

WEST AFRICA CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

SCHOOL OF BIOLOGICAL SCIENCES

COLLEGE OF BASIC AND APPLIED SCIENCES

UNIVERSITY OF GHANA, LEGON

***IN VITRO* MECHANISTIC STUDY OF ANTI-LEISHMANIAL ACTIVITY OF NOVEL**

TETRACYCLIC IRIDOID COMPOUNDS ISOLATED FROM *MORINDA LUCIDA*

BENTH

BY

AZERIGYIK FAUSTUS AKANKPERIWEN

(10598918)



**THIS THESIS/DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA,
LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD
OF MASTER OF PHILOSOPHY IN MOLECULAR CELL BIOLOGY OF INFECTIOUS
DISEASES DEGREE.**

JULY, 2018.

TABLE OF CONTENTS

LIST OF FIGURES	III
LIST OF TABLES	IV
DECLARATION	V
DEDICATION	VI
ACKNOWLEDGEMENT	VII
ABSTRACT	VIII
CHAPTER ONE	1
1.0 INTRODUCTION.....	1
1.1 SIGNIFICANCE.....	5
1.2 RATIONALE.....	6
1.3 HYPOTHESIS	8
1.4 AIM:.....	8
1.5 SPECIFIC OBJECTIVES:.....	8
CHAPTER TWO	9
2.0 LITERATURE REVIEW	9
2.1 <i>Leishmaniasis</i>	9
2.2 <i>Sand fly vectors in leishmaniasis</i>	10
2.3 <i>Life cycle of Leishmania species</i>	14
2.4 <i>Diagnosis of leishmaniasis</i>	15
2.6 <i>Outlook of Leishmania and its control in Ghana</i>	17
2.7 <i>Traditional plant medicine</i>	20
CHAPTER THREE	27
3.0 MATERIALS AND METHODS.....	27
3.1 <i>Materials</i>	27
3.2 <i>Methods</i>	27
3.3 <i>Parasite and cell culture conditions</i>	28
3.4 <i>In vitro anti-leishmania activity of compounds against Leishmania spp</i>	29
3.5 <i>Cytotoxicity analysis of compounds on human cell lines</i>	29
3.6 <i>Mechanism of cell death and cell cycle analysis</i>	30

3.7. <i>KMP expression and morphometric analysis</i>	31
3.8. <i>Time course analysis of compounds on promastigote forms</i>	32
3.9. <i>Anti-leishmania activity of compounds on intracellular amastigotes</i>	32
3.10. <i>Statistical analysis</i>	33
3.11. <i>Ethics</i>	34
CHAPTER FOUR	35
4.0 RESULTS	35
4.1. <i>Activity of tetracyclic iridoids against Leishmania spp.</i>	35
4.2. <i>Anti-amastigote activity of tetracyclic iridoids.</i>	36
4.3. <i>Cytotoxicity of tetracyclic iridoids on normal cell lines.</i>	36
4.4. <i>Tetracyclic iridoids induced apoptotic-like cell death in Leishmania donovani.</i>	37
4.5. <i>ML-F52 and Molucidin induced cell cycle arrest with enhanced arrest at G2-M.</i>	38
4.6. <i>KMP immunohistochemistry assessment and time-course analysis.</i>	41
4.7. <i>Morphometric characterization of compound-treated promastigotes.</i>	41
CHAPTER FIVE	48
5.0 DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS	48
5.1 <i>DISCUSSIONS</i>	48
5.3 <i>CONCLUSIONS</i>	56
5.4 <i>RECOMMENDATIONS</i>	56
REFERENCES	57

LIST OF FIGURES

Figure 1	Distribution of cutaneous leishmaniasis in West Africa in 2010	8
Figure 2	Sand fly	9
Figure 3	Life cycle of <i>Leishmania</i> species	13
Figure 4	Promastigote and Amastigote forms	14
Figure 5	<i>Leishmania tropica</i> -induced bilateral pretibial ulcerations.....	15
Figure 6	Communities in the Volta Region of Ghana with reported cases of cutaneous <i>Leishmania</i>	18
Figure 7	<i>Morinda lucida</i> plant	22
Figure 8	Chemical structure of iridoids isolated from <i>M. lucida</i>	29
Figure 9	Nexin assay	37
Figure 10	Cell Cycle Analysis	39
Figure 11	Immunohistochemistry diagrams	41
Figure 12	Morphometric Analysis	44
Figure 13	Time-Course Analysis	46
Figure 14	DAPI view and Differential interference contrast (DIC) view of parasites	48

LIST OF TABLES

Table 1	Geographical distribution of medically important vectors and susceptible <i>Leishmania</i> spp.	12
Table 2	<i>In vitro</i> anti-proliferative activity of compounds against <i>Leishmania</i>	35
Table 3	IC ₅₀ of compounds on intracellular amastigotes of <i>L. donovani</i>	37
Table 4	Cytotoxicity and SI values of compounds screened against Cell lines	38

DECLARATION

I, Azerigyik Faustus Akankperiwen, do hereby declare that this submission is my own work towards the award of Master of Philosophy degree, except where due acknowledgement has been made in the text for references to other people's work, this report is the product of my own research carried out at the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, Ghana and Department of Parasitology, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, Ghana, under the supervision of Dr. Theresa Manful Gwira and Professor Mitsuko Ohashi.

Faustus Akankperiwen Azerigyik

(Student)

.....

(Signature)

.....

(Date)

Certified by:

Dr. Theresa Manful Gwira

(Supervisor)

.....

(Signature)

.....

(Date)

Professor Mitsuko Ohashi

(Co-supervisor)

.....

(Signature)

.....

(Date)

DEDICATION

This work is dedicated to the Almighty God and to my parents, Mr. And Mrs Azerigyik who have supported me all through my MPhil Programme. This is also to my awesome siblings, Richard and Abraham who have wished me well in my studies, without their prayers I would not come this far. Special thanks to Prof. Mitsuko Ohashi for the support and to all who immensely supported and contributed to my achievement.

ACKNOWLEDGEMENT

Special thanks go to the Almighty God, for His goodness and mercies always exceed my expectations and understanding. I would also like to say Thank you to the Japan Agency for Medical Research and Development (AMED) project, NMIMR, for funding this study and to the West African Centre for Cell Biology and Infectious Diseases (WACCBIP) for the Platform to carry out my MPhil Training. I also want to show appreciation to Dr. Theresa Manful Gwira and Professor Mitsuko Ohashi, my supervisors, for their patience and kindness. To my mentors and boss' Dr. Irene Ayi and Prof. Mitsuko Ohashi, I am grateful and consider myself blessed to have had the privilege of meeting you. To the Head and members of the Department of Parasitology, I am eternally grateful for your assistance in making this project a success. I will also like to say special thank you to Michael Amoa-Bosompem, Ms. Thelma Tettey, Mr. Owusu Baffour-Awuah, Ms. Georgina Djameh, Mr. Senyo Botchie, Mr. Emmanuel Blay, Mr. Kofi Kwofie, Mr. Joshua Adjah and Mr. Aboagye Kwarteng Dofuor all of whom played an invaluable role during my study. Last but not least, I thank my family for the inspiration.

ABSTRACT

Leishmaniasis is widely considered among neglected tropical disease and threatens about 350 million people globally. In spite of advances in drug discovery, high toxicity, and drug resistance limits current drugs. With current emphasizes on use of medicinal plants worldwide, three novel tetracyclic iridoids, Molucidin, ML-2-3 and ML-F52 were identified from *Morinda lucida*, a medicinal plant, to have anti-*trypanosomal* activity. This study sought to determine the activity of iridoids against *Leishmania* spp. and to determine the mechanism of action of active compounds. The activity of tetracyclic iridoids against *Leishmania donovani* (D10) and *L. major* (NR48815) were studied using promastigotes and intracellular amastigotes. Infectivity and cytotoxicity assays were performed with RAW 264.7 macrophage cells using Amphotericin as reference drug. The mechanisms of action were analyzed by performing Nexin Assay, Immunohistochemistry (IHC), and Cell cycle analysis. A 50% inhibitory concentration of compounds was determined by Alamar blue assay. Molucidin and ML-F52 inhibited the growth of promastigote in *L. donovani* (Molucidin; $IC_{50} = 2.94 \pm 0.60 \mu\text{M}$, ML-F52; $IC_{50} = 0.91 \pm 0.50 \mu\text{M}$) and *L. major* (Molucidin; $IC_{50} = 1.85 \pm 0.20 \mu\text{M}$, ML-F52; $IC_{50} = 1.77 \pm 0.20 \mu\text{M}$). ML-F52 had a 10-fold cytotoxic effect on parasites relative to normal cell lines. Against intracellular forms, Molucidin and ML-F52 inhibited intracellular amastigote replication and infectivity. Amphotericin B, Molucidin and ML-F52, induced a dose-dependent apoptotic effect on promastigotes. Molucidin and ML-F52 induced apoptotic mechanism of cell death in 38.9 % and 12.8% of promastigote culture, respectively. Both compounds inhibited cytokinesis and induced phenotypic changes in promastigote forms. Molucidin also induced ‘‘nectomonad-like’’ forms and loss of kDNA, ML-F52 induced ‘cell-rounding’ with loss of flagellum. Molucidin further induced cell growth arrest at G₂-M phase (54.5

%). A significant induction of apoptosis ($P = 0.05$) was shown by an enhanced peak in the sub-G₁ confirming the apoptotic inducing properties of Molucidin and ML-F52. This study shows the anti-*leishmania* activity of tetracyclic iridoids which could be further investigated for the development of new chemotherapy against leishmaniasis.

CHAPTER ONE

1.0 INTRODUCTION

Leishmaniasis is a protozoan infection transmitted by an infected female phlebotomine sand fly during blood meal. Leishmaniasis remains an important health condition in humans and other vertebrates because of its global prevalence and socioeconomic repercussions on the health of humans and animal life. Geographically, leishmaniasis is endemic in the tropical, subtropical, and southern Europe, in areas such as the deserts in Western Asia and the rain forests of the Americas. It is approximated that three hundred and fifty million people are threaten by the disease with 12 million in about 98 countries currently burdened by the disease (Vos et al., 2016, Organization, 2009, Barrett and Croft, 2012) resulting in approximately 20 to 50 thousand deaths annually (Organization, 2013, Lozano et al., 2012). *Leishmania* is classified among the NTDs (Neglected Tropical Diseases) by the WHO and infects both humans and animals such as dogs, rodents, etc. (Organization, 2013).

Sand flies are the known vectors of transmission of leishmaniasis via the bite of the female sand fly infected with the protozoan, which inoculates the protozoa *Leishmania* via the skin (Organization, 2013). *Leishmania species* go through development in the vector stage, the promastigote forms, a stage where they differentiate in the mid-gut of the sand fly, and the amastigote stage; a host related stage where they can be found engulfed by macrophages. During blood meal by sandflies, *Leishmania* from an infected host (Wang et al., 2016) is ingested by the sand fly. The protozoan migrates to the midgut of the sand fly, here they differentiate as promastigotes (Sundar and Chakravarty, 2013), which replicate and subsequently transform into

the infective metacyclic promastigotes. These forms then move to the proboscis (Dorlo et al., 2012) of the sand fly where they are transmitted during blood feeding. Infective metacyclic forms when injected reach the puncture wound into the lower dermis of the skin and are internalized by resident macrophages (Organization, 2013) transforming into intracellular forms (Barrett and Croft, 2012) inside the macrophages. Amastigotes replicate in infected macrophages and can damage various tissues depending on the site of infection and the species of *Leishmania* (Organization, 2013) resulting in the different clinical presentations associated with the various forms of the disease.

The clinical symptoms of leishmaniasis include the severe visceral form (more disseminating), mucosal infections and self-curing cutaneous leishmaniasis. Clinical presentations of leishmaniasis is however dependent on the strain of *Leishmania* (Dawit et al., 2013). Cutaneous conditions are often painless and can however be colonized by opportunistic bacteria resulting in complications. Globally it is estimated that 90 % of a 1.5 million cases of Cutaneous leishmaniasis (CL) occur in Brazil, Algeria, Saudi Arabia, Iran, Syria, Peru, and Afghanistan (Choi and Lerner, 2001, Desjeux, 2004). In developing nations in Africa, countries bordering the leishmaniasis belt of East Africa, West, Central, North, and the Horn of Africa are predominantly endemic with leishmaniasis (Fryauff et al., 2006). Most cases of *Leishmania* distribution in the North African countries including Algeria, Morocco, Egypt and Libya, is transmitted via the *P. papatasi* vector. Correspondingly the distribution across the North from the Canary Islands to Egypt (Kimutai et al., 2009) is caused by *L. tropica*. In the Eastern part of Africa, cutaneous leishmaniasis cases occur in a certain parts of Kenya, Ethiopia, and North Sudan with *L. aethiopica* being the prevalent parasite (Consortium, 2010). The known major causative species of CL in West African is *Leishmania major* with CL appearing to be in epidemic proportions in different countries such as

The Gambia, Nigeria, Mauritania, Cameroon and Senegal (Kimutai et al., 2009). However, the prevalence of the disease in some regions increased over a period of time and reduces drastically in relation to the number of reported cases (Boakye et al., 2005).

There have been sporadic reports of leishmaniasis infection in Ghana since the first reported cases in 1999 during a health surveillance in the Volta region of Ghana (Boakye et al., 2005). However, a study by (Kwakye-Nuako et al, 2015) have isolated a new strain of *Leishmania* DNA (*L. enrietti*) in sand flies collected from the Volta Region of Ghana. This therefore highlights the need for active surveillance and to identify potential ongoing infection even on a low scale.

Mucocutaneous leishmaniasis is an unusual manifestation yet very similar to the cutaneous manifestation. This form is described as being marked by progressive degradation of the mucosal linings (Chappuis et al., 2007). *Leishmania* spp. which causes this form of infection invade the mucus membranes for several years inducing acute destruction of skin and tissues of the nasopharyngeal cavity. The processes of this form of infection is inadequately understood, though believed to be as a result of poorly managed cutaneous infection. Mucocutaneous infection involves the formation of lesions which spread and subsequently leads to severe mutilation of the face with accompanying fevers, fatigue and general malaise. In severe situations, certain complications such as secondary infectious and complete deformations to face and other sites of infection will result when poorly managed.

