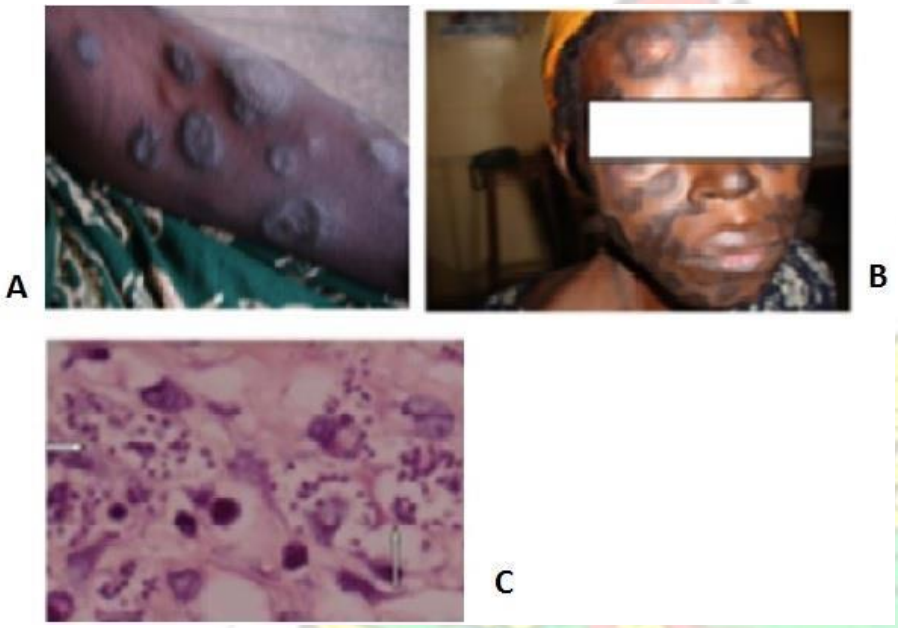

Figure 1: Clinical manifestation of Leishmaniasis (Lartey *et al*., 2006)
A:Hand lesions B: facial lesions C: clusters of free *Leishmania* in intestinal tissue and sub-cutaneous fat as shown in the haematoxylin and eosin stain

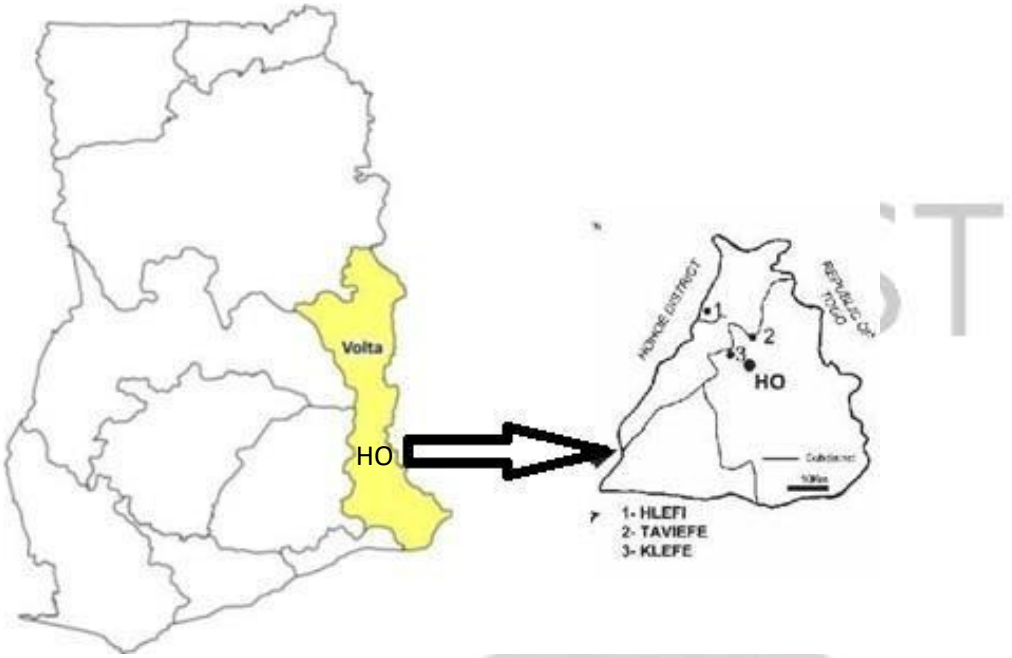

Figure 2: Leishmaniasis endemic zone in Ghana (Ho, district). Outlet highlights areas where human cutaneous Leishmaniasis has occurred (Fryauff *et al*., 2006; Landau, 2008).

2.2 *LEISHMANIA* SPP.

2.2.1 Morphology of the *Leishmania* Parasite

Leishmania spp. are heteroxenous (digenetic) parasites whose life cycle involves two hosts; an invertebrate and a vertebrate host. The invertebrate host is the sand fly. The life cycle stage of *Leishmania* found in the sandfly is referred to as promastigote. Promastigotes are 15-30µm in length and 5mm in width. They are extracellular, longitudinal, motile and divide by binary fission at 27ºC in the sand fly (Gossage, Rogers, & Bates, 2003). The life cycle stage found in the vertebrate host is the amastigote. Amastigotes are non-motile forms of the parasite that divide by longitudinal binary fission at 37ºC. They are 3-6µm in length and 1.5-3µm in width.

Amastigotes are flagellated intracellular parasites whose flagella do not protrude beyond the body surface and cannot be seen under the light microscope. Thus amastigotes were previously thought to lack flagella. The flagellum has however been found to be a very important organelle in the survival of the amastigote and the completion of the parasite's life cycle (Gluenz, Ginger, & McKean, 2010). While the promastigote flagellum is used for motility, cell division, proliferation and invasion of macrophages in the mammalian host, the amastigote flagellum, which has an axoneme structure similar to primary cilia (with 9 microtubule doublets) rather than a shortened promastigote flagellum, is considered as a sensory organ (cellular antenna) involved in cellular organization, cell to cell signaling, regulation of molecular traffic and sensory perceptions critical for intracellular survival of the *Leishmania* parasite within the macrophage (Gluenz *et al*., 2010; Santrich *et al*., 1997). Figure 3 shows promastigote and amastigote forms of *L. donovani* with the flagella labeled.

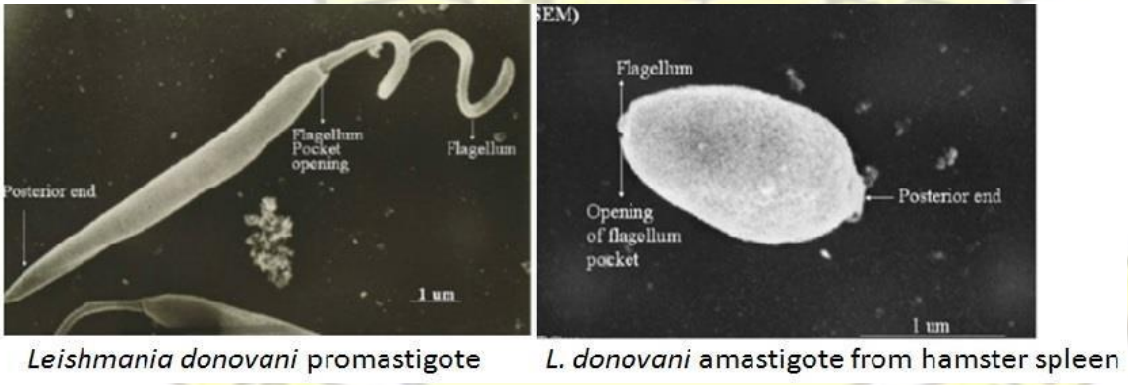

Figure 3; *Leishmania donovani* promastigote and amastigote (Gluenz *et al*., 2010)

2.2.2 Distribution of different species of *Leishmania*

All *Leishmania spp.* infect mammals and are most commonly found in humans, dogs and rodents. Although Leishmaniasis is confined to the tropics, different species of the parasite cause the disease in the New world (that is Central and South America) as opposed to the Old World (that is Africa and the Middle East) (Pratlong *et al*., 2009). In the Old World cutaneous Leishmaniasis is caused mainly by *Leishmania major*, *Leishmania tropica* and *Leishmania aethiopica* (Pratlong *et al*., 2009). *Leishmania donovani* and *Leishmania infantum* are minor causers of cutaneous Leishmaniasis in the Old World (Pratlong *et al*., 2009). The species of *Leishmania* recorded in Ghana as the causative agent of cutaneous Leishmaniasis is *L. major* (Landau, 2008; Villinski *et al*.,