A severe form of *Leishmania* is the visceral leishmaniasis which when not quickly diagnosed for faster and proper treatment or management can lead to high fatality (Barrett and Croft, 2012). The parasite in an infected host moves to the intramural organs such as the bone marrow, spleen as

well as the liver. The visceral manifestations are systemic with other symptoms or presentations such as anemia, fever and hepatosplenomegaly (Boelaert et al., 2007). Poor management of these presentations subsequently can lead to death. The major species associated with visceral leishmaniasis include but not limited to *L. donovani*, *L. chagasi* and *L. infantum*. There have been reported cases of visceral leishmaniasis co-infection with HIV in 25%- 70% of patients in Southern Europe (Sundar and Rai, 2002).

In the diagnosis of leishmaniasis, microscopy remains the gold standard. This technique is rather laborious to perform considering it requires a lot of time and skill in both sample collection and observation. It is also less specific or effective in detecting low concentrations of parasitemia (Bensoussan et al., 2006) and can also be subjective. Other techniques that are currently used in the diagnoses of leishmaniasis are antigen/antibody-detection based rapid test kits, ELISA and Polymerase Chain Reaction (Boelaert et al., 2007). The detection of *Leishmania* DNA in biopsy taken from a host can be done employing various PCR tests with the most sensitive methods using minicircle kinetoplast DNA found in the parasite (Barrett and Croft, 2012).

In the management of leishmaniasis, early diagnosis and treatment as well as an integrated vector control, control of reservoirs and education is critical. Treatment with a combination of paromomycin, and pentavalent antimonials are routinely used as recommended (Sundar and Chakravarty, 2013). However, disease control is complicated by the geographical diversity of the parasites, vectors, and reservoirs. This is further compounded by the absence of vaccine control. In the past decades, drug toxicity and resistance issues associated with current drugs necessitate a continued effort to identify alternative chemotherapy against *Leishmania*.

Due to recent prominence or extensive applications associated with medicinal plant usage, plants serve as a common source for the derivation of leads for further development for the treatment of various NTDs among indigenous communities in Africa (Ofori et al., 2011, Organization, 2013). An estimated 80 % of people living in developing countries resort to the use of traditional remedies (Organization, 2013), which include plants with a gradual increase in the patronage by the Western world (Ofori et al., 2011). In some parts of Africa, including Ghana, traditional medicine is the first line of therapy against various diseases. There have been reports of the anti-parasitic effect of some orthodox drugs which derive their source from medicinal plants such as artemisinin (Miller and Su, 2011). Some of these medicinal plants from many studies exhibit anti-*Leishmania* activity also (De Medeiros et al., 2011, Peraza-Sánchez et al., 2007). Despite some reports showing the anti-parasitic qualities of these medicinal plants, there is however inadequate scientific evidence to back these claims. This study therefore seeks to screen some selected compounds isolated from Ghanaian medicinal plant, *Morinda lucida* for anti-*Leishmania* properties on the major developmental stages of *Leishmania*.

1.1 SIGNIFICANCE

There is growing concern of opportunistic infections from bacteria culminating in the severity of leishmaniasis especially in immunocompromised patients with CL. In Ghana, especially in the Volta Region, leishmaniasis is endemic in some communities (Lartey et al., 2006). Also studies by Kwakye-Nuako et al. (2015) have identified a new strain of *Leishmania* DNA in the mid-gut of sand flies in this region known as *L. enrietti* which suggest a possible risk of ongoing infection. The absence of a vaccine or drugs for mass drug administration due to high toxicity and resistance

issues therefore necessitates the continuous efforts to identify alternative drugs. In spite of recent advances in drug discoveries, *Leishmania* infections can be complicated by the absence of vaccines, resistance to current drugs, drug toxicity and continuous presence of vectors and reservoirs. Therefore, this study will potentially lead to the identification of alternative therapy for leishmaniasis.

1.2 RATIONALE

There is growing concern of opportunistic infections from bacteria culminating in the severity of leishmaniasis especially in immunocompromised patients with CL. In Ghana, especially in the Volta Region, Leishmaniasis is endemic in some communities. A study by (Mba-tihssommah, 2016) identified 13 species of the vectors capable of transmission from 3 communities in the Volta Region which could suggest ongoing transmission since there has been no study on current status of transmission or infections since the last report in 2009. There has been a single report on cutaneous leishmaniasis and HIV co-infection (Lartey et al., 2006). Another study by (Kwakye-Nuako et al., 2015) identified a new strain of *Leishmania* DNA in the mid-gut of sand flies in this region known as *L. enriettii* which seem to suggest a possible risk of infection if not an ongoing transmission.

In spite of recent advances in drug discoveries, the *Leishmania* infections can be complicated by the absence of vaccines, resistance to current drugs, drug toxicity and continuous presence of vectors and reservoirs. There is therefore the need to identify alternative chemotherapy against leishmaniasis. It is estimated that about 80 % of people living in middle income countries, especially, resort to the use of traditional medicinal plant recipes with a gradual increase in the patronage by the Western world (Ofori et al., 2011). Despite some reports of the anti-parasitic

qualities of some of these medicinal plants, there is however inadequate scientific backings to some of these claims. In Ghana, plants with phytochemical properties suitable as treatment have been used for the management of various conditions among indigenous communities including the use of *Morinda lucida* as medication for the treatment of trypanosomiasis and leishmaniasis (Busari et al., 2014, Lawal et al., 2012). The mechanism of action or active components are however not known for most of these medicinal plants despite increased reliance on them. The efficacy of *Morinda lucida* against various parasite including *Trypanosomes*, *Plasmodium* and *Leishmania* (Lawal et al., 2012, Busari et al., 2014) have been extensively studied and our group had previously isolated novel tetracyclic iridoids compounds, Molucidin, ML-2-3 and ML-F52, from *Morinda lucida* leaves to have anti-trypanosomal and anti-microbial activity against some bacteria strains (Kwofie et al., 2016, Antwi et al., 2017).

1.3 HYPOTHESIS

Tetracyclic iridoids have been shown to have potent anti-trypanosomal activity against a *Trypanosoma brucei*, a kinetoplastid, suppressing the expression of paraflagellar rod protein and will therefore have anti-leishmanial activity.

1.4 AIM:

To screen novel tetracyclic compounds isolated from the leaves of *Morinda lucida* for anti-*Leishmania* properties and to investigate the possible mechanism of action.

1.5 SPECIFIC OBJECTIVES:

- (I) To determine the effect of Molucidin, ML-F52 and ML-2-3 on intracellular amastigotes and promastigotes forms of *L. major* and *L. donovani*
- (II) To determine the cytotoxicity and S.I of compounds against mammalian cell lines.
- (III) To determine the possible mechanisms of action of active tetracyclic iridoids by Nexin, Flow cytometry, and Immunohistochemistry.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is plagued in about eighty-eight countries, with millions of suffering various complications, predominantly those in the tropical and subtropical regions. Annually, 2 million new cases in humans are reported (Organization, 2013). Cutaneous leishmaniasis constitutes three quarters of these cases, with about one quarter being visceral leishmaniasis (VL) (Organization, 2013).

More than over 20 species of the genus *Leishmania* cause leishmaniasis of which different species cause peculiar forms of the diseases in humans (Barrett and Croft, 2012). *Leishmania donovani*, *L. chagasi* and *L. infantum*, cause VL; whereas *L. Mexicana*, *L. pifanoi* and *L. major* cause CL; Species associated with mucocutaneous leishmaniasis include *L. panamensis*, *L. guyanensis*, and *L. braziliensis*. The more disseminating VL, usually progresses to the reticulohistiocytic system (Barrett and Croft, 2012), affecting the liver, spleen and bone marrow.

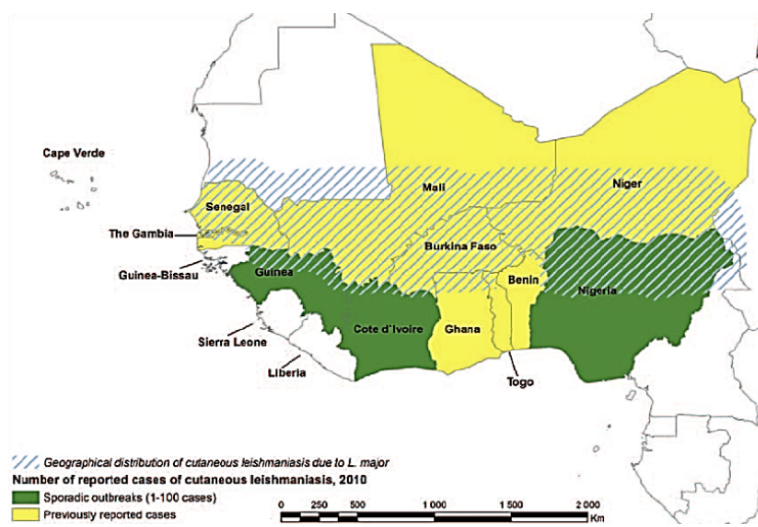


Figure 1. Distribution of cutaneous *Leishmania* in West Africa in 2010 (WHO, 2010).

2.2 Sand fly vectors in leishmaniasis

It is estimated that there are about 900 species of sand fly vectors subsumed under two categories known as the Old World and New World into five genera. These include *Sergentomyia* (about 258 species) and *Phlebotomus* (94 species) of the Old World, and *Warileya* (5 species), *Brumptomyia* (23 species) and *Lutzomyia* (379 species) under the New World.



Figure 2. Sand fly (<https://ecdc.europa.eu/en/disease-vectors/facts>)

However, an estimated seventy species in 2 genera, *Lutzomyia* and *Phlebotomus* have been shown as the proven implicated vectors of *Leishmania* and are considered as the definitive hosts of the causative agent (Bates, 2007, Kato et al., 2010). These two genera of vector species are known to support the survival and transmission of specific *Leishmania* species (Bates, 2007). Sand flies are frail insects that exhibit a typical hopping and landing movement from one point to a host. Usually sand flies are spry at night time, and hide in crevices of homes; especially straw roofing and caves during the day (Kato et al., 2010).

The female sand flies are the main carriers of *Leishmania* parasites which they transmit during blood meal from a host. Characteristic of small Diptera, they measure between 2-3mm in length and coloured black to silver grey with wings folded in a V-shape when at rest (Kato et al., 2010).

The parasites usually overcome various barriers to enable its establishment and complete development in both the vector and host. There are two main developmental forms of *Leishmania* parasites, the promastigote form, which usually is found in the gut of the sand fly, adhere firmly to the gut walls of the vector so as to provide stability and restrict their expulsion by movements in the gut. This allows for multiplication, transformation and differentiation into infective metacyclic forms which are subsequently transmitted to host during blood meal picked up by host macrophages. Inside the macrophages, promastigote forms undergo developmental transformation into the intracellular amastigote forms (Sacks, 2001; Killick-Kendrick, 1999). The transmission of infective forms is usually facilitated by the promastigote factor PSG produced by the vector (Rogers, 2012) which plays an important function in the complete development of *Leishmania* at the vector stage, and is also responsible for the incrimination status of the sand fly as the competent vector of transmission. The competency of a sand fly as vector of transmission has been shown in previous studies with *Phlebotomus duboscqi* and *P. papatasi*; the main natural vectors responsible for *L. major* transmission, *P. argentipes*; being the vector of *L. donovani* transmission and *P. sergenti*; the vector of *L. tropica*, all of the Old World. Vectors which constitute the New World include *Lutzomyia verrucarum* and *Lu. Longipalpis* (Kamhawi et al., 2004). Others of medical importance are highlighted in Table 1.

There have been reports of midges as potential vectors of *Leishmania* in Australia with the most prevalent sand fly being *Sergetomyia queenslandi* (Dougall et al., 2011). Although most midges have not been associated with infection, a moderate prevalence of infection were associated with *Forcipmyia midges* inhabiting the kangaroo *Leishmania* parasites. This new speculated vectors as well as the previously known 18 sand fly species, belonging to the *Phlebotomus* or *Sergentomyia* genera are known to feed off smaller mammals and reptiles (Dougall et al., 2011, Lewis and Dyce, 1982).

Table 1: Geographical distribution of medically important vectors and susceptible *Leishmania* spp. (Rogers, 2012, Ashford, 2000).

Sand fly Species	Geographical location	<i>Leishmania</i> spp. transmitted
<i>P. guggisbergi</i> <i>P. papatasi</i> <i>P. duboscqi</i> <i>P. longipes</i> <i>P. pedifer</i> <i>P. perniciosus</i>	Africa	<i>L. major</i> <i>L. aethiopica</i> <i>L. donovani</i>
<i>P. sergenti</i> <i>P. papatasi</i> <i>P. argentipes</i>	Asia	<i>L. tropica</i> <i>L. donovani</i>
<i>Lu. flaviscutellata</i> Lu. <i>umbratilis</i> <i>Lu. whitmani</i> <i>Lu. anduzei</i> <i>Lu. ayrozai</i> <i>Lu. paraensis</i>	Caribbean	<i>L. amazonensis</i> <i>L. guyanensis</i> <i>L. naiffi</i>
<i>P. ariasi</i> <i>P. perniciosus</i>	Europe	<i>L. donovani</i> <i>L. infantum</i> <i>L. gerbilli</i>
<i>P. papatasi</i>	Middle East	<i>L. major</i> <i>L. Arabica</i>
<i>Lu. anthophora</i>	North America	<i>L. mexicana</i>
<i>Lu. longipalpis</i> <i>Lu. evansi</i> <i>Lu. olmeca</i> <i>Lu. faviiscutellata</i> <i>Lu. wellcomei</i> <i>Lu. ubiquitalis</i> <i>ayacuchensis</i> Lu. <i>whimani</i>	South America	<i>L. infantum</i> <i>L. mexicana</i> <i>L. amazonensis</i> <i>L. pifanoi</i> <i>L. braziliensis</i> <i>L. lainsoni</i> <i>L. panamensis</i> <i>L. peruviana</i> <i>L. shawi</i>

2.3 Life cycle of *Leishmania* species

Promastigotes are the forms of the parasite found in the midgut of an infected sand fly which replicate via binary fission. Their development in the vector usually takes 8-20 days when ingested from an infected organism and then move to the pharynx as metacyclic promastigotes which are ready for onward transmission during blood feeding (Chatterjee, 1964).