2008). There are reports of a new species of Leishmania infecting people living in the Eastern Ghanaian community of Taviefe in the Volta Region (Villinski *et al*., 2008). The new species of Leishmania has been reported to be new members of the *Leishmania enriettii* complex (Kwakye-nuako *et al*., 2015). Visceral Leishmaniasis on the other hand is transmitted solely by *L. donovani* and *L. infantum* in the old World. These cutaneous and visceral Leishmaniasis causing parasites in the Old World are transmitted mainly by Sand flies of the genus *Phlebotomus* (Pratlong *et al*., 2009). Currently the sand fly species implicated in transmission of Leishmaniasis in Ghana is *P. duboscqi*. In the New World cutaneous Leishmaniasis is caused mainly by *L. mexicana*, *L. peruviana* and *L. braziliensis* (Pratlong *et al*., 2009). Visceral Leishmaniasis in the New World is caused by *L. donovani Leishmania infantum* and *L. chagasi*. Leishmaniasis in the New World is transmitted by sand flies of the genus *Phlebotomus* and *Lutzomyia* (Pratlong *et al*., 2009).

2.2.3 *Leishmania* life cycle

The *Leishmania* life cycle begins when an infected sand fly injects the infective stage of the parasite (promastigotes) into a human (vertebrate) host during a blood meal. Injected promastigotes are phagocytized by macrophages. Although the promastigotes specifically interact with the surface of the host cells, there are varying reports of they not actively penetrating the host cell (Chappuis *et al*., 2007) (Figure 4) or actively doing so (Santrich *et al*., 1997). Once the promastigotes have been engulfed, they are fused with lysosomes in the macrophage. Within the macrophages, the promastigotes transform into amastigotes in the phagolysosome. The amastigotes multiply in the infected cells and may affect different tissues depending on the *Leishmania spp.* Continuous replication of the amastigotes within the macrophages leads to the rupture of heavily parasitized macrophages and the release of amastigotes. The released amastigotes are phagocytized by new macrophages. When a sand fly takes a blood meal from an infected person, they ingest parasitized macrophages. Within the sand fly, the *Leishmania* develop into several distinct developmental stages as they migrate anteriorly to the stomodeal valve, which forms a junction with the foregut. The promastigotes then attach to the midgut-hindgut epithelium in the sand fly where they divide by binary fission. The development within the sand fly takes 8 to 20 days after ingestion. The parasite then moves from the midgut-hindgut into the pharynx where they produce a partial or complete blockage of the sucking apparatus. When the sand fly wants to feed, it regurgitates a bolus of metacyclic promastigotes and this is injected into the bite wound and the cycle begins again (Figure 5) (Almeida *et al*., 2004; Gluenz *et al*., 2010; Gossage *et al*., 2003).

For experimental purposes, the promastigote stage of the parasite's life cycle can be grown in axenic cultures at 25-27°C. Metacyclic promastigotes appear at the late stationary phase and are infective to the mammalian host. They however rapidly lose their virulence in culture. Continuous axenic cultured amastigotes can be achieved at 37°C. However, extensive work has not been done on axenic amastigotes (Gossage *et al*., 2003).

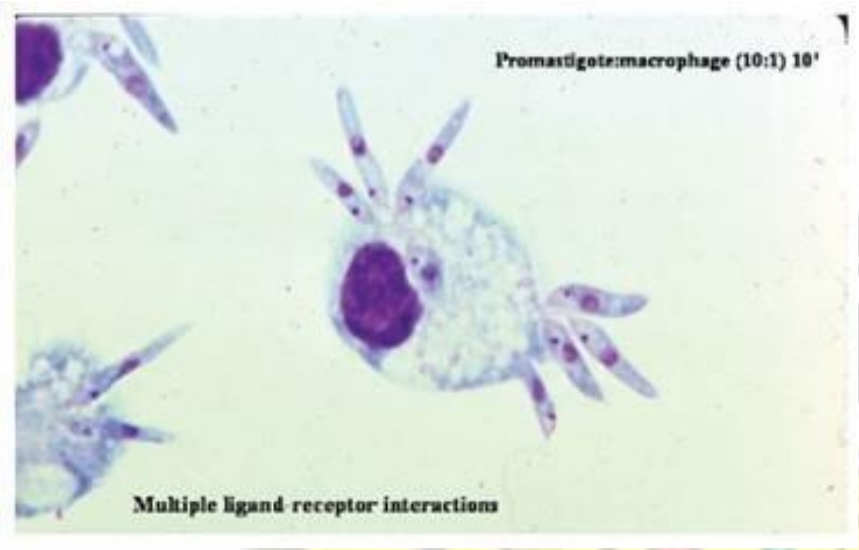

Figure 4: *L. donovani* promastigotes binding to macrophages (Gossage *et al*., 2003)

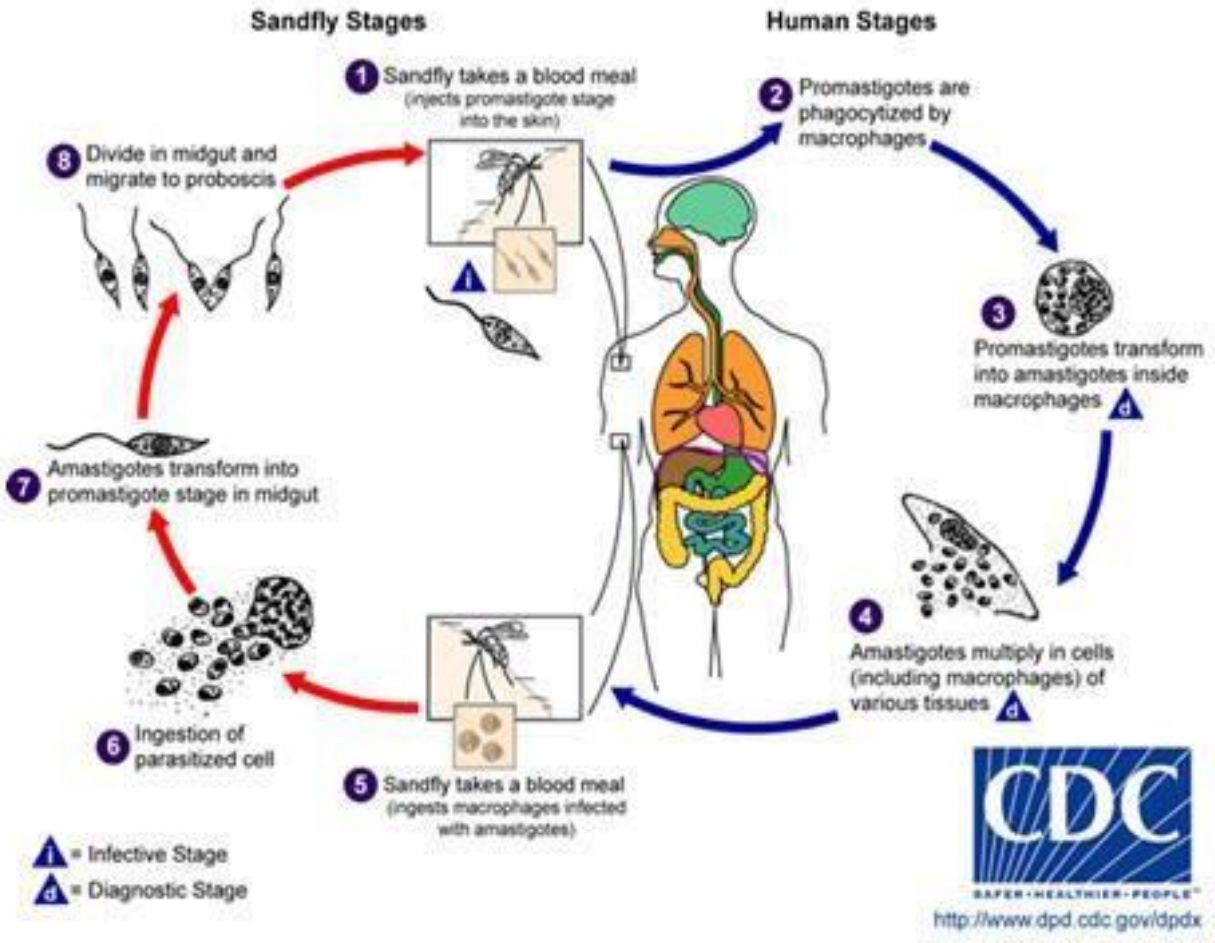

Figure 5: Life cycle of *Leishmania* spp (Chappuis *et al*., 2007).