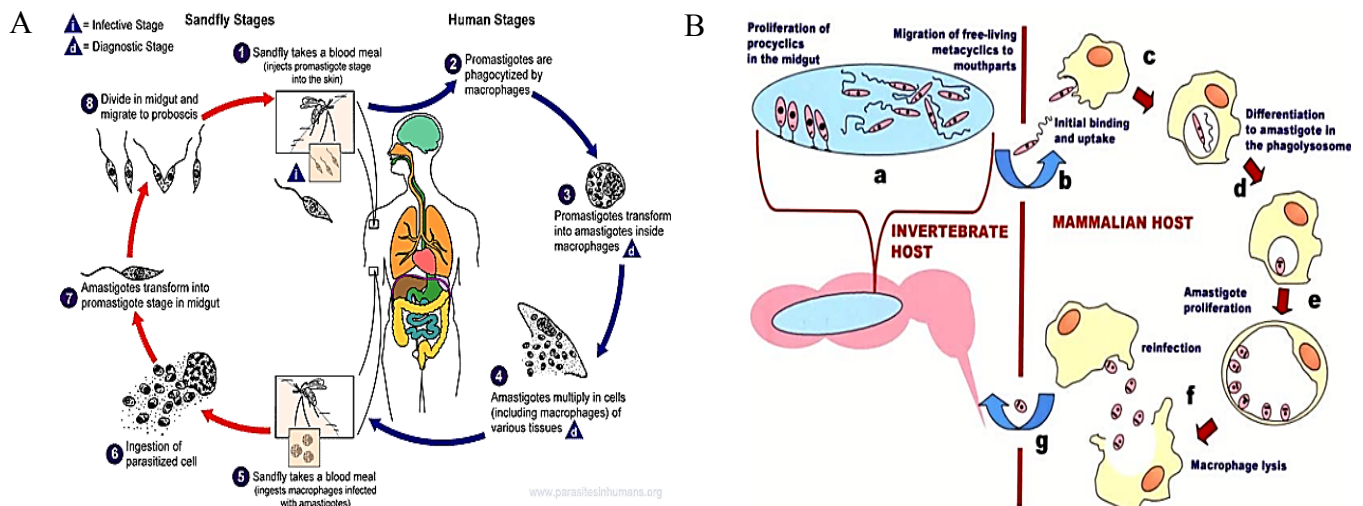


Figure 3. (A) Life cycle of *Leishmania* species (Control and Prevention, 2013), (B) Mechanism of promastigote macrophage invasion (Vannier-Santos et al., 2008): Procyclic promastigotes differentiate into infective metacyclic forms (a) which are inoculated in the mammalian host during blood feeding (b). In the dermal layer, promastigotes are recognized by receptors of macrophage and phagocytosed (c). A parasitophorous vacuole of newly internalized parasite, transforming into round, non-motile amastigotes (d) and dividing in the phagolysosome (e). Infected macrophages releasing free amastigotes which will infect neighboring macrophages (f). Sandflies ingest amastigotes which differentiate into the procyclic promastigotes (a).

The vectors salivary glands are not infected, hence, its mode of infection occurs as the fly regurgitates a bolus of metacyclic parasites into the wound created showing an unparalleled fashion of transmission with tsetse fly in trypanosomiasis transmission where the salivary gland is actually infected with the parasites. The inoculated promastigote forms are then phagocytized the macrophages in the dermis where they differentiate into the intracellular amastigotes. Many studies have investigated the molecular mechanisms leading to macrophage invasion by the

promastigotes. However, these studies have led to conclusions that there is a redundancy in the entry process as the parasites tend to employ different receptor-ligand interactions for invasion (Alexander et al., 1999). Other studies have postulated membrane lipid rafts and cholesterol as essential for *Leishmania donovani* and some intracellular parasites adhering to the plasma membrane of host cells and invading (Pucadyil and Chattopadhyay, 2007, Chattopadhyay and Jafurulla, 2011). The parasite's life cycle in mammalian host is that of an obligate intracellular parasite in monocytes or macrophages as the pathogen has developed specialized mechanisms to prevent normal macrophage destruction of its phagocytized components.

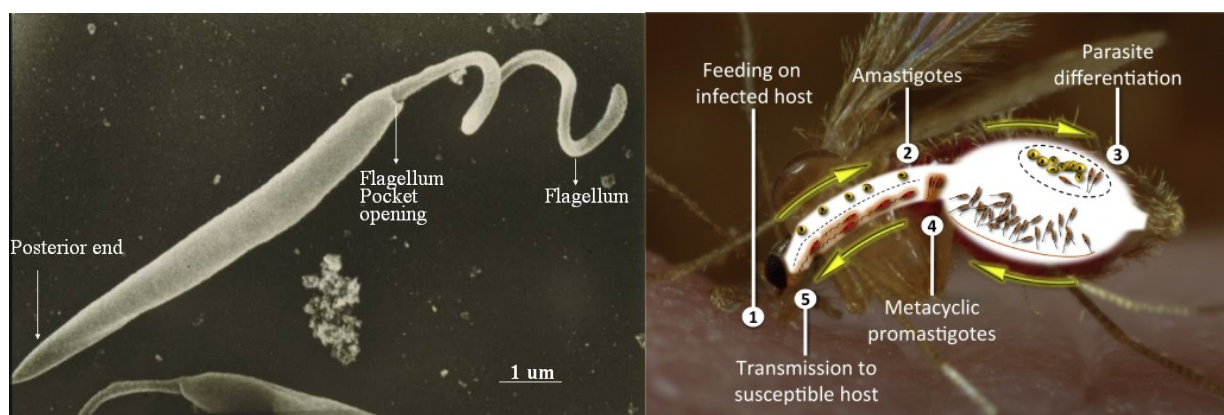


Figure 4: Promastigote form (<http://dna.kdna.ucla.edu/parasite>) and Amastigote form (Mondragon-Shem and Acosta-Serrano, 2016).

2.4 Diagnosis of leishmaniasis

2.4.1 Culture and microscopic observation

Conclusive diagnosis of VL is imperative for adequate treatment or management of the disease. Therefore, a conclusive diagnostic method should be able to detect the parasites in clinical specimen (Elmahallawy et al., 2014). Culture and microscopy are used as diagnostic strategy and splenic and or bone marrow aspirates are the samples usually used. A limitation of microscopy is that, where there is low parasite load, diagnosis is subjective to the technician carrying out the test. It is also a less specific method compared to other techniques such as Polymerase chain reaction.

Culturing of the pathogen improves diagnostic sensitivity, however, this technique is tedious, expensive and a waste of time to implement.



Figure 5: *Leishmania tropica*-induced bilateral pretibial ulcerations (Weiss et al., 2009).

2.4.2 Serological diagnosis

The basis for serological diagnosis is the fact that a specific humoral response can be tapped based on antigen-antibody interactions. The current serological technologies include indirect fluorescent antibody (IFA), direct agglutination test, and other Enzyme-linked immunosorbent assays. Despite the sensitivity of serological methods of diagnosis, they almost often suffer the same setback which is either cross-reactivity or the fact that antigens or antibodies produced during an infection remain in circulation for some time even after pathogen clearance.

2.4.3 Polymerase Chain Reaction (PCR)

PCR protocols for the detection of the protozoan in samples such as spleen, lymph node aspirates or bone marrow involves the extraction of genomic DNA from these samples and subsequent detection with primers, which amplify, conserved allele regions in the parasite genome. PCR is

more specific as it has the ability to differentiate between the different species of the pathogens causing the infection. Its sensitivity depends on the type of sample used (Lachaud et al., 2000, Singh et al., 1999, Osman et al., 1997).

2.5 Treatment of leishmaniasis

The current recommended chemotherapy for leishmaniasis treatment are the pentavalent antimonials (Haldar et al., 2011). Over the past decades, new discoveries have been made in terms of alternative chemotherapy for the treatment of the disease, however these have been licensed for administration in some countries whilst others are yet in various clinical trial phases (Long et al., 2012). There still remain significant challenges with respect to the design of a single chemotherapy for all forms of the disease due to differences in the sites of infection posing different pharmacokinetic requirements as well as the intrinsic differences in the sensitivity of the drug on the species and host. Some of the drugs currently in use include miltefosine, paramomycin, and amphotericin B.

2.6 Outlook of *Leishmania* and its control in Ghana

2.6.1 *Leishmania* in Ghana

The control or management of leishmaniasis across the globe requires a combination of intervention strategies which highlights the need for early diagnosis and treatment. In Ghana, the Health Service carried out insecticide spraying exercise in Ho, Hohoe and Kpando in the Volta Region when there was an outbreak of leishmaniasis some years ago (Amoa-Bosompem, 2016). However, as a result of the neglect of the disease in West Africa, there has not been an organized control effort in most regions except during outbreaks. The prevalence of *Leishmania* in Ghana

has over the years been significantly low, which can be attributed to the poorly resourced health facilities in some of these communities to diagnose the condition (Ofori et al., 2011), and sometimes the self-curative nature of the cutaneous form of the condition resulting in low reporting to point of care health facilities.

Despite the low level of reporting on the condition in Ghana, a study conducted by Mba, et al., 2015 identified 13 species of the vector of transmission in 3 communities in the Volta Region.

Studies by (Kwakye-Nuako et al., 2015) isolated DNA from *L. tropica* and a new *L. enriettii* strain in the midgut of sandflies sampled from these communities, which highlights possible ongoing transmission or risk of transmission (Amoa-Bosompem, 2016). Some suspected cases of CL were first reported in some communities of the Volta Region, specifically in the Ho Municipality in 1999 (Kweku et al., 2011). Although the moist semi-deciduous forest ecosystem of this region is known not to be a typical climatic environment that supports leishmaniasis, these communities share borders with Burkina Faso on the West African CL belt.

The Ho Municipality spans an area of approximately 2,564 square kilometres with an estimated population of about 249,000 (estimations from the 2000 population census data). It is bordered in the East by the Ghana-Togo border, West by the Kpando district, the South by the North-Tongu and Akatsi Districts and Hohoe Municipality on the North (Kweku et al., 2011). The settings of the Volta Region are mainly forest and forest savannah and presents two major climatic seasons: a dry season and wet season. The major rains come in the wet season in the region between the periods of May to August (main rains) and October to November (Minor rains) with the rest of the season being relatively dry (absence of rain).

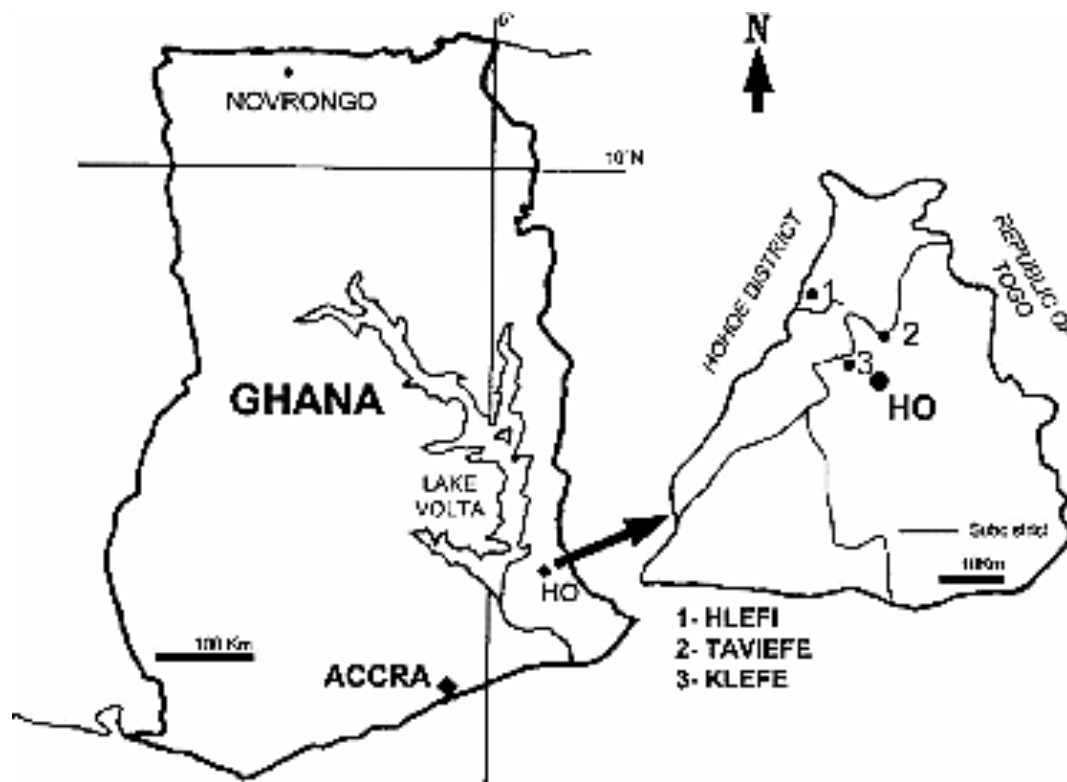


Figure 6: Communities in the Volta Region of Ghana with reported cases of cutaneous *Leishmania* (Fryauff et al., 2006).

2.6.2 Control of leishmaniasis in Ghana

Due to the relatively low prevalence in Ghana, there has been an apparent neglect of Leishmaniasis with no control programs against either the vector or the parasite. There was mass spraying in endemic communities since the first reported cases with insecticides and this could explain the low incidence reported. This possibility helped in the control of leishmaniasis at the time but not a sufficient measure for complete elimination (Amoa-Bosompem, 2016). The isolation of a new strain of *Leishmania enriettii* in sand flies in Ghana is evident of the presence of *Leishmania* in Ghana. The relatively high costs of drugs which otherwise would have offered relief to infected persons has led to lot of self-medication on alternatives which might not be efficient in treating the condition (M. Kweku personal communication). This notwithstanding highlights the need for

continuous efforts for alternative drugs as the currently available drugs are challenged by toxicity to the host and also reported cases of resistance to drugs (Sundar and Chakravarty, 2013).