2.3 DIAGNOSES AND TREATMENT

2.3.1 Diagnoses

The World Health Organization established a symptom based diagnoses of visceral Leishmaniasis to include prolonged fever (> 2weeks) and splenomegaly in individuals living in endemic areas. Although both signs were thought to be present in most cases, splenomegaly is absent in a number of cases. This led to the addition of other clinical signs by some visceral Leishmaniasis programs for the diagnoses of Leishmaniasis.

These signs included wasting, anemia and lymphadenopathy all of which lacked

specificity as they were present in other illnesses such as malaria which was also present in the endemic regions (Boelaert & Bhattacharya, 2007). Another limitation to the symptom based diagnoses was the observation that the symptoms differed from region to region (Boelaert & Bhattacharya, 2007).

Due to the high cost and toxicity of available drugs, there is the need to carry out confirmatory diagnostic tests especially in first line health centers where the probability of the disease is lower than referral centers. Confirmatory diagnostic tests are designed to detect and/or confirm the presence of the parasite (amastigotes) in tissue smears/swabs or cultures. The sensitivity of parasite detection in samples is dependent on the type of aspirate used. The most sensitive is the splenic aspirate with a sensitivity of up to 98.7%. Other very sensitive aspirates are the bone marrow aspirates and the lymph nodes aspirates, sensitivities of 85% and 58% respectively. Collection of all three samples can only be done under highly controlled conditions and by highly skilled personnel. They are therefore not recommended in decentralized use in first line health centers (Boelaert & Bhattacharya, 2007). Peripheral blood and tissue swabs require relatively less skill and can therefore be safely performed in first line health centers. These however have very low sensitivity especially in apparently immune-competent individuals with low parasite levels (Chappuis *et al*., 2007). The gold standard for diagnoses or parasite detection in tissue swabs, cultures, aspirates or blood is microscopy. Once the samples have been collected, they are stained with giemsa and observed under the microscope. Positive samples are those that contain monocytes or macrophages invaded by amastigotes. The limitation of microscopy as a diagnostic tool is that the identification of amastigotes requires expertise and training and the accuracy is dependent on the experimenter.

Due to the inadequacies associated with microscopy, researchers are working on developing alternate diagnostic tools to improve the diagnoses of Leishmaniasis. One of such is the detection of specific anti-*Leishmania* antibodies in the serum of patients.

Examples of anti-body based tests include the Direct Agglutination Test (DAT) and the Immuno Chromatographic Test (ICT) (Boelaert & Bhattacharya, 2007). The limitation to this method of diagnoses is that although treatment results in a decrease in serum antibody levels, anti-*Leishmania* antibodies may remain at detectable level several years post-treatment/cure. It is therefore difficult to distinguish a relapse from a cured patient using serum antibodies. Considering the high toxicity of available drugs, treatment is restricted to diseased individuals and not asymptomatic individuals (Chappuis *et al*., 2007). However, between <10% to >30% of apparently healthy individuals living in low/moderately endemic regions to highly endemic regions could be positive for anti-*Leishmania* antibodies (Chappuis *et al*., 2007). Antibody based tests can therefore not be used in isolation but in combination with standard clinical case determination for Leishmaniasis.

Theoretically, antigen detection tests are generally more specific than antibody detection tests. This is because in antigen detection there is little to no chance of cross reactivity and can accurately distinguish between infections from past infections (Guerin *et al*., 2002). For this reason, antigen based diagnostic kits have been developed for the diagnoses of visceral Leishmaniasis especially. This diagnostic tool involves the detection of a heat stable low molecular weight carbohydrate antigen in the urine of visceral Leishmaniasis patients (Chappuis *et al*., 2007). Several studies conducted in East Africa and the Indian sub-continent showed good specificity but low to moderate sensitivity. An example is a

clinical trial performed in Nepal on suspected patients in a visceral Leishmaniasis endemic area where the selectivity was disappointingly low (Boelaert & Bhattacharya, 2007; Chappuis *et al*., 2007). With respect to specificity, the urine has to be boiled to lower the chances of false positives. Differentiating weakly positive samples from negative samples poses a significant challenge (Boelaert & Bhattacharya, 2007).

Although work is still ongoing to improve antigen-detection as a diagnostic tool, several researchers are also focusing on polymerase chain reactions (PCR) as a diagnostic tool. The PCR techniques currently in use include the Kinetoplast DNA (KDNA) PCR, the rRNA gene Internal Transcribed Spacer 1 (ITS1) and the spliced leader mini-exon PCR (Bensoussan & Nasereddin, 2006). KDNA PCR has been found to be the most sensitive in diagnosing cutaneous Leishmaniasis with 98.7% sensitivity. ITS1 PCR and spliced leader mini-exon PCR have 91% and 53.8% sensitivity respectively (Bensoussan & Nasereddin, 2006). PCR in general has been found to have a 45%-93% sensitivity of detecting visceral Leishmaniasis depending on the test sample (Abbasi *et al*., 2013). Peripheral blood is the test sample with the least sensitivity and is therefore suitable for first line screening only. The test samples with high sensitivity are bone marrow and splenic aspirates but are limited by the skill needed to collect samples and the risk factor (Abbasi *et al*., 2013). Other limitations associated with PCR as a diagnostic tool is that PCR is a very expensive technique relative to microscopy. PCR is also unable to distinguish asymptomatic cases from disease cases. With the current situation of highly toxic available drugs, this would pose a huge dilemma to physicians (Abbasi *et al*., 2013). None of the above mentioned techniques are employed in the hospitals and health centers in Ghana. There is currently no diagnoses for Leishmaniasis in the Ghana (Landau, 2008).

2.3.2 Treatment

Despite the obvious problems associated with the diagnoses of Leishmaniasis, individuals positively diagnosed with the Leishmaniasis (usually by microscopy) and exhibiting symptoms of Leishmaniasis are treated with the available anti-*Leishmania* drugs, although these drugs have been reported to have high toxic effects (St George, Bishop, Titus, & Selitrennikoff, 2006).

The treatment of Leishmaniasis involves the use of specific anti-*Leishmania* drugs and the aggressive management of any bacteria or parasitic co-infection, hypovolemia (decreased blood volume) and malnutrition (Gupta, 2011). Although alternate chemotherapy are in existence and are being developed against Leishmaniasis, pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate remain the first line drugs for the treatment of visceral Leishmaniasis in many endemic areas (Gupta, 2011). The pentavlent antimonial based drugs are used for the treatment of all clinical forms of Leishmaniasis in the Old World. Although the branded forms of antimonials are expensive, there are cheaper generic forms of these antimonials available (Kobets *et al*., 2012). The efficacy of the antimonials has however been reported to be dependent on not only the clinical form but also the species of *Leishmania* as well as the geographical region (Kobets *et al*., 2012).

The major limitation to the use of antimonials even in the regions that have reported them to have high efficacy is their high level of toxicity which results in frequent and sometimes life threatening side effects (Singh & Sundar, 2014). The side effects may include cardiac arrhythmia and acute pancreatitis (Gupta, 2011). There are reports that suggest that children under the age of two and adults above 45 years with signs of advanced disease and/or malnutrition are the groups with the highest risk of fatality post antimonial

treatment. This is because of the drug toxicity, slowness of drug action, visceral Leishmaniasis associated complications or a combination of the above reasons (Gupta, 2011). In terms of efficacy, there have been several reports of resistance of the *Leishmania* parasite against the antimonials in India rendering them useless in the country that has the highest incidence of visceral Leishmaniasis in the world (Guerin *et al*., 2002). Also antimonials have been found to have little to no efficacy against *L. donovani* (Kobets *et al*., 2012).