2.7 Traditional plant medicine

The use of plants has been emphasized worldwide due to the medicinal properties associated with their use and their ability to cure many diseases. Also traditional medicinal plants have served as beneficial sources for the derivation of novel compounds with antimicrobial activity making them a safe source compared to artificial colloids (de Menezes et al., 2015). Due to the fact that traditional medicinal plants are cheaper source of therapy for many indigenes on the African continent, they have become an indispensable source of treating many neglected tropical diseases (Ofori et al., 2011). The employment of traditional medicine from plant sources is not restricted to Africa but goes far beyond the peripheries of Africa. It is estimated that 50% of the world's population resort to the use of traditional medicine for treating poor health conditions (Sadeghi-Nejad et al., 2011).

Some medicinal plants have been identified as potential sources of drugs for the treatment of many neglected tropical diseases for many years. Based on previous findings, WHO has laid emphasis on investigating plants sources for anti-*Leishmania* agents which can lead to the identification of promising leads for leishmaniasis (Ozbilgin et al., 2014). These promising findings were however not entirely unexpected because medicinal plants have been demonstrated to be a rich source of antimicrobials and anti-*protozoans* agents for the treatment of many ailments of the past decade with over 25% of recommended drugs being plant derivatives with or without supplementary adjustments (Miller and Su, 2011, Sadeghi-Nejad et al., 2011, Lee, 2002). Additionally, about 28% of 1,184 new drugs enlisted between 1981 and 2000 were plant products and their derivatives

(Wink, 2012). Despite contemporary advancements in the screening of plants with medicinal properties against different pathogens by various research groups, there is a burgeoning inadequacy in the translation of these findings into clinical practice (Wink, 2012). Therefore, the need to authenticate these results with scientific evidence to advance the use of medicinal plants has led various research groups to introduce measures through proven research backings.

In Ghana, a typical excuse for the reliance on self-treatment and as such the indulgence in herbal concoctions for cure can be associated with various factors including the high costs of orthodox drugs, and the poor attention received at point of care health facilities (medical doctors to patients, as reported in 2012, was 1:6000) (Ofori et al., 2011). This highlights the obvious shortcomings associated with such a burden (Ofori et al., 2011). The use of traditional medicine is common as it is highly recommended by rural folks in spite of obvious limitations regarding the lack or inadequate documentation of indigenous knowledge on their usage, dosage requirements, mechanism of action and side effects/toxicity. The methanolic fractions of *Eryngium thorifolium*, a Turkish medicinal plant, have been reported as a promising lead for the treatment of *Leishmania tropica* infections (Ozbilgin et al., 2014). Also, medicinal plants such the leaves of *Aloe vera*, *Hyptis pectinata*, and the stem of *Chenopodium ambrosioides* and *Pfaffia glomerata* have been used in the treatment of CL in North Eastern Brazil (De Queiroz et al., 2014). There have also be reports of both the methanol and ethanol extracts of *Satureja khuzestanica*, an Iranian medicinal plant, having anti-proliferative effect against *Leishmania major* promastigotes with reported 50% inhibitory concentrations of 4.8mg/ml and 2.4mg/ml, respectively (Sadeghi-Nejad et al., 2011). *Jurinea dolomiaea*, a medicinal plant from Pakistan has also been shown to be potent against *Leishmania* (Shah et al., 2014). Similarly, methanol extracts from the seeds of *Annona*

senegalensis have been reported to be active against *Leishmania major* promastigotes but with no significant activity observed against *Leishmania donovani* promastigotes (Sahpaz et al., 1994).

The anti-*leishmania* activity of *Morinda lucida* has been demonstrated against *Leishmania donovani* (Okpekon et al., 2004). However, there is no documented evidence of any plant medicine used in the treatment of leishmaniasis in Ghana. Amoa-Bosompem et al., (2016) and Antwi et al., (2017) showed the anti-*Leishmania* and antimicrobial activity of *Morinda lucida*, a Ghanaian medicinal plant, on *Leishmania* and bacteria isolates. In Ghana, similar studies have led to the isolation of novel tetracyclic iridoids isolated from the leaves of *Morinda lucida* which was shown to have anti-*trypanosomal* activity (Kwofie et al., 2016).



Figure 7: *Morinda lucida* plant (<http://www.liberianfaunaflora.org/liberian-flora/rubiaceae>)

2.7.1 Natural products with anti-*leishmania* properties

2.7.1.1 Alkaloids

Alkaloids are characteristic naturally occurring compounds which contain nitrogen atoms within the chemical structure and with some derivatives being weak acids or neutral compounds

(McNaught and McNaught, 1997, Manske and Holmes, 2014). Apart from the mostly common carbon, hydrogen and nitrogen atoms within their chemical composition, alkaloids may also contain other elements including oxygen, sulfur, phosphorus, bromine and chlorine. In traditional and modern medicine today, alkaloids have found use as the foundation for drug discovery. This importance is associated with their non-exhaustive pharmacological properties including their use as analgesics, antibacterial, antimalarial, anticancer, antiasthma, etc. (Kittakoop et al., 2014).

Steroidal alkaloids such as Sarachine (Solanaceae), has been shown to completely inhibit the growth of *L. braziliensis*, *L. amazonensis* and *L. donovani* promastigotes (Moretti et al., 1998) Also alkaloids extracted from the leaves of *Holarrhena curtisii* (Apocynaceae) were shown to exhibit anti-promastigote activity against *L. donovani* (Kam et al., 1998).

2.7.1.2 Quinones

The quinones are classified as a group of organic compounds formed from aromatic compounds by the transformation of $-\text{CH}=\text{}$ groups into $-\text{C}(=\text{O})-$ groups (IUPAC and Wilkinson, 1997). Both synthetic or natural quinones have found use in the medical and or pharmaceutical industry as some of them have been used for their anti-tumoral, antimicrobial and anti-parasitic activities (Liu, 2011). Such is the case with diospyrin, a metabolite extracted from the bark of *Diospyros montana* which was found to be potent against *L. donovani* promastigotes. Its hydroxylated derivative inhibited 73.8% of intracellular amastigotes growth (Muñozb et al., 1992).

2.7.1.3 Indole analogues

Indoles are aromatic heterocyclic organic compounds with a bicyclic structure made up of six-membered benzene rings fused to a five-membered pyrrole ring. They are wide spread in the environment and are produced by various bacteria (Seigler, 2012). Indole alkaloids, such as harmaline, the main constituent of some plants, have been shown to possess anti-protozoal activity

and used in indigenous medicine to treat leishmaniasis by its ability to intercalate DNA interrupting amino acid metabolism (Wright and Phillipson, 1990, Mahiou et al., 1994).

2.7.1.4 Iridoids

Iridoids are usually synthesized in plants as glycosides mostly bound to glucose and serve mainly as defense agents for the plant against herbivores and microorganisms (Geu-Flores et al., 2012). Some works on iridoids have shown their leishmanicidal effect on *L. donovani* such as Arbortristosides A, B, C and 6- β -hydroxyloganin, extracted from the seeds of *Nyctanthes arbortristis* (Tandon et al., 1991).

2.7.1.5 Sesquiterpenes

These are a class of terpenes consisting of 3 isoprene units with a molecular formula, C₁₅H₂₄. Although sesquiterpenes can be synthesized by oxidation or biochemical rearrangements are also found naturally in insects and some plants as semiochemicals produced as defensive agents or pheromones (Muñozb et al., 1992). Studies on Dehydrozaluzeanin C, a sesquiterpene lactone extracted from the leaves of *Munnozia maronii* has been found to have anti-promastigote activity against 11 species of *Leishmania* at concentrations of between 2.5 and 10 $\mu\text{g/ml} \pm 1.61$ (mean \pm standard deviation).

2.7.1.6 Diterpenes

Diterpenes are well known to be tumor-inducing and highly cytotoxic metabolites of plants. 12-O-tetradecanoyl phorbol-13-acetate (TPA), a diterpenoid phorbol ester, has been found to inhibit *L. amazonensis* via a variety of structural alterations in the parasite (Wright and Phillipson, 1990, Schmeda-Hirschmann et al., 1996, Vannier-Santos et al., 2008). Others include jatrophone and jatrogrossidione (Schmeda-Hirschmann et al., 1996).

2.7.1.7 Triterpenes

The triterpenes are compounds which are made up of 3 terpene units which are naturally produced by plants, animals and some fungi (Xu et al., 2004). Triterpenes with anti-leishmanial activity include betulin-aldehyde and ursolic acid, extracted from the stem of *Doliocarpus dentatus* and the stem bark of *Jacaranda copaia*, respectively (Sauvain et al., 1996). It is claimed that the anti-protozoal activity is associated with the presence of its carboxylic acid group in the structure (Camacho et al., 2003).

2.7.1.8 Saponins

Various plant species are the main sources of saponin which are classified pharmacologically under amphipathic glycosides due to their soap-like foaming properties when shaken under aqueous solutions (Hostettmann and Marston, 1995). Although saponins are known widely to be produced from plant sources, another source is sea cucumber, a marine organism (Hostettmann and Marston, 1995, Riguera, 1997). Saponins are produced in plants which serve as anti-feedants (Forester and Hartmut, 2006) and to provide protection against fungi and microbes. Some saponins including α -hederin, β -hederin and hederagenin possess anti-*leishmania* effect against promastigotes of *L. tropica* and *L. infantum*, and their activity associated with their ability to alter the external membrane of the protozoan (Majester-Savornin et al., 1991, Delmas et al., 2000).

2.7.1.9 Chalcones

Chalcones are aromatic ketone derivatives of phenolic compounds and occur in various plants species (Mahapatra et al., 2017). The chalcone (E)-1-[2,4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-3-[4-hydroxy-3-(3-methylbut-2-enyl)phenyl]-prop-2-en-1-one 73 has shown

toxicity to *L. donovani* promastigotes although its use in the treatment of leishmaniasis has been suggested to have some suppressive effect on host immune system (Christensen et al., 1994).

2.7.1.10 Flavonoids

Flavonoids are phenolic derivatives and a class of secondary metabolites produced by some plants and fungi (McNaught and McNaught, 1997). They may be produced as chemical messengers, Cell cycle inhibitors, and physiological regulators. The compound 5,7,4 -trihydroxyflavan, a flavonoid, has been shown to have a toxic effect on the amastigote forms of *L. amazonensis* (Cordell, 1995).

2.7.1.11 Acetogenins

Acetogenins are natural products produced in plants (family: Annonaceae) and are classified as polyketides (Li et al., 2008). These polyketides are characterized by a 32 or 34 linear carbon chain with oxygenated functional groups such as ketones, hydroxyls, tetrahydrofurans, epoxides, and tetrahydropyrans (Mangal et al., 2016).

Acetogenins isolated from the seeds of *Annona senegalensis* have been investigated for anti-*leishmania* activity against promastigotes of *L. major* and *L. donovani* with activity varying between 25 and 100 µg/ml (Sahpaz et al., 1994). Acetogenins have also been shown to exhibit anticancer properties (Mangal et al., 2016), however its potential therapeutic use has been limited by its neurotoxic effect (Levine et al., 2015, Potts et al., 2012, Preedy et al., 2011, Coria-Tellez et al., 2018, Le Ven et al., 2011). Other acetogenins including annonacin A and goniothalamycin, isolated from the seeds of *Annona glauca*, and rolliniastatin-1, isolated from the bark of *Rollinia emarginata* have demonstrated to have anti-proliferative effect against promastigote forms of *Leishmania spp* including *Leishmania donovani*, *L. amazonensis*, and *L. braziliensis* (Waechter et al., 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Materials

Medium 199, Gentamycin, Methanol, Dimethylsulfoxide (DMSO), Trypsin Solution 10X, BME Vitamins Solutions, Bovine Serum Albumin (BSA), obtained from Sigma Aldrich, Phosphate Buffered Saline (PBS) and Fetal Bovine Serum (FBS) from Gibco-BRL, Guava Cell Cycle Reagent and Guava Nexin Reagent from EMD Millipore Corp., Alamar Blue from Invitrogen, Amphotericin B from Add Pharma Gh. Ltd.

3.2. Methods

3.2.1 Plant materials and compound preparations

The leaves of *Morinda lucida* Benth were collected from the Centre for Plant Medicine Research, Akuapim-Mampong, and compounds extracted following detailed protocols established previously (Kwofie et al., 2016). Briefly on the processes involved, about 1100 g of air-dried *Morinda lucida* leaves were grinded into powder and extracted with 2.0 liters 50% aqueous ethyl alcohol (EtOH) at 40°C with sonication. Extraction solvent was removed by evaporation and residue (203 g) suspended in a liter of water. This was subsequently partitioned with ethyl acetate (EtOAc), hexane and CHCl₃ to obtain soluble fractions of EtOAc (3.6 g), hexane (2.1 g) and CHCl₃ (3.8 g). The most active fraction identified from previous studies to have anti-trypanosomal activity, CHCl₃ fraction, was then put through a silica gel column (45 by 350 mm) fractionation with hexane-EtOAc (2:1, vol/vol) as the transportable or mobile phase. Fractions determined to have anti-trypanosomal activity were further processed for the isolation of compounds. Fractions

obtained were loaded onto a reverse-phase column with MeOH-H₂O (3:2, vol/vol) and purified to obtain ML-2-3 (a colorless crystal, 35 mg), Molucidin (colorless crystal, 50 mg), and ML-F52 (55 mg) was obtained from the fractions. The structure of the compounds including stereochemistry was determined by means of the optical rotation value and Nuclear Magnetic Resonance spectra. The structure of the extracted compounds, Molucidin, ML-2-3 and ML-F52 are as shown in Fig.8. DMSO was used as solvent system in the dissolution and preparations of stock concentrations of compounds. Working concentrations were then prepared from the stock volume using Medium 199 (M199: from Sigma-Aldrich) for compound dilutions and assays.