The second line drugs used in the treatment of Leishmaniasis are amphotericin B and pentamidine. Amphotericin B is fast replacing the antimonials as the first line drug in regions where the antimonials have recorded high failure(Chappuis *et al*., 2007). The anti-*Leishmania* activity of amphotericin B is attributed to its selective affinity for ergosterol, a constituent of the *Leishmania* plasma membrane but not cholesterol, the primary sterol component in mammalian cells (Kobets *et al*., 2012). In the *Leishmania* parasite, amphotericin B induces the formation of aqueous pores in the parasite plasma membrane and therefore has an excellent leishmanicidal effect (Kobets *et al*., 2012). Amphotericin B has however been reported to have very low solubility and bioavailability (Kobets *et al*., 2012). Other than these limitations, Amphotericin B, like the antimonials, has also been reported to have adverse side effects associated with its usage. These side effects may include infusion related fever chills and rigors (a universal side effect) and life threatening adverse side effects such as hypokalemia/low potassium levels in blood, nephrotoxicity and first-dose anaphylaxis (Chappuis *et al*., 2007). Besides these adverse side effects, amphotericin B is very expensive and has a very complex regimen. For example treatment with amphotericin B requires 15 slow

infusions on alternate days (Chappuis *et al*., 2007; Kobets *et al*., 2012; Pink *et al*., 2005).

The toxicity of amphotericin B has been attributed to the lipid formulations of the drug. This toxic effect has been reduced by replacing deoxychelate in amphotericin B with other lipids that facilitate its preferential uptake by the reticulo endothelial cells ensuring a targeted drug delivery to the parasite increasing efficacy while reducing toxicity (Kobets *et al*., 2012). Three such lipid associations of amphotericn B that are currently available are liposomal amphotericin B (L-AMB), amphotericin B lipid complex (ABLC) and amphotericin B colloidal dispersion (ABCD) (Gupta, 2011; Kobets *et al*., 2012). From the three formulations, L-AMB is the one considered by some experts as the best existing drug against visceral Leishmaniasis (Gupta, 2011; Singh & Sundar, 2014). Although the high cost of L-AMB has proven to be a considerable limitation, the major limitation to the use of L-AMB is that its efficacy is dependent on either the genetics of the population in different endemic areas and/or the species of *Leishmania* in the different endemic areas (Kobets *et al*., 2012).

Pentamidine on the other hand is progressively being abandoned as a second line therapyfor visceral Leishmaniasis. This is because the use of pentamidine is not only challenged by its high toxicity but also the emergence of parasite resistance in India (Guerin *et al*., 2002). The adverse side effects of pentamidine may include nephrotoxicity, cardiotoxicity, diabetes mellitus-like state and hypoglycaemia (Demagos, Baltus, & Höfle, 1981; Kobets *et al*., 2012). Although there are reports suggesting that pentamidine is still used in treatment of cutaneous and muco-cutaneous Leishmaniasis, the World Health Organization (WHO) has recommended that pentamidine be used only when there are no other options available (Kobets *et al*., 2012).

Paramomycin is an aminoglycoside developed as an anti-*Leishmania* agent in the 1960's but ignored until the 1980's when topical formulations were found to be effective against cutaneous Leishmaniasis leading to the development of the parenteral formulation for visceral Leishmanaisis (Kobets *et al*., 2012; Singh & Sundar, 2014). Although Paramomycin is not expensive and has relatively lower toxicity, its administration has to be done parenterally and its efficacy is dependent on the species of *Leishmania* (Guerin *et al*., 2002; Singh & Sundar, 2014).

Although a wide range of anti-*Leishmania* drugs have been developed in more recent times with associated limitations, miltefosine has proven to be the most promising candidate (Kobets *et al*., 2012; Sundar & Olliaro, 2007). Miltefosine is an alkyl phospholipid derivative and the first orally effective anti-*Leishmania* agent (Sundar & Olliaro, 2007). In terms of mechanism of action, miltefosine has been shown to induce apoptosis-like death in *L. donovani* promastigotes (Kobets *et al*., 2012). There has also been prevalent but not fully established hypothesis that miltefosine inhibits the synthesis of Phosphotidyl choline (PC), an essential element in the synthesis and integrity of parasite cellular membranes and a source of signaling molecules (Sundar & Olliaro, 2007). In terms of anti-*Leishmania* efficacy, it has been reported that when miltefosine is given daily under medical supervision for 4 weeks, it cures 94% of patients (both children and adults) and has a relatively low toxicity (Sundar & Olliaro, 2007). This therapy is however challenged by two major limitations; the first is adherence mainly because of the long course of the drug. The major fear associated with lack of adherence is the emergence of resistant strains of the parasite. Some experts suggest that the problem of adherence could be reduced or solved with combination therapy as this might reduce the longevity of the treatment and

encourage adherence (Seifert, 2011). The second and possibly the most important limitation is that miltefosine has been reported to be teratogenic; inhibiting the development of the embryo of fetus resulting in either a halt of the pregnancy or the induction of a congenital malformation (birth defect) (Seifert, 2011). This means that pregnancy should be avoided during treatment with miltefosine and the first two months after. The distribution of this drug must therefore be done under strict supervision with structures put in place to protect females of child bearing age (Seifert, 2011). None of the above mentioned drugs are available to people suffering from Leishmaniasis in Ghana. There is no treatment for Leishmaniasis in Ghana (Landau, 2008).

2.4 Medicinal Plants

The use of traditional medicine (medicinal plants) is an indispensable factor in the treatment of diseases in Africa. Other than the fact that it is a cheaper form of therapy for many people across the African continent, there are also so many rural communities in Africa where health care facilities are unavailable or at best very poor (Darko, 2012). In Ghana, the ratio of medical doctors to patients, as reported in 2012, is 1:6000. This highlights the overburdened health facilities as well as the obvious shortcomings associated with such a burden. This is a typical example of a reason why people would resort to traditional medicine (Darko, 2012). Another reason traditional medicine is so popular is that it is recommended highly by the rural folk despite the obvious limitation of having little or no documentation of the indigenous knowledge on the use of plant medicine. Other than the absence of proper documentation on the use of plant medicine, there is also little or no knowledge on the active ingredients, the possible toxicity nor the mode of action. The use of traditional medicine is not limited to Africa alone but goes far

beyond the borders of Africa along with the associated limitation in documentation as well as scientific data. The World Health Organization (WHO) has estimated that

50% of the worlds' population use traditional medicine for their health care (Saki, Khademvatan, & Nanaei, 2011). The part of the plant used in the preparation of traditional medicine differs between diseases as well as localities.

With respect to infectious diseases, medicinal plants have been used in treatment for many years. It is for this reason that WHO laid emphasis on the need to investigate the anti-*Leishmania* effect of plants used in traditional medicine which subsequently led to some interesting/promising findings (Ozbilgin *et al*., 2014). These findings were not totally unexpected because plants have been used in the treatment of various ailments for many centuries with over 25% of recommended drugs being plant derivatives with or without supplementary adjustments (Shah, Khan, & Nadhman, 2014). Furthermore, 28% of 1184 new drugs registered between 1981 and 2000 were plant products or their derivatives (Wink, 2012). An extra 24% of these drugs had pharmacophores (functional groups with pharmacological activity) derived from plant products (Wink, 2012).

Although many more promising results have been obtained by various research groups working on medicinal plants, the translation of these results into clinical practice still remains a neglected field (Wink, 2012). This has led various research groups to put steps in place to back the efficacy of medicinal plants available in their countries with scientific evidence to facilitate the use of these medicinal plants as a cheaper and more readily available alternate as well as a possible source of active compounds to be used in the production of orthodox drugs.

The methanol extracts of *Eryngium thorifolium*, a turkish medicinal plant, has been reported to be a promising candidate to be used for the treatment of *Leishmania tropica* infections (Ozbilgin *et al*., 2014). Also, 5 medicinal plants, *Hyptis pectinata* (Leaves) Poit, Aloe vera (leaves), *P faffia glomerata* (spreng) Pedersens and *Chenopodium ambrosioides*, used in the treatment of cutaneous Leishmaniasis indigenously in Alagoas, North Eastern Brazil were reported to have *in vitro* anti-*Leishmania* activity at 100 µg/ml (Queiroz *et al*., 2014). Methanolic and ethanolic extracts of *Satureja khuzestanica,*an Iranian medicinal plant has activity against *Leishmania major* promastigotes with IC_{50} (concentration of the extracts that inhibits parasite proliferation by 50%) values of 4.8mg/ml and 2.4mg/ml respectively (Saki *et al*., 2011). The methanolic extract of *Jurinea dolomiaea*, a medicinal plant from Pakistan has been reported to be potent against *Leishmania* parasites (Shah *et al*., 2014).

The methanol extract of *Annona senegalensis* seeds were reported to be active against *Leishmania major* promastigotes but not *Leishmania donovani* promastigotes at an IC_{50} of 200 µg/ml (Sahpaz *et al*., 1994). However, acetogenins from *A. senegalensis* seeds have been reported to be active against both *L. major* and *L. donovani*, at IC_{50} values of 50µg/ml and 25 µg/ml respectively (Akendengue, Ngou-Milama, Laurens, & Hocquemiller, 1999).

In Ghana, There is no report of tests conducted on any Ghanaian medicinal plant for anti-*Leishmania* activity. There is also no documented evidence of any plant medicine used in the treatment of Leishmaniasis in Ghana.

2.5 Control of Leishmaniasis in Ghana

In Ghana, there is no control program for Leishmaniasis. There was however a mass spraying with insecticides when Leishmaniasis was first detected in Ghana and this is believed to have played a role in the control of Leishmaniasis although it is far from sufficient (Landau, 2008).

CHAPTER THREE

3.0 Materials and Methods

3.1 Plant Extract Collection

Plants were collected and identified by experts at the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong. Plants' extracts were prepared with 50% ethanol at

CSRPM and transferred to the Noguchi Memorial Institute for Medical Research (NMIMR) in the form of paste, powder or pellets and stored at 4ºC until used.

3.2 Plants' Extract Preparation for anti-*Leishmania* activity Assays

Plants' extracts (extracts) were weighed and dissolved with 50% ethanol to a concentration of 10 mg/ml and filtered in a biosafety cabinet to remove all contaminants. Extracts once dissolved were used on the day of preparation. The extracts were diluted with growth culture medium (M199, GIBCO®) to a concentration of 400 µg/ml in a 24 well plate and then tested for anti-*Leishmania* activity.

3.3 *Leishmania* Promastigote Cell culturing

About 500 µl of promastigote *Leishmania donovani* cultures donated by the US Naval Medical Research Unit-3 (NAMRU-3) and stored in liquid nitrogen was thawed rapidly by rubbing between the palms of the hands. The thawed sample was left at room temperature for 30 minutes. About 10 µl of the stock solution was viewed under the microscope to assess the viability of the parasites based on their motility. The rest of the stock was placed in a 25 cm^2 culture flask. About 5 ml of promastigote culture medium (M199 GIBCO®) (pH=7) added drop wise and very slowly, mixing after each drop. A drop of the culture was put on a slide and the viability re-checked. The culture was incubated at 25ºC for 3 days to confirm growth and the absence of contaminants. The parasites were sub-cultured when they were confluent i.e. concentration reaches about $13x10^7$ cells/ml to establish a stable population.

3.4 DRUG ASSAYS

3.4.1 Test for the anti-*Leishmania* activity of plants' Extracts

The alamar Blue® assay (Invitrogen, USA) is a cell viability assay that was used to test for the efficacy of the extracts to inhibit proliferation of *Leishmania donovani* promastigotes. The test was performed in a 96-well plate. About 2.5 x 10^6cells/ml of parasites were seeded with varied concentrations, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml, of ethanolic plants' extracts. The final concentration of ethanol in the preparation was below 1%. Negative controls were similar concentrations of parasite but without extracts. Positive control was varying concentrations (2 µg/ml, 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml) of amphotericin B. Each sample was done in triplicates. The setup was incubated at 25°C for 44 hours, then 10% Alamar blue dye was added and incubated for 4 hours at 25°C in the dark. The plate was read for absorbance at 540nm using the Wako Spectrophotometer (TECAN Sunrise, USA) (Figure 9A, Appendix). IC_{50} values of extracts were calculated by the pro of inhibition curve. Activity of extracts was categorized by a range of IC_{50} values as varying degrees of positivity (+++, ++, +) or negative (-). Eight of the extracts with very strong anti-*Leishmania* activity, IC_{50} values ranging from 10.1 µg/ml to 18.2 µg/ml, were subjected to mechanistic studies.

3.5 Mechanistic studies

3.5.1 Determination of the Apoptosis Inducing Properties of Active Extracts by FACS

The nexin apoptosis assay was used to determine the apoptosis inducing properties of the active extracts. Seeding and incubation of parasites with extracts were done under the same condition of Alamar Blue assay as described above (section 3.4.1). The set-up was incubated for 24 hours. The Guava reagent for Nexin was added to each well in the ratio

of 1:1 and mixed properly. The set-up was incubated in the dark for 20 minutes at room temperature. The plate was read using the Millipore guava easyCyte 5HT FACS machine according to the manufacturer's instruction Figure 9B, appendix).

3.5.2 Determination of the Effect of the Active Extract on Parasite Morphology To determine whether or not the crude extract induced morphological changes in the parasites, 50 µg/ml of each of the eight active extracts were incubated with parasites and then subjected to immunohistochemistry using DAPI which stains the nucleus and the kinetoplast of the parasite. After incubation with or without extracts for 24 hours, parasites were harvested and fixed with 70 % ethanol on an 8 well chamber slide at 20^{o}C for an hour. The parasites were washed twice with 500 µl of PBS for 5 minutes each and PBST (0.1 % Triton X 100 in PBS) at room temperature for 15 minutes. About 500 µl of blocking reagent (3 % BSA in PBS) was added and incubated for 30 minutes at room temperature. The nucleus and kinetoplasts were stained with 4,6-Diamidino-2Phenylindole, DAPI, (5 µg/ml DAPI in PBS) for 10 minutes. The slides were then washed again as described above, mounted using parmafluor mounting reagent and covered with cover slips. The slides were observed under the Olympus fluorescent microscope at a magnification of X100 (Figure 9C, appendix).

3.6 Data analysis

The data obtained from the alamar Blue® and Apoptosis assays were analyzed by comparing the mean activity between the test groups and the negative (solvent) control. In the alamar Blue® assay, a dose-dependent curve was used to determine the IC_{50} value of each extract. The IC_{50} values are used to determine the anti-*Leishmania* activity of each extract. The higher the IC_{50} value, the lower the activity and vice versa.

In the apoptosis assay, the parasites were grouped into four (4) quadrants by the FACS machine. Parasites in the lower left quadrant were intact or viable. The lower and upper right quadrants represented parasites in the early and late stages of apoptosis while the upper left quadrant represented parasites undergoing necrosis. A comparison of the distribution of the extract treated parasites to the non-extract treated parasites was used to determine the apoptosis inducing properties of each extract.

In immunohistochemistry, the stained parasites were observed under the fluorescent microscope for characteristic fluorescence. The extract treated parasites were compared to the negative control to determine differences in morphology as well as differences in the nucleus and kinetoplast.

CHAPTER FOUR

4.0 Results

4.1 Anti-*Leishmania* effect of ethanolic crude extracts

Out of 96 crude extracts tested, 66.67 %, had no activity and had IC_{50} values above 100 µg/ml, while 33.33 %, had varying degrees of activity (Figure 6, detail in table 3 in appendix). The extract with the highest activity, 045L, had an IC_{50} value of 10.1 µg/ml while the extract with the least activity had an IC_{50} value of 88.9 µg/ml (details in Table 3

in appendix). Eight extracts with high activity were further analyzed for mechanistic activity. The Eight extracts worked on extensively were from seven different plants (Table 1).

4.2 Mechanism of action of selected active ethanolic crude extracts

4.2.1 Determination of the Apoptosis Inducing Properties of extracts

All eight extracts showed no apoptotic signals within the concentration range tested (Figure 7). 0.5 µg/ml of amphotericin B (five times the IC_{50}), showed slight induction of apoptosis with 2.3% of parasite cells, compared to 0.0% in the negative control. The parasite cells are represented by the red dots in the dot plot (Figure 7).