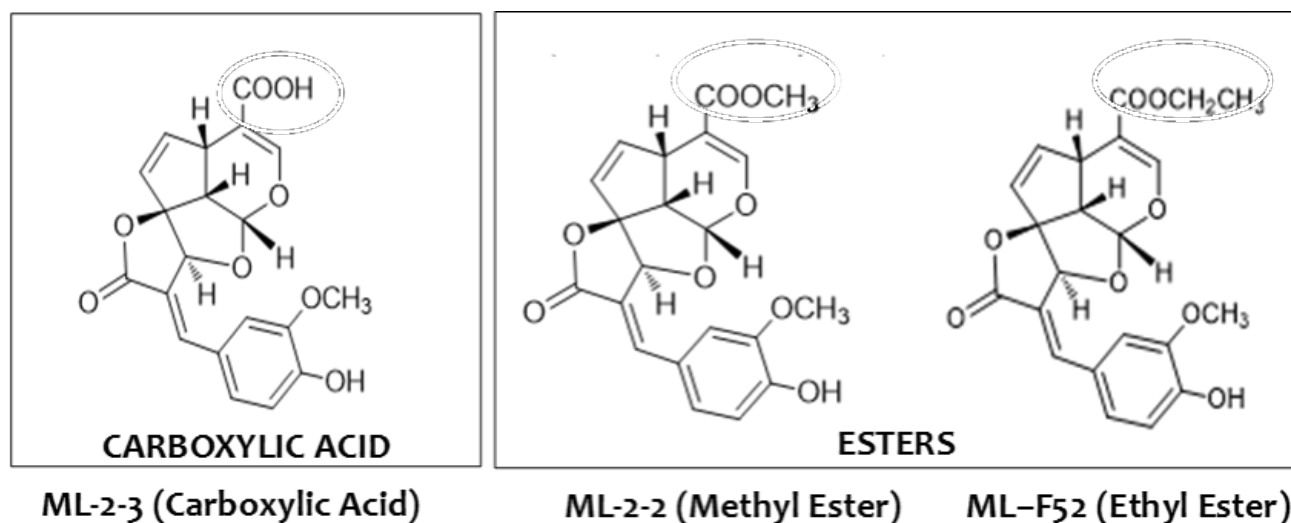


Figure 8: Chemical structure of iridoids isolated from *M. lucida*. ML-2-3 (R= H), Molucidin (R= CH₃), and ML-F52 (R= CH₂CH₃).

3.3. Parasite and cell culture conditions

In vitro cultures of *L. donovani* (D10 strain) and *L. major* (NR48815) parasites, were maintained in M199 medium supplemented with 10% heat-inactivated FBS, gentamycin (1.25 ml/500 ml medium) and BME vitamins (5 ml/500 ml) and passaged every 72 hours at a mean density of 1.0x10⁶ cells/ml in fresh medium. Details of the procedure was a modification of previously

established protocol (Mottram, 2008). Murine macrophages (RAW 264.7) were seeded at a mean cell density of 1.0×10^6 cells/ml in M199 medium (pH 7.4). The medium was supplemented with 10 % heat-inactivated FBS cells cultured for 72 hours at 37°C in 5 % CO₂. Confluent flasks of RAW 264.7 cells were then sub-cultured in fresh M199 medium at a mean density of 1.0×10^6 cells/ml.

3.4. *In vitro* anti-*leishmania* activity of compounds against *Leishmania* spp.

This was performed by a growth inhibition assay using the alamar blue cell viability assay. The IC₅₀s (half maximal inhibitory concentration) values were determined as previously established (Islamuddin et al., 2014). Briefly, promastigotes at a cell density of 3×10^6 cells/ml were seeded in 96-well plates and incubated for 48 hours at 28°C with or without compounds in two-fold serial dilutions. Each experiment was done in triplicates (0 - 50 µM). Amphotericin B was used a positive control. A volume of 10 µl of alamar blue reagent was 4 hours prior to end of treatment time point and absorbance read at 540 nM using a microtitre-plate reader (Tecan Sunrise Wako spectrophotometer). The IC₅₀ values were determined by graphical extrapolation after plotting the mean percentage absorbance versus the log concentration of the compounds using the Graph Pad Prism software. IC₅₀s were reported as mean ± standard deviations.

3.5. Cytotoxicity analysis of compounds on human cell lines

Cytotoxicity assay was performed to identify the toxicity of compounds on other mammalian cell lines *in vitro*. This assessment will provide information on the site-effects of tetracyclic iridoid compounds relative to current treatment (Control: Amphotericin). The cytotoxic effect of compounds was assessed on four human cell lines including Hs888Lu (lung) procured from the

European Collection of Authenticated Cell Cultures (ECACC), NB1RGB (skin fibroblast) and HF-19 (lung fibroblast) was from the RIKEN Bio Resource Center Cell Bank (Japan) and Chang Liver cells. NB1RGB and HF-19 cells were maintained in minimum essential medium- (MEM) supplemented with 10% FBS and 1% penicillin-streptomycin. Chang Liver and Hs888Lu cells were seeded in Eagle's minimum essential medium (EMEM) and Roswell Park Memorial Institute (RPMI) medium 1640, respectively, and supplemented as stated before. Murine macrophages (RAW 264.7) were cultured in same medium as parasites (M199 supplemented with gentamycin (5ml/500ml M199), BME vitamins and 10 % FBS. Cultures were then incubated at 37°C under 5% CO₂. Cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) assay. The cell lines were treated with Molucidin, ML-2-3, or ML-F52 at two-fold serial dilutions of concentrations from 0-50 µM for 48 hours. Cells were then plated at a density of 0.5 x 10⁴ cells/well into 96-well plates and after 24 hours of incubation, were treated with compounds for 48 hours. Four hours prior to end point of treatment, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added and incubated in the dark for 4 hours and then measured at a wavelength of 595 nm using the microplate reader (Immuno Mini NJ-2300; Nihon InterMed, Tokyo, Japan). Cytotoxicity was determined as a percentage of live cells relative to the untreated control. The selectivity index (SI) was expressed as the ratio of the IC₅₀ obtained for mammalian cells and the IC₅₀ against *Leishmania* shown below:

$$SI = \frac{IC_{50} \text{ of Cell lines}}{IC_{50} \text{ of parasite}}$$

3.6. Mechanism of cell death and cell cycle analysis

Assessing the apoptotic inducing properties of the tetracyclic iridoids, the externalization of phosphatidylserine (PS) on the outer membrane of compound-treated promastigotes was analyzed

using Guava Nexin reagent (EMD Millipore Corp.). The protocol by (Mukherjee et al., 2009) was adapted with some modifications. Briefly, mid-log phase promastigotes (3×10^6 cells/ml) were incubated in the presence or absence of tetracyclic iridoids at concentrations of 0-50 μ M for 48 hours at 37°C under 5% CO₂ for 48 hours. Amphotericin B was the drug of reference as it had been shown to induce apoptosis in *Leishmania spp.* (Lee et al., 2002). Parasites, both treated and untreated group, were harvested after 48 hours of incubation by centrifugation (17000 rpm, 10 min) and supernatant discarded. The pelleted cells were subsequently washed in 1x PBS with subsequent re-suspension of pellets in 70 % ice-cold ethanol, added drop wise, whilst vortexing at minimum speed. The pellets were then refrigerated at 4°C for at an hour prior to staining with Nexin reagent per the manufacturer's instructions (EMD Millipore). Cells were resuspended in 1 ml 1x PBS with further centrifugation. A volume of 200 μ L Guava Cell Cycle Reagent was added with mixing and incubated at room temperature for 30 minutes in the dark. Samples were then transferred into microcentrifuge tubes and read using the Flow analyzer (BD LSRFortessa™ X-20). Acquired data was analyzed using the FlowJo Software (version 10).

3.7. KMP expression and morphometric analysis

Gene-knockout studies of KMP-11 in *T. brucei* showed basal body segregation, accumulation of multi-unequal sized nuclei (Li and Wang, 2008). Molucidin and ML-F52 treated promastigotes, were taken through further analysis to assess their effect on KMP levels and on the cellular morphology as well cytokinesis (Cell division). Promastigotes at a concentration of 3.0×10^6 cells/ml were incubated in the presence of tetracyclic iridoid compounds at half-IC₅₀, IC₅₀ and 2xIC₅₀ value. An untreated group was setup as negative control. After 48 hours of incubation with compounds, cells were fixed with 4 % PFA on a polylysine slide and immuno-stained using anti-

KMP primary antibody (secondary antibody: goat-anti-mouse FITC) and DAPI according to the manufacturer's manual. Promastigotes were examined for cellular morphology changes such as slender body, elongated or rounded, as well as the number of nuclei DNA, kinetoplast DNA and flagella under fluorescent and phase-contrast objectives. A minimum of 10 fields were viewed for each sample group (treated and untreated) and analysis of these parameters carried out on the Adobe PhotoShop CS6 Extended platform.

3.8. Time course analysis of compounds on promastigote forms

Investigating the sequence of cell division events (Mitosis of nuclei, kinetoplast and flagella) and how this could provide possible insight into mechanism (s) of action, the time course effect of Molucidin and ML-F52 on treated cultures were investigated. Promastigotes were incubated with compounds at IC₅₀ concentrations for 0.5, 1.5, 3, 6, 12, 24 and 48 hours and subsequently subjected to Nexin and Immunohistochemistry analyses. These time intervals were chosen based on the generation time of 30 minutes for parasite growth.

3.9. Anti-*leishmania* activity of compounds on intracellular amastigotes

The anti-amastigote activity of the compounds was further assessed on intracellular amastigote forms. Briefly, a concentration of 2×10^5 cells/ml of murine macrophages (RAW264.7) were plated in M199 medium (10% FBS, complete medium) in 8-chamber well plates and then incubated at 37 °C in 5 % CO₂ overnight. After 12 hours, adhered macrophages were infected with stationary-phase promastigotes at a ratio of 10:1 (parasites: macrophages) for 4-12 hours. Promastigotes that were not internalized were washed off and then incubated for further 24 hours with or without compounds at two-fold serial dilution of compounds (0-25 μM). Amphotericin B was used as the

positive. Media and compounds were washed off, after 24 hours, fixed in 4% PFA, DAPI-stained and observed under the fluorescence microscope. Concentration of infected macrophages per well were recorded employing two methods, the first as describes by (Islamuddin et al., 2014) with slight modifications. The IC₅₀ values were then extrapolated from a graph using the GraphPad Prism (Dutta et al., 2008). The percentage inhibition was then determined using the formulas below:

$$\% \text{ inhibition} = 100 - \frac{\% \text{ macrophages infected in treated cultures}}{\text{mean \% macrophages infected in untreated controls}} \times 100$$

and

$$\% \text{ Inhibition} = 100 - \frac{PT}{PC} \times 100$$

where PT = average number of amastigotes/macrophages in treated wells and PC= average number of amastigotes/macrophage in control wells.

3.10. Statistical analysis

The activity of the compounds was computed as IC₅₀ values using GraphPad Prism (Version 7.0) from a non-linear regression curve to the computed reports from microplate reader. The 50% inhibitory concentration (IC₅₀) values were then extrapolated from the dose response curves. The IC₅₀ values were reported as the mean ± (SEM) of three independent assays from data points of three independent experiments set up in triplicate. Cytotoxicity data of compounds, percentage of cells with induced apoptosis, cell cycle arrest, and morphological variations observed from Immunohistochemistry data obtained were reported as statistical significance with p-value of less than 0.05.

3.11. Ethics

In this study, ethical clearance was obtained from Review Board of the Noguchi Memorial Institute for Medical Research, (NMIMR-Reference number 066/15-16).

CHAPTER FOUR

4.0 RESULTS

4.1. Activity of tetracyclic iridoids against *Leishmania* spp.

The activity of the three tetracyclic iridoids compounds, ML-2-3, Molucidin and ML-F52 (Fig.8) were investigated for their anti-*leishmania* potential as shown in table 1. Absorbance was read at 540 nm after 4 hours of incubation with Alamar blue in the dark. It was observed that ML-F52 and Molucidin had anti-proliferative effect on promastigotes with IC₅₀ values of 0.91 μ M and 2.94 μ M against *L. donovani*, respectively, and an IC₅₀ value of 1.77 μ M and 1.85 μ M against *L. major*, respectively (Table 1). However, ML-2-3 showed no significant activity against either species of the *Leishmania* tested (IC₅₀ >50 μ M). There were no significant variations in the susceptibility (IC₅₀ value) between the two species to compounds ($p > 0.05$) although both species were generally more susceptible to ML-F52 relative to Molucidin ($p < 0.05$).

Table 2: *In vitro* anti-proliferative activity of compounds against *Leishmania* spp.

Promastigotes	IC ₅₀ (μ M)			
	Amphotericin B	Molucidin	ML-2-3	ML-F52
<i>L. donovani</i>	0.43 \pm 0.07	2.94 \pm 0.06	>100	0.91 \pm 0.05
<i>L. major</i>	0.32 \pm 0.02	1.85 \pm 0.02	>100	1.77 \pm 0.02

IC₅₀ values reported as mean \pm SEM

4.2. Anti-amastigote activity of tetracyclic iridoids

The intracellular amastigotes of *L. donovani* using murine macrophages (RAW264.7) was established (Fig. 14) and the anti-amastigote activity of the compounds was assessed. Both treated and untreated controls were DAPI-stained and observed under the phase contrast and DAPI objectives.

Molucidin and ML-F52 showed anti-amastigote activities with IC₅₀ values reported in table 2. It was observed that intracellular amastigote forms were more susceptible to ML-F52 relative to Molucidin. However, no significant activity was observed with respect to the ML-2-3 treatment in intracellular amastigote forms (>100 µM).

Table 3: IC₅₀ values of compounds on intracellular amastigotes of *L. donovani* after 48 hours of incubation.