4.2.2 Effect of eight active extracts on the morphology of the *Leishmania* promastigote

Out of the eight extracts tested four caused nuclei fragmentation in the *Leishmania* parasite while one extract increased the number of nuclei in a single parasite (Figure 8, Table 2). One of the extract caused kinetoplast disintegration while four had no observable effect on the kinetoplast mainly because of the fragmented nuclei. Each extract had a different effect on the parasite flagella ranging from multiple flagella on parasites to the absence of a prominent flagellum (Figure 8, Table 2). Parasites in the negative control were long and slender with prominent flagella. They were evenly distributed with an intact nucleus and kinetoplast.

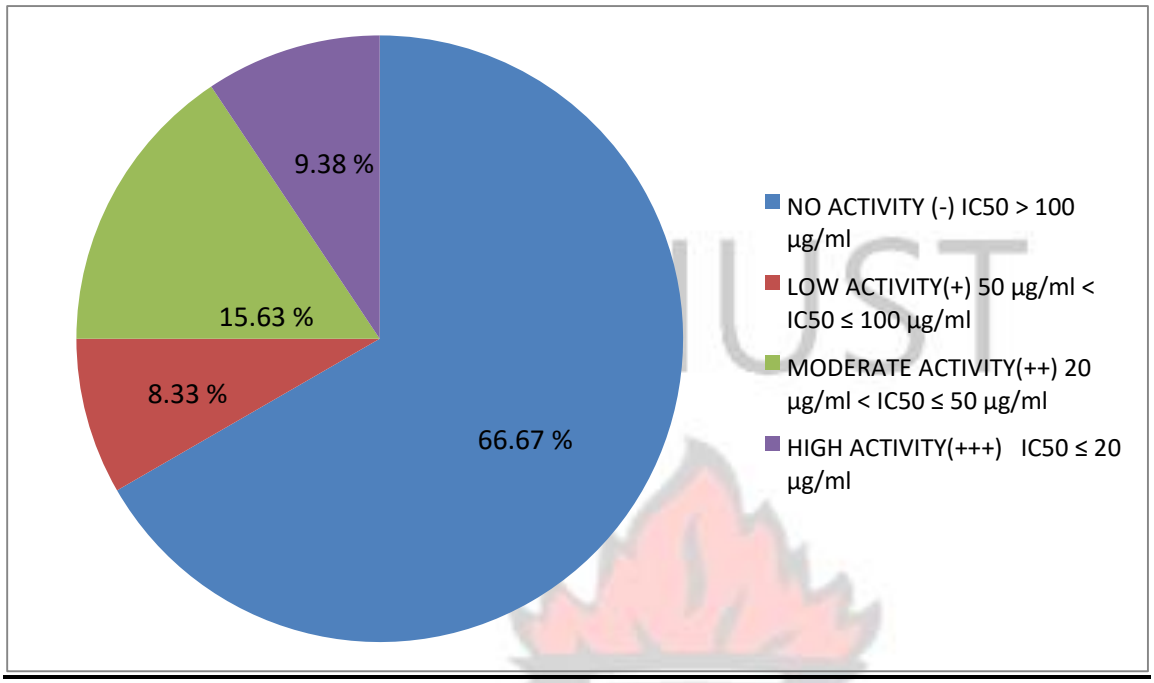

Figure 6: Proportion of plants' extracts with varying levels of anti-*Leishmania* activity

Table 1: eight active extracts used for mechanistic analysis

CODE OF EXTRACT	PLANT NAME	PLANT PART	IC_{50}(µg/ml)
1. 032L	*Cola cordifolia*	Leaves	18.2
2. 035L	*Annona senegalensis*	Leaves	10.8
3. 036R	*Clausena anisata*	Roots	12.1
4. 038L	*Bridellia ferruginea*	Leaves	16.5
5. 045L	*Cassia alata*	Leaves	10.1
6. 050SBL	*Parkia clappertoniana*	Stem back and Leaves	17.6
7. 050L	*Parkia clappertoniana*	Leaves	17.3
8. 064R	*Zanthoxylum xanthoxyloides*	Roots	13.5

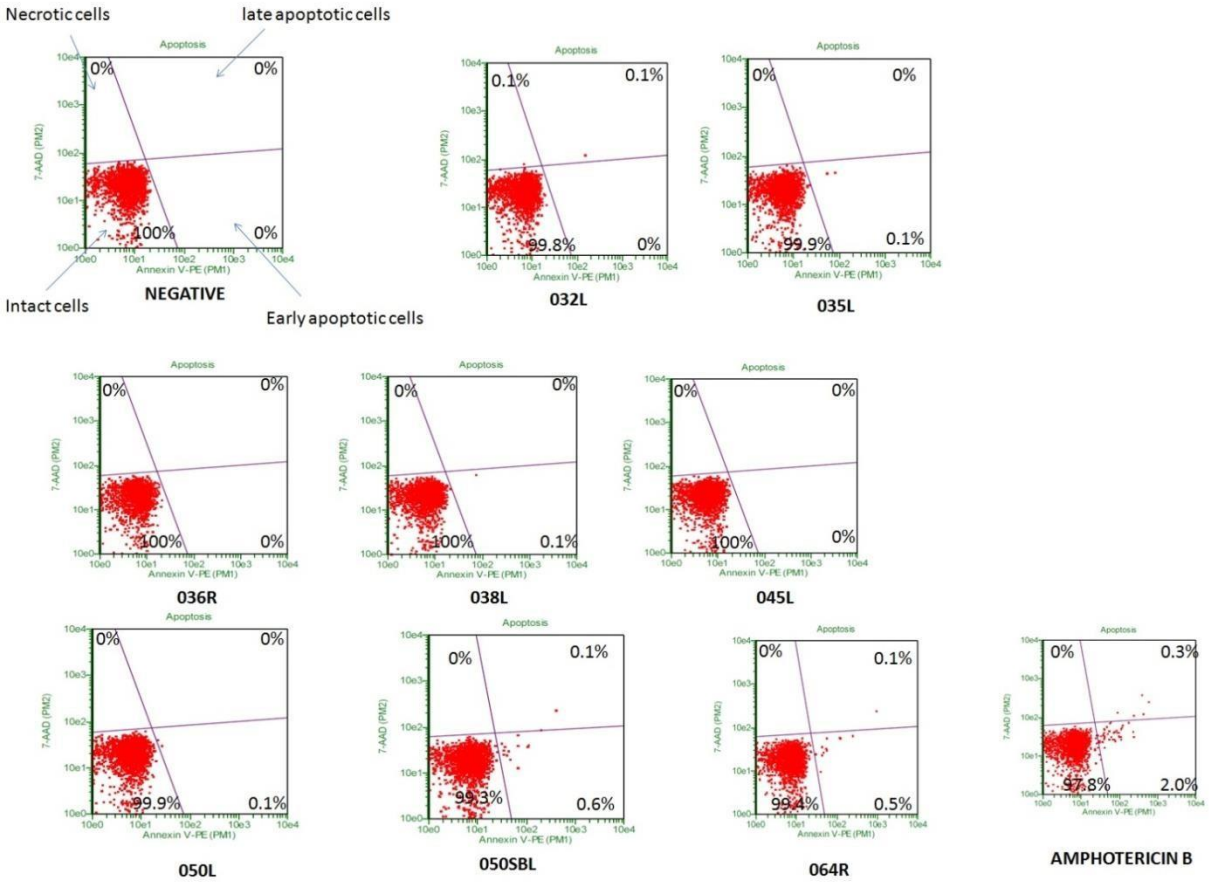

Figure 7: Dot plot of nexin apoptosis assay. Plants' extracts did not induce apoptosis at the concentration range tested.

Red dots represent the *Leishmania* parasites; Negative means parasites cultured without plant extracts, 032L … 064R are the codes of the plants' extracts. Amphotericin B is the positive control.