Amastigotes	IC ₅₀ (µM)		
	Molucidin	ML-F52	Amphotericin B
No. of parasites/macrophage	1.418 ± 0.26	0.627 ± 0.02	0.177 ± 0.08
No. of infected macrophages/field	4.654 ± 0.46	1.928 ± 0.19	2.251 ± 0.62

IC₅₀ values reported as mean ± SEM

4.3. Cytotoxicity of tetracyclic iridoids on normal cell lines

The cytotoxic effect of compounds was determined using normal human cell lines. The cytotoxic effect against murine macrophages (RAW 264.7) was also assessed by MTT assay as described earlier. Selectivity Indices (SI) are tabulated in table 4. ML-F52 was determined to have S.I. of 10-fold. However, Molucidin was relatively toxic to all the cell lines tested (indicated by the S.I. <10).

Table 4: Cytotoxicity and SI values of compounds screened against Cell lines.

Cell type	Cell line	IC ₅₀ (μM)			S.I.		
		Molucidin (2.94*)	ML-2-3 (>1000*)	ML-F52 (0.91*)	Molucidin	ML-2-3	ML-F52
Normal Skin fibroblasts	NBIRGB	7.11	>50	4.74	2.42	>20	5.21
Normal lung fibroblasts	HF-19	14.24	>50	10.94	4.84	>20	12.02
Normal lung	Hs-888Lu	9.29	>50	8.89	3.16	>20	9.77
Normal liver	Chang Liver	9.34	>50	18.13	3.18	>20	19.92
Macrophage	Raw 264.7	6.54	37.06	9.80	2.22	>20	10.77

Anti-*leishmania* activities and cytotoxicity are represented by IC₅₀ values obtained from Alamar Blue (*L. donovani* and *L. major*) and MTT (RAW 264.7) assays. * Represents IC₅₀ values of compounds against *L. donovani* promastigotes.

4.4. Tetracyclic iridoids induced apoptotic-like cell death in *Leishmania donovani*

Flow cytometry analysis was done to determine the effect of the compounds on the parasites. Analysis was carried out via gating of cells into four characteristics, healthy cells were gated in the lower left quadrant; lower right quadrant being cells undergoing early apoptosis; cells in the late phase of apoptosis were gated in the upper right quadrant; and the left upper quadrant were cell undergoing necrosis (Fig. 9A). The induction of apoptosis was observed to be dose dependent (Fig.9b). As the concentration of compounds were increased, a significantly higher percentage of cells ($p < 0.05$) were observed to be in the late apoptotic phase. These observations suggest that both ML-F52 and Molucidin caused cell death reminiscent of apoptotic-induced mechanism, as indicated by increased Propidium iodide incorporation and annexin V binding. Molucidin induced a significant apoptotic effect in the parasite cultures (38.9 % of cell population, $p < 0.05$) relative to ML-F52 (12.8 %) and Amphotericin B (1.2 %) at twice IC₅₀ after 48 hours of incubation.

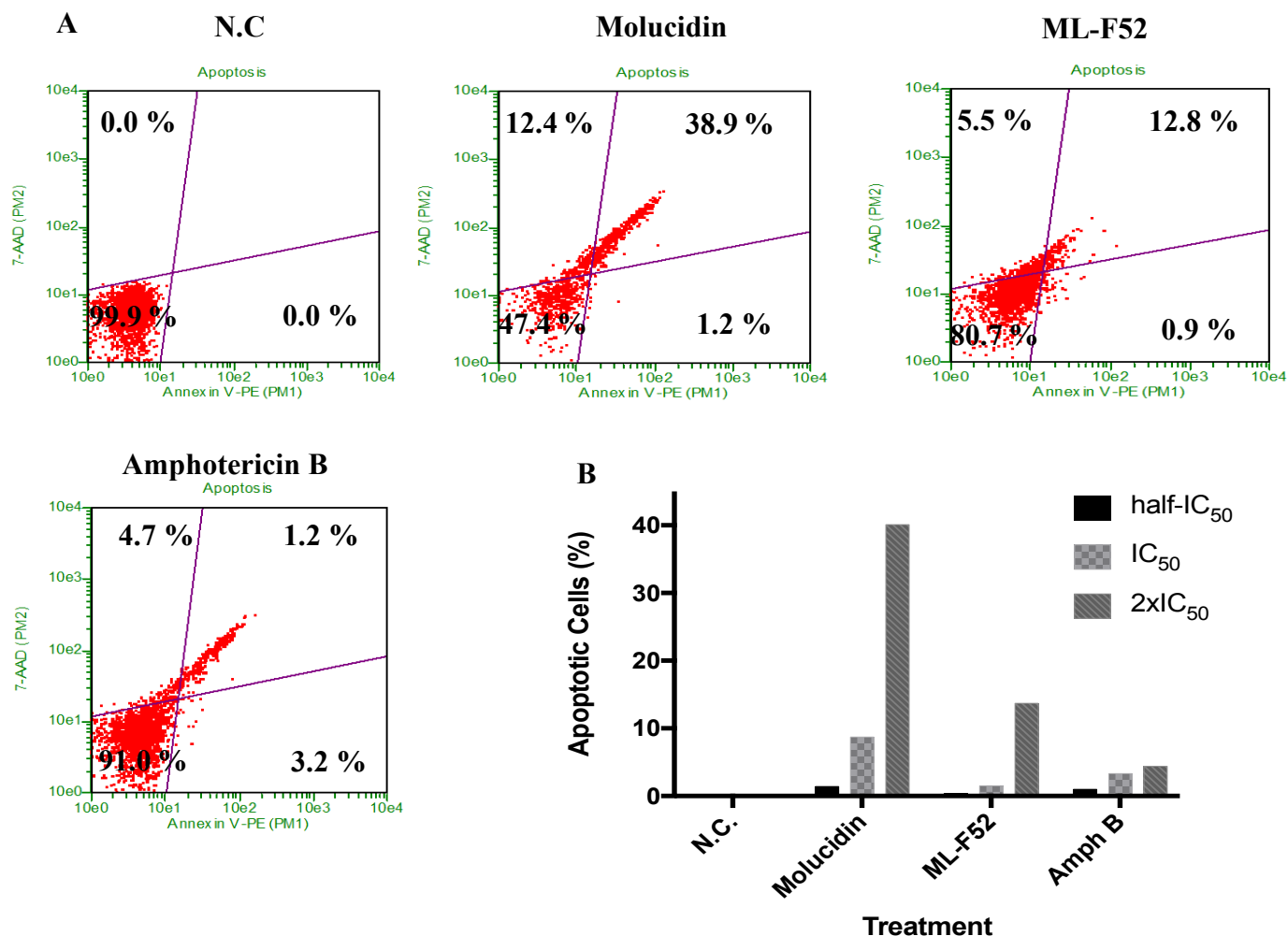


Figure 9: Nexin assay (A) Effect of compounds on apoptosis Assay of *L. donovani*. Active compound, Molucidin induced strong apoptotic effect (38.9 %) relative to ML-F52 (12.8 %). The negative control group constituted parasites not challenged with compounds (N.C). (B) Bar graph of Apoptotic cells (%) against compounds. Plot shows a dose dependent apoptotic-effect of compounds on promastigotes. ML-F52, Molucidin and Amphotericin B induced apoptosis with increasing concentration of compounds.

4.5. ML-F52 and Molucidin induced cell cycle arrest with enhanced arrest at G₂-M

Flow cytometry was carried out to assess the growth effect of compounds on cellular events within the parasites. Figure 10 shows the quantification of DNA in treated and untreated controls. For a given *Leishmania* parasite, the amount of PI corresponded to the amount of DNA. Accordingly, DNA fragmentation in apoptotic cells translated into a sub-G₀-G₁ phase. The peaks at each growth

phase indicates the cell cycle arrest of parasite population captured in a particular stage of the cellular division. In the treated groups, compounds induced a significant effect on the cell cycle of *L. donovani* relative to the negative control ($p < 0.05$) as shown below (Figure 10 B&C). Molucidin induced cell cycle arrest at G₂-M phase indicated by the significantly higher peak relative to ML-F52 and negative control ($p < 0.05$). ML-F52 was observed to induce significant cell cycle arrest at the S-phase relative to Molucidin and negative control ($p < 0.05$). However, both compounds were observed to induce apoptosis in *L. donovani* confirming data obtained from nexin assay as shown by the increased displacement at subG₁ (damaged DNA content).

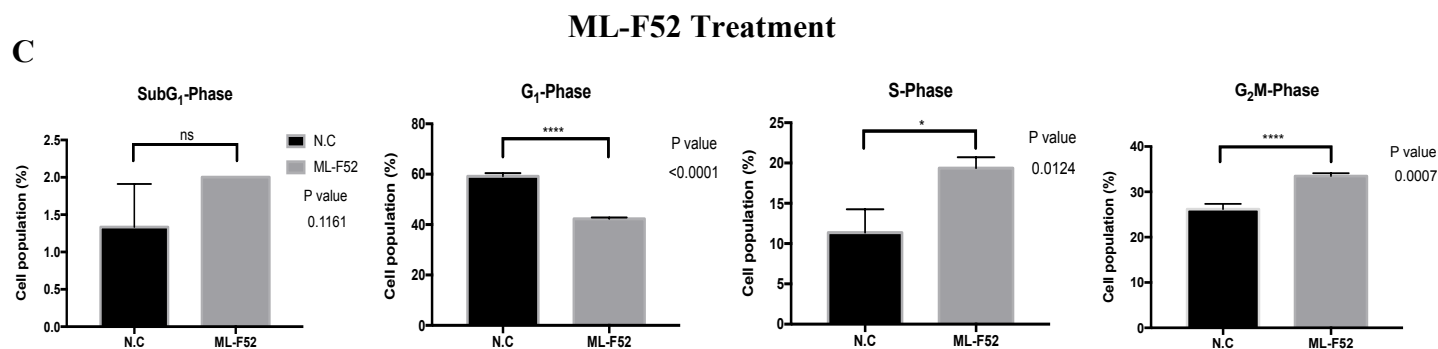
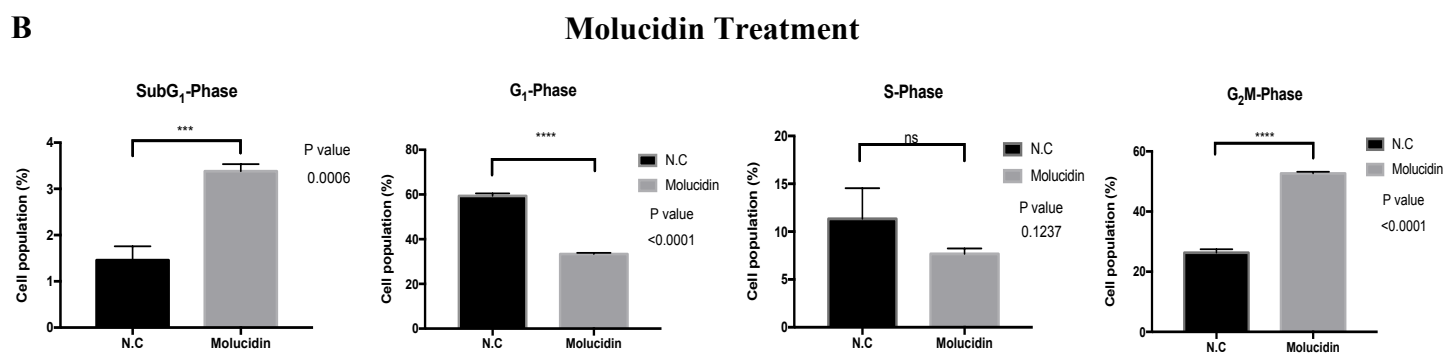
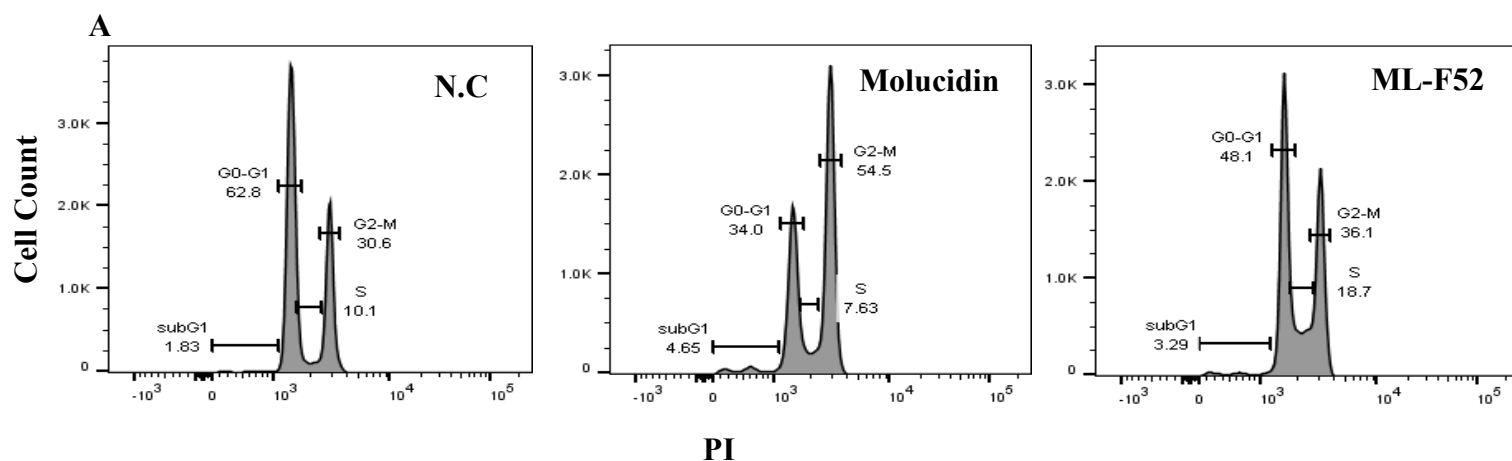


Figure 10: Cell Cycle Analysis (A) Effect of compounds on promastigote *Leishmania donovani*. Cells were analyzed and a histogram obtained from a plot of cell count against Propidium iodide incorporation (Quantity of Cell DNA). Peaks in the histogram indicate the percentages of cell cycle arrest relative to the amount of DNA content. B and C show statistical significance of the effect of tetracyclic iridoids on *L. donovani* relative to the untreated group (N.C).