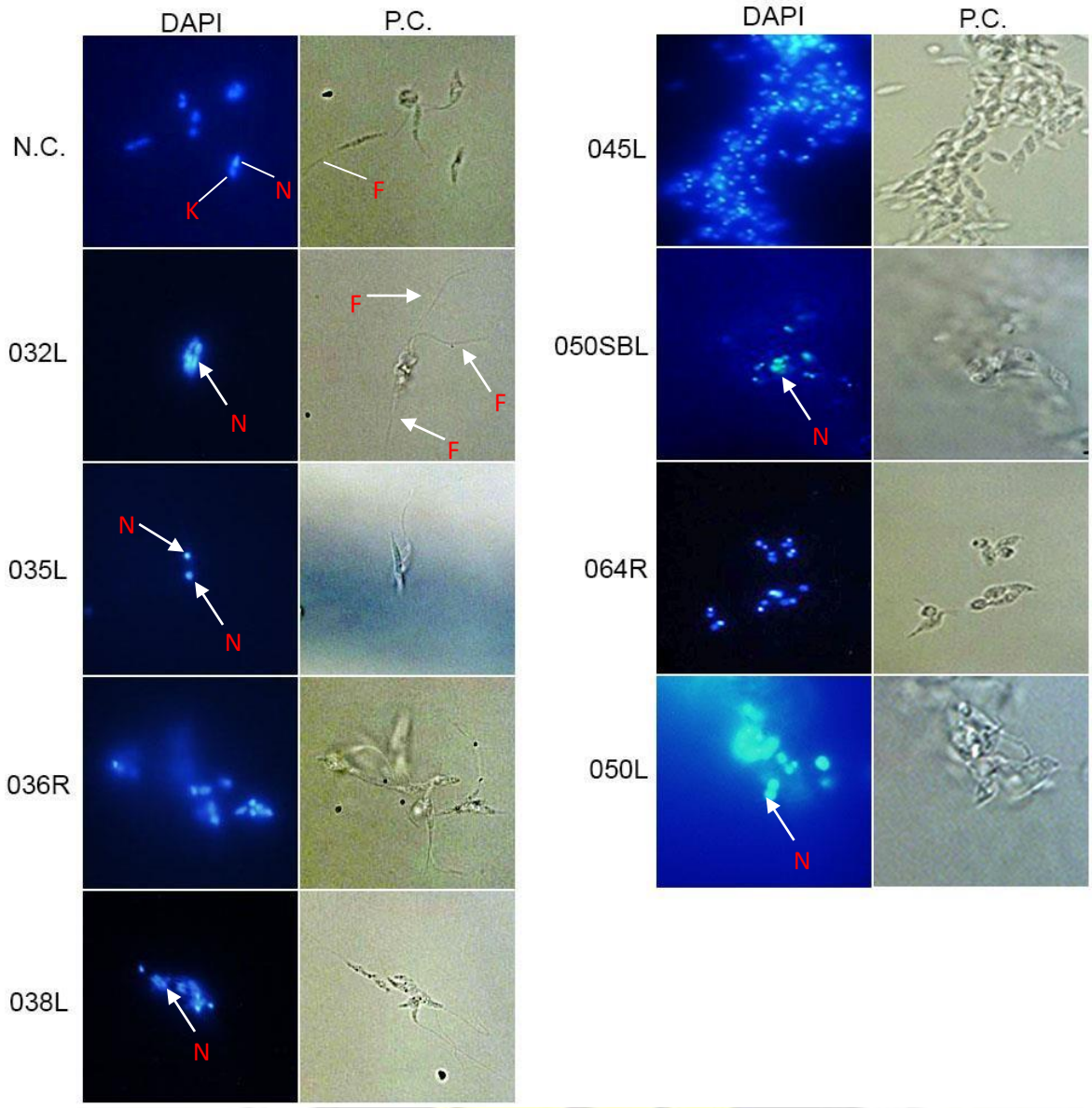

Figure 8: Immune-fluorescence assay showing the effect of plants' extracts on the nucleus, kinetoplast and morphology of the parasite. P.C. is the phase contract view, DAPI is the fluorescence view, N is the nucleus, K is the kinetoplast and F is the flagella. Pictures were taken with a 100X magnification and exposure time of 3.92 ms.

Table 2: Effect of extracts on parasite morphology, nucleus and kinetoplast

EXTRACT	NUCLEUS	KINETOPLAST	MORPHOLGY/ DISTRIBUTION	FLAGELLA
032 L	fragmented	No observable effect	Incomplete division of parasites	Multiple flagella
035L	Intact	Absent / disintegrated	Partly fused daughter parasites	Not prominent
036R	Intact	Intact	Aggregated with abnormal morphology	Prominent
038L	Fragmented	No observable effect	Parasites linked to each other	Prominent flagella
045L	Multiple per parasite	Multiple per parasite	Short and stumpy with large size aggregation	Not prominent
050SBL	Fragmented	No observable effect	Small sized aggregations	Shortened
064R	Intact	intact	Short and stumpy with small sized aggregations	Not prominent
050L	Fragmented	No observable effect	Small sized aggregations	Shortened

CHAPTER FIVE

5.0 Discussion

The aim of the study was to identify anti-*Leishmania* properties of selected Ghanaian medicinal plants and investigate molecular mechanisms such as apoptosis and morphological changes induced by the crude extracts.

In this study, 2 extracts, 035L and 064R, found to have high anti-*Leishmania donovani* activity were extracted from 2 plants, *Annona senegalensis* and *Zanthoxylum xanthoxyloides,* that had previously been reported by separate studies to have some anti*L. maj*or and/or *L. donovani* activity (Akendengue *et al*., 1999; Sahpaz *et al*., 1994). The current study did not include *L . major* parasites because of availability.

The methanol extract of *Annona senegalensis* seeds reported to be active against *Leishmania major* promastigotes but not *Leishmania donovani* promastigotes had an IC_{50} of 200 µg/ml (Sahpaz *et al*., 1994). However Akendengue *et al*. (1999) reported that acetogenins from *A. senegalensis* seeds had activity against both *L. major* and *L. donovani*, at IC_{50} values of 50 µg/ml and 25 µg/ml respectively. The current study found 50% ethanolic extracts of *A. senegalensis* leaves (035L) to have activity against *L. donovani* promastigotes at an IC_{50} value of 10.8 µg/ml which is lower than the IC_{50} values in the reported studies. The current study further showed that 035L had no observable effect on the nucleus of the *L. donovani* promastigotes but caused kinetoplast disintegration. Promastigotes treated with 035L were also observed to be paired without observable flagella which implied that nuclear division had occurred to produce two daughter cells but the absence of the kinetoplast inhibited flagella formation. The absence of the flagella prevent the new daughter cells from dividing completely from each other to form two distinct daughter cells and subsequently leads to the death of the parasites.

Zanthoxylum xanthoxyloides root extracts (064R) have been reported to have activity against amastigote *L. major* parasites (Maximin, 2007). The current study also found 064R to have activity against *L. donovani* promastigotes with an IC_{50} value of 13.5 µg/ml and in addition induced short and stumpy form parasites. It has been reported that in some

kinetoplastids, such as *Trypanosoma brucei sp*, transformation from long slender forms to short stumpy forms in the mammalian host facilitates death and prolong the life span of the host (Debrabant & Nakhasi, 2003). Extract 064R also caused small aggregation of parasites. The aggregation may also be due to the loss of the prominent flagella of the promastigote parasites, resulting in suppression of their motility and ability to divide into distinct cells which may subsequently lead to parasite death.

In this study, none of the 8 extracts showed apoptotic effect on the parasites in the concentration range tested indicating that the 8 extracts did not inhibit parasite growth and proliferation through the apoptotic pathway. *Leishmania spp*. have been shown to use the apoptosis mechanism for successful survival (Shaha, 2006). There are reports indicating that Hydrogen peroxide (a non treatment chemical) and miltefosine (an anti*Leishmania* treatment drug) induce apoptosis in *Leishmania spp*. (Berman, 2005; Das, Mukherjee, & Shaha, 2001). It was also observed in this study that amphotericin B induced apoptosis in *Leishmania donovani* parasites however no such reports were found in existing literature.

Parasites treated with *Cola cordifolia* leaf extracts (032L) had nuclear fragmentation. Extract 032L treated parasites had multiple prominent flagella but did not appear to be undergoing cell division. This may mean that there is kinetoplast division resulting in the formation of new flagella. The nuclear fragmentation however prevents cell division from happening resulting in a single parasite cell having multiple flagella and a distorted morphology. These parasites are unable to produce new daughter cells and eventually die.

Clausena anisata root extract (036R) treated parasites were irregularly shaped and with minor aggregations. Although there was no observable defect on the nucleus or kinetoplast, the irregular shaped 036R treated parasites aggregated because of their inability to move rapidly. This aggregation subsequently led to their death preventing them from passing this undesirable trait to their progeny.

Bridellia ferruginea leaf extract (038L) treated parasites were observed to have undergone nuclear fragmentation. The 038L treated parasites were slender with observable flagella. The parasites were however observed to have undergone partial cell division forming a colony of cells joined at the posterior end. This inhibition of cell division might have been as a result of the fragmented nuclei and could result in parasite death. Nuclear fragmentation has been reported to be one of the occurrences that lead to the induction of apoptosis (Debrabant & Nakhasi, 2003). Apoptotic signals were however not observed in 036R nor 038L treated parasites indicating that nuclear fragmentation might have induced through a pathway other than the apoptotic pathway.

Parasites treated with *Cassia alata* leaf extract (045L) had formed relatively the largest aggregation of parasites. There was a large number of parasites in the aggregations that had double nuclei and kinetoplasts. This may be due to the parasites undergoing kinetoplast and nuclei division but not progressing into cytokinesis to form two distinct daughter cells.

Parasites treated with *Parkia clappertoniana* extracts (050SBL/050L) had formed aggregations made up of rounded cells with no flagella and stumpy cells with a shortened flagella. There was no observable effect on the nucleus or kinetoplast of some of the parasites in the aggregation while others had fragmented nuclei. In this group as well, the

absence of apoptotic signals may imply that the fragmentation of nuclei and the subsequent cell death maybe occurring via a different pathway.

Over all, most of the eight plants' extracts on which extensive work was done affected the flagella to varying degrees. In the promastigote stage of the parasites, the flagellum plays an irreplaceable role, including parasite motility, division, proliferation and invasion of the macrophages in the mammalian host (Santrich *et al*., 1997). This implies that any defect on the flagellum results in the inability of the parasite to function optimally and results in its death. It also means that once the promastigote *Leishmania* loses its flagellum its ability to invade the mammalian macrophage is hindered therefore preventing the progression of the parasite infection to the disease state.

In addition, the flagellum of amastigote *Leishmania* parasites plays an irreplaceable sensory role which is key to the parasites survival (Gluenz *et al*., 2010). These facts strongly suggest that although the function of the flagellum differs in each stage of the life cycle, suppression of flagellum function does hinder their ability to survive.

CHAPTER SIX

6.0 Conclusion

Out of eight extracts worked on extensively, this study reports on the anti-*Leishmania* activity of six of them for the first time.

Although all eight active ethanolic plants' extracts are promising candidates for the development of a treatment drug for Leishmaniasis, *Cassia* alata (045L) had the highest activity and inhibited cytokinesis.

6.1 Study Limitation

- The study was carried out using promastigote Leishmania parasites.

6.2 Recommendations

- The eight active extracts should be tested on amastigote *Leishmania* parasites. This is because, the amastigote forms of the parasite is the most common stage in mammals and would therefore give an idea as to whether the active extracts are better suited as prophylactic drugs or curative drugs

- The eight active extracts should undergo fractionation and purification to determine the active compound or component. This could facilitate the characterization of the active component.
- *In vivo* efficacy of the crude extracts as well as the active compounds which are an important step in drug development should be determined to ascertain the suitability of the crude extracts and/or the active compounds as good candidates for herbal medicine or orthodox drug development.

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Appendix

Table 3: IC_{50} values of 96 ethanolic crude extracts from Ghanaian medicinal plants

CODE OF EXTRACT	IC_{50}(µg/ml)	ACTIVITY
1. 001L	>1000	-

2. OO1R	>1000	-
3. 002SB	>1000	-
4. 002L	>1000	-
5. 002R	>1000	-
6. 003L	>1000	-
7. 003R	138.9	-
8. 003SB	784	-
9. 004L	>1000	-
10. 004SB	>1000	-
11. 005SB	60.2	+
12. 005L	>1000	-
13. 006SB	>1000	-
14. 006L	>1000	-

CODE OF EXTRACT	**IC_{50}(µg/ml)**	**ACTIVITY**
15. 007SB	>1000	-
16. 008R	>1000	-
17. 008SB	>1000	-
18. 008L	>1000	-
19. 009L	>1000	-
20. 010L	>1000	-
21. 011SB	>1000	-

22. 012L	>1000	-
23. 013S	>1000	-
24. 013WP	>1000	-
25. 013L	>1000	-
26. 015L	81.5	+
27. 018F	173.6	-
28. 018L	542.86	-
29. 019SB	37.0	++
30. 019L	88.89	+
31. 020R	66.0	+
32. 020SB	68.6	+
33. 022L	443.2	-
34. 023R	142.6	-
35. 023L	62.9	+
36. 024SB	159.4	-

CODE OF EXTRACT	**IC_{50}(µg/ml)**	**ACTIVITY**
37. 024L	673.1	-
38. 025SB	>1000	-
39. 0026L	>1000	-
40. 026LB	149.99	-
41. 027R	130.14	-

42. 028SB	>1000	-
43. 028L	631.0	-
44. 029SB	601.4	-
45. 029L	137.0	-
46. 030SB	843.7	-
47. 030R	79	+
48. 030L	41.5	++
49. 031SB	31.1	++
50. 032SB	25.1	++
51. 032L	18.2	+++
52. 033SB	44.8	++
53. 034WP	18.6	+++
54. 035SC	27.8	++
55. 035L	10.8	+++
56. 036R	12.1	+++
57. 037L	21.9	++
58. 037SB	28	++

CODE OF EXTRACT	**IC_{50}(µg/ml)**	**ACTIVITY**
59. 038L	16.5	+++
60. 039SB	43.2	++
61. 040SB	34.4	++

62. 041WP	119.4	-
63. 043SB	77.1	+
64. 045L	10.1	+++
65. 046L	>1000	-
66. 047WP	162.24	-
67. 048SBL	23.2	++
68. 048L	24.9	++
69. 049LSB	47.8	++
70. 050SBL	17.6	+++
71. 050L	17.3	+++
72. 051ST	36.1	++
73. 051L	>1000	-
74. 052L	>1000	-
75. 052R	>1000	-
76. 052SB	>1000	-
77. 054WP	>1000	-
78. 055L	>1000	-
79. 05LA	>1000	-
80. 055SC	>1000	-
CODE OF EXTRACT	**IC_{50}(µg/ml)**	**ACTIVITY**
81. 056L	>1000	-

82. 057SB	>1000	-
83. 058RB	>1000	-
84. 058SB	>1000	-
85. 059S	>1000	-
86. 060S	>1000	-
87. 060	>1000	-
88. 062SB	>1000	-
89. 063S	>1000	-
90. 064L	>1000	-
91. 064R	13.5	+++
92. 064SB	45.2	++
93. 065SB	>1000	-
94. 066SB	>1000	-
95. 066L	>1000	-
96. 067R	>1000	-

The degree of activity of each active extract is represented with plus signs.

$IC_{50} \leq 20\mu g/ml$; very active (+++). $20\mu g/ml < IC_{50} \leq 50\mu g/ml$; moderately active (++). $50\mu g/ml < IC_{50} \leq 100\mu g/ml$; low activity (+). All inactive extracts are represented by a minus sign (-). The IC_{50} of amphotericin B (the positive control) was 0.1µg/ml.

B

A

C

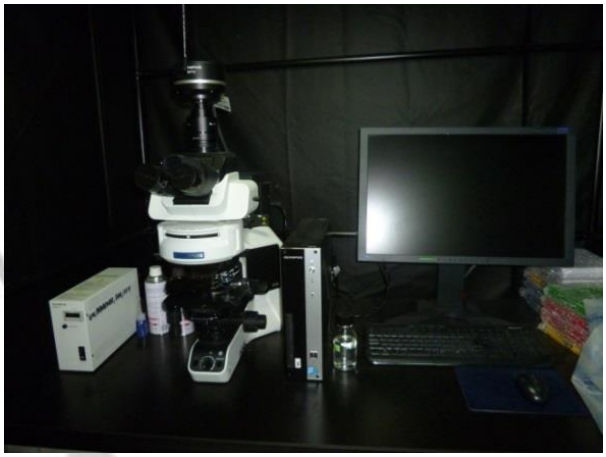

Figure 9: Instruments used in performing experiments. A is the spectrophotometer used in the alamar Blue® assay, B is the FACS machine used in the Nexin apoptosis assay and C is the Fluorescent microscope used in immunohistochemistry.