4.6. KMP immunohistochemistry assessment and time-course analysis

Kinetoplastid Membrane Protein (KMP) gene-knockout studies have shown the important role of this membrane protein to *Leishmania* development which includes the parasites' inability to regulate cell division, inhibition of basal body segregation, and accumulation of multiple unequal sized nuclei when this gene was knocked out (Li and Wang, 2008). The level of KMP-11 expression in treated groups was assessed by immunofluorescence analysis (Fig. 11). No change in KMP expression between the treated and untreated group was observed, though compounds induced variations in the phenotypic transformations observed. The effect of tetracyclic iridoid treatment-time on the cultures was also monitored. It was observed that Amphotericin, Molucidin and ML-F52 showed effect on 10 % of promastigotes after 0.5, 6.0 and 12 hours' period of treatment, respectively (Fig.13).

4.7. Morphometric characterization of compound-treated promastigotes

Molucidin induced slender elongated forms ('nectomonad-like') in the cultures at $\frac{1}{2}$ IC₅₀ (1.47 μ M= 32 %) and mid-mitosis forms at twice IC₅₀ (5.88 μ M= 67 %, Fig. 11C). ML-F52 treatment induced significantly higher population of rounded or oval promastigotes with loss of flagellum at twice IC₅₀ (1.82 μ M= 90 %, Fig. 11D) relative to the untreated group (12 %). Both Molucidin and ML-F52 inhibited cellular division in cultures at IC₅₀ (0.91 μ M) shown by the increase in incomplete cytokinesis (high number of mid-mitotic forms) as shown in Fig.12A. In the treated groups, Molucidin induced significantly high nectomonad-like forms (approximately 13 μ in size, characterized as non-replicating promastigote forms, Fig.11B). However, ML-F52 inhibited progress of cellular events within parasites as shown by the increased population of mid-mitotic or generally rounded morphological forms in promastigote. Parasites were also observed to form clusters (aggregations) after being challenged with high concentrations of ML-F52 (Fig. 13B),

evident of cells under stress conditions (Li and Wang, 2008). In the treated group, significantly lower number of cells undergoing cellular division of nuclear and kinetoplastid DNA was observed. There were less number of cells with 2N:2K in the treated groups relative to the untreated group (Fig.12B). We further investigated mitochondrion-kDNA loss by staining treated parasites with DAPI (data presented in Fig.12B). It was observed that Molucidin triggered significant loss of kDNA in *Leishmania* after 48 hours of incubation suggesting possible damage to the kinetoplastid-DNA of the cell. Comparison of the affected forms of promastigotes showed significant defect in cellular processes in parasite cultures when treated with Molucidin (Fig. 12C)

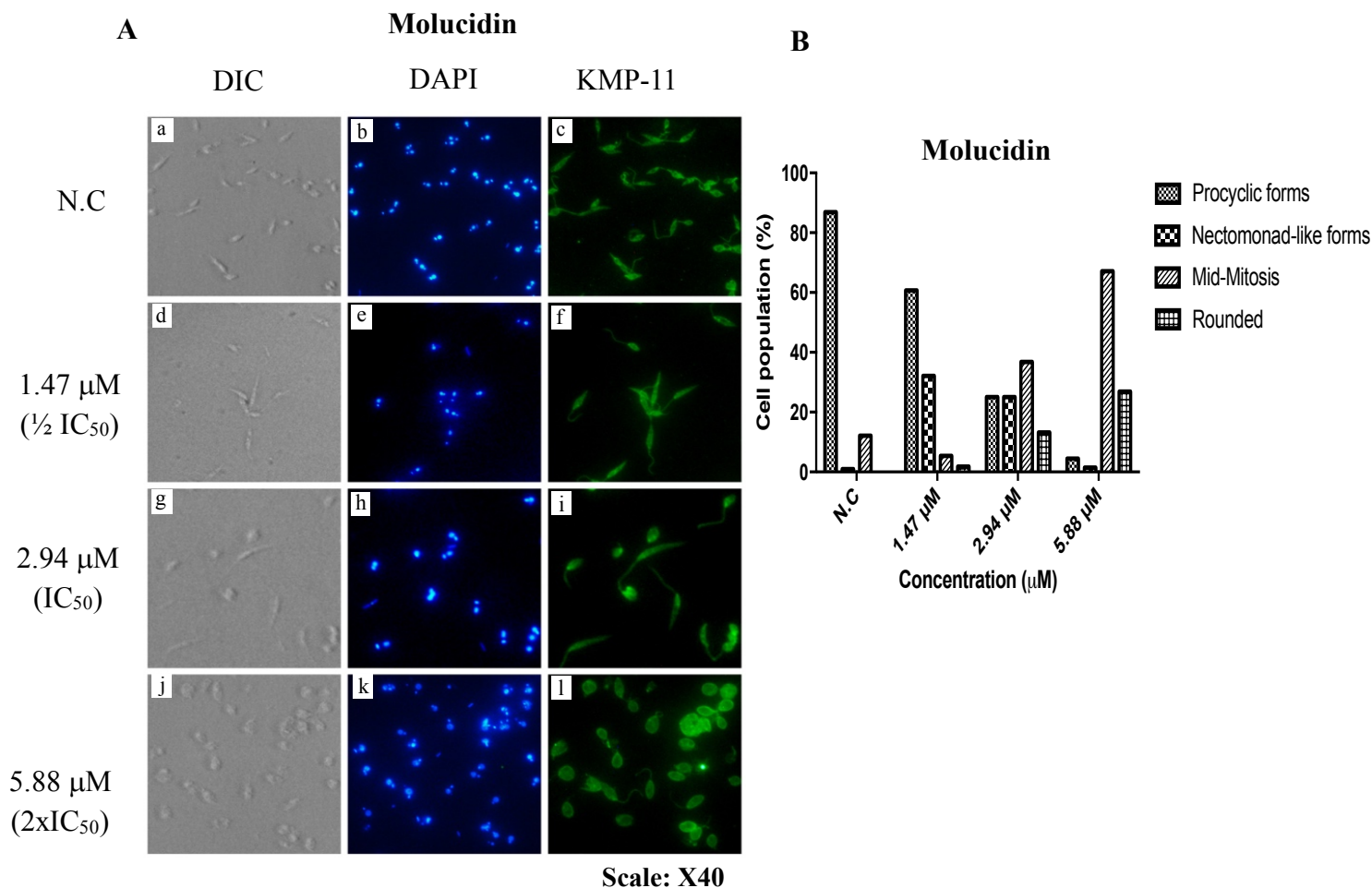


Figure 11: Immunohistochemistry diagrams (A & B) Plots of percentage cell (parasite) population verse morphological forms observed with increasing treatment. Nectomonad-like forms indicated by red arrows.

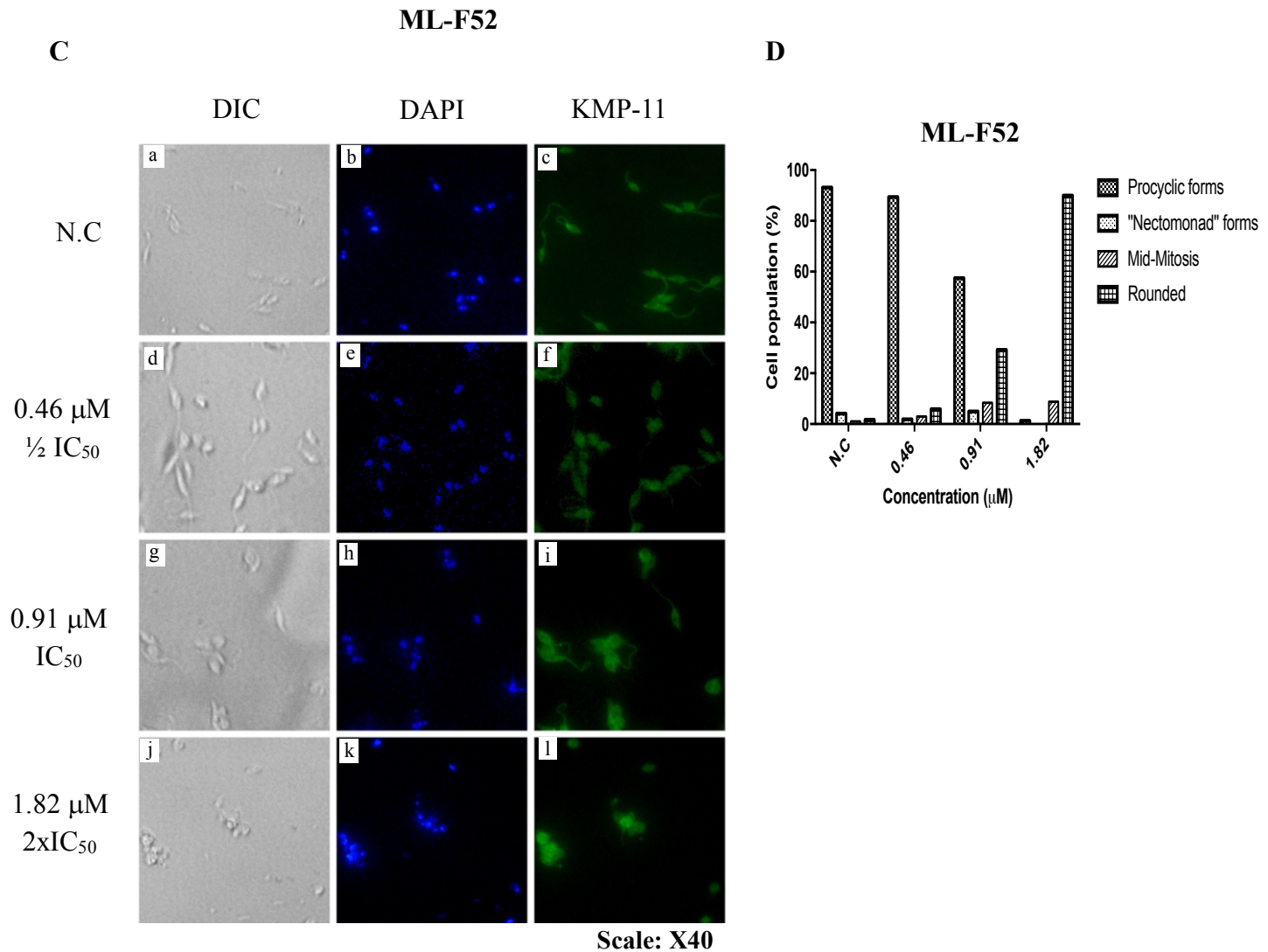


Figure 11 (C & D) Immune-fluorescence assay showing the effect of compounds on the morphology of *L. donovani* promastigote forms. DIC is the differential interference contrast view, DAPI and KMP-stained are the fluorescence view. Images were captured at 40X magnification and exposure time of 3.92 ms. Nectomonad-like forms indicated by red arrows.

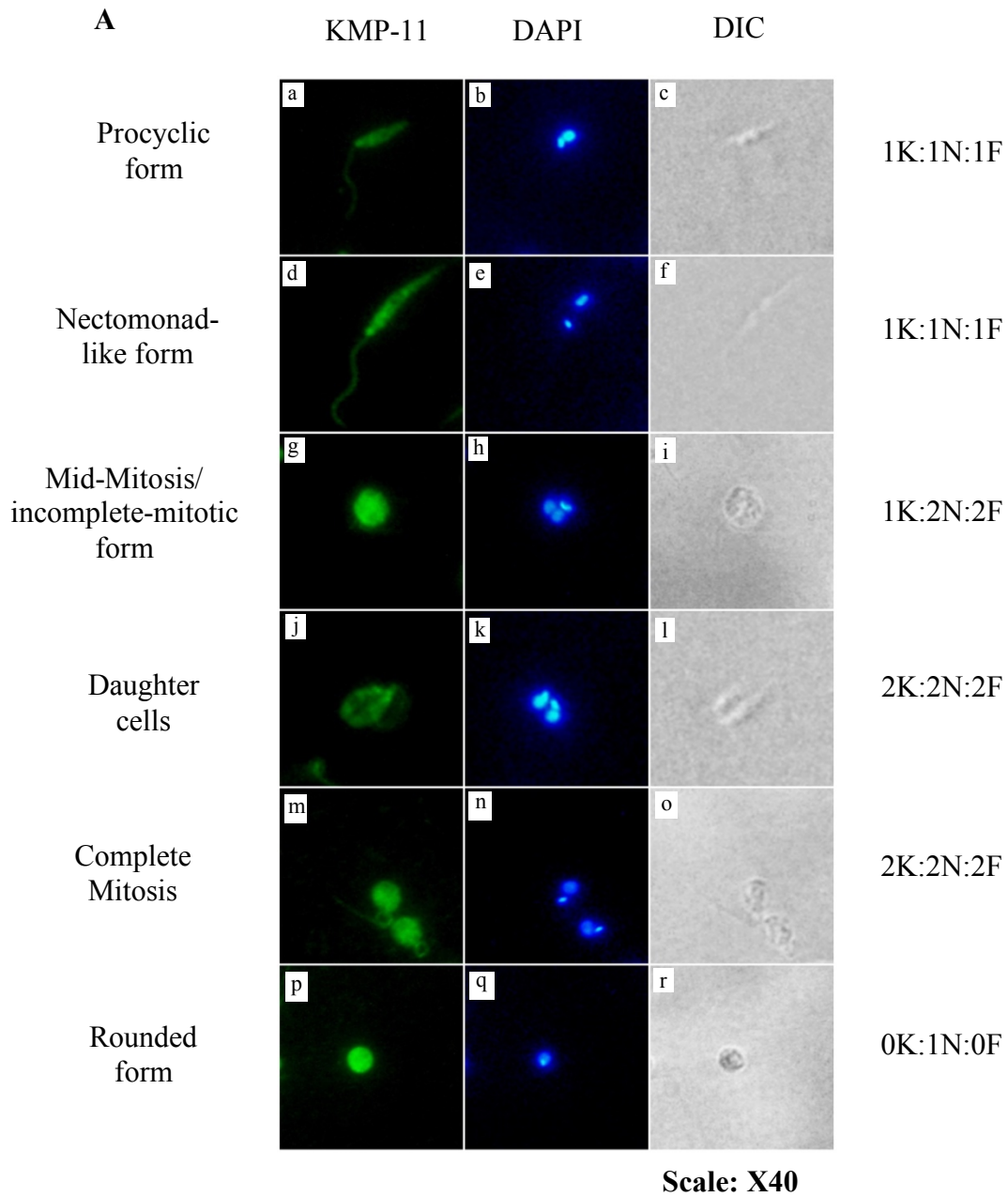
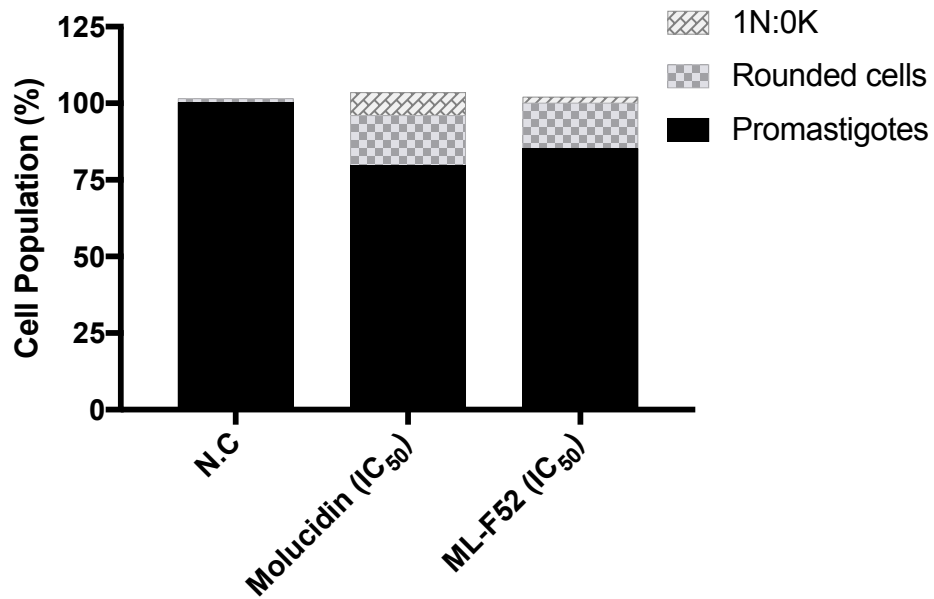


Figure 12: Morphometric analysis (A) Immunohistochemistry showing different morphological forms of *L. donovani* promastigotes after treatment at IC₅₀. KMP-11 stains basal body of parasites and DAPI stains nuclear and kinetoplast DNA of parasites, DIC is the differential Interference contrast view. (A–R) Images of cells observed in assays with tetracyclic iridoids and the cellular configuration (K: N: F).

B



C

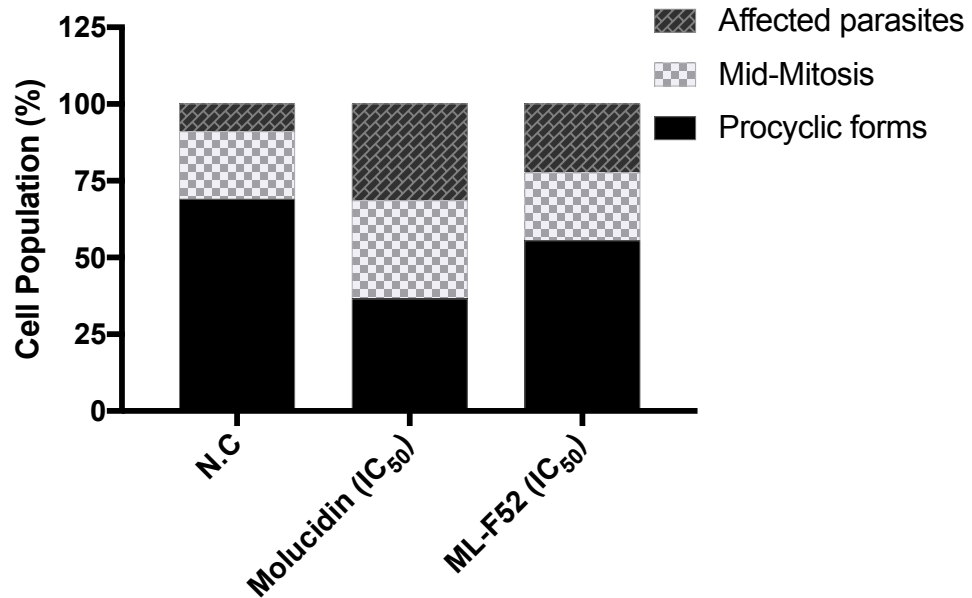


Figure 12 (B) A bar plot showing effect of compounds on promastigote phenotypes observed (C) A bar plot of effect of compounds against procyclic population.

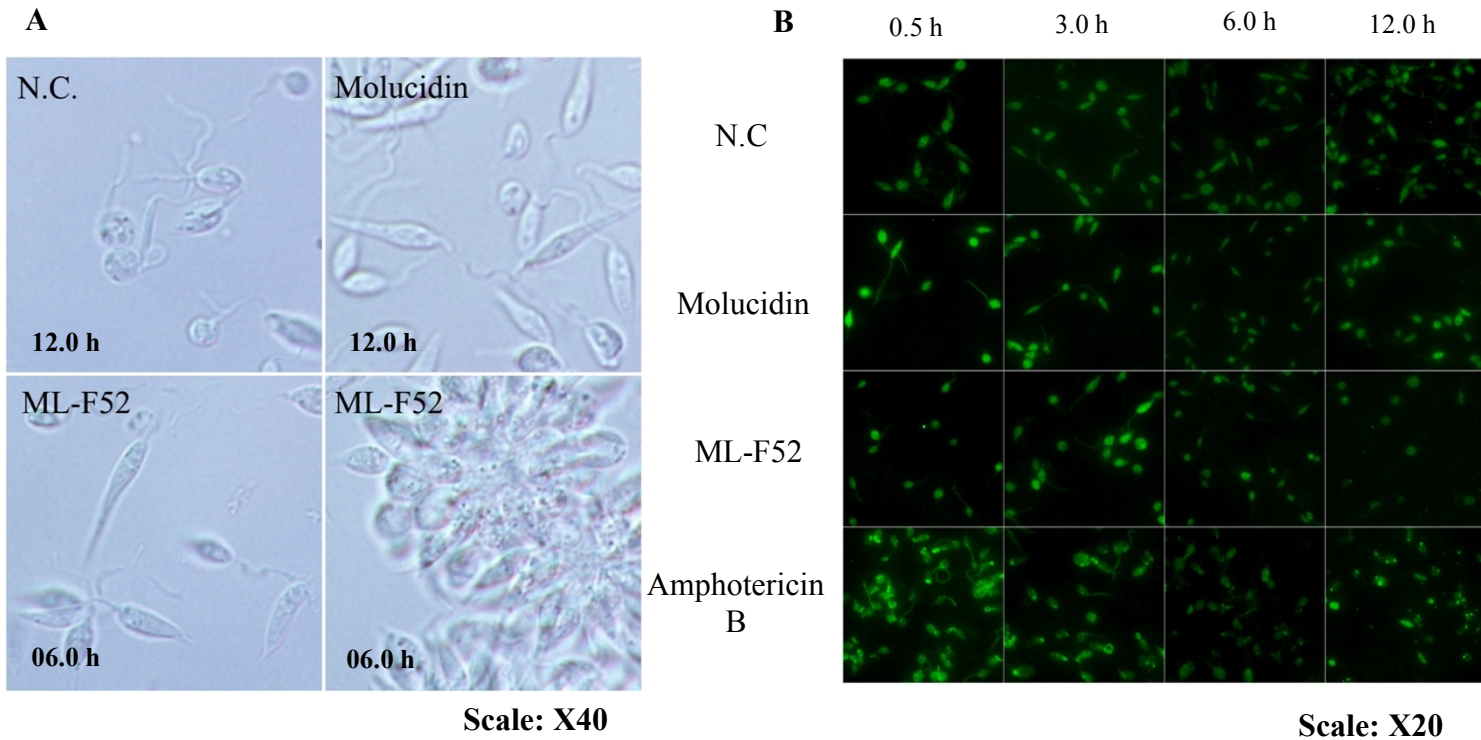
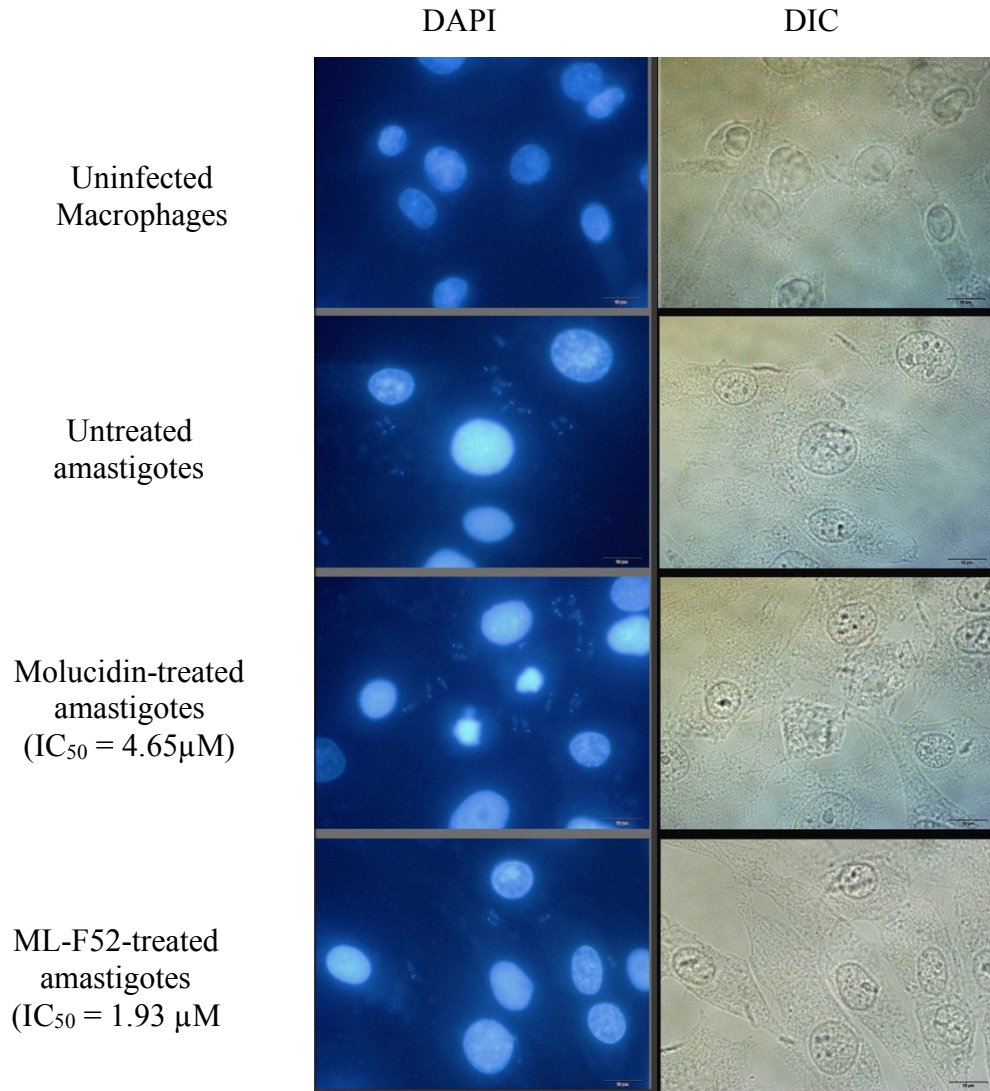


Figure 13: Time-course analysis (A & B) Differential Interference contrast view and KMP stained *L. donovani* promastigotes showing changes in forms in treated groups relative to untreated group with time. (C) Plot of parasite concentration against treatment time for each compound.



Scale: X100

Figure 14: DAPI view and Differential interference contrast (DIC) view of Parasites. Uninfected macrophages and infected macrophages were assigned as controls.

CHAPTER FIVE

5. 0 DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSIONS

Morinda lucida is a traditional medicinal plant which is commonly used in African folk medicine. Its medicinal properties have been shown through various studies. Some attributes highlighted in these studies include anti-inflammatory, antibacterial and antimalarial activities (Chukwujekwu et al., 2005, Fakoya et al., 2014). The biological and pharmacological properties of *M. lucida* extracts have also been assessed using various *in vitro* models. Studies by (Kwofie et al., 2016), led to the isolation of tetracyclic iridoid compounds, Molucidin, ML-2-3 and ML-F52, from the leaves of *M. lucida* and authors showed the anti-trypanosomal activity of these compounds. This study therefore aimed at investigating the anti-leishmanial effect of these three compounds on *Leishmania* species and to determine the mechanism of action of active compounds.

5.1.1 Activity of tetracyclic iridoids against promastigotes

The *in vitro* anti-leishmanial activity of tetracyclic iridoid compounds, ML-2-3, Molucidin and ML-F52, were assayed against the promastigote and intracellular forms of *L. donovani* and *L. major*. Two tetracyclic iridoids, ML-F52 and Molucidin, were shown to have significant leishmanicidal effect on *L. donovani* promastigotes, with IC₅₀ values of 0.91 µM and 2.94 µM, respectively, and against *L. major* promastigotes, with IC₅₀ values of 1.77 µM and 1.85 µM, respectively. Previous studies of iridoids isolated from the bark of *Peperomia galoides*, grifolin and piperogalin have been shown that they exhibit anti-*leishmania* activity against 3 species of *leishmania*, *L. braziliensis*, *L. donovani* and *L. amazonensis* by causing their total lysis when tested

at a concentration of 100 $\mu\text{g/mL}$ (Mahiou et al., 1995). This therefore demonstrates the ability of iridoid derivatives such as Molucidin and ML-F52 to inhibit promastigote activity. It was however shown that ML-2-3 had no observable effect on *Leishmania* promastigote. This effect of the compound may be related to the structure of the compounds as it was observed that promastigotes were increasingly susceptible to compounds by the modification of the side group chain (-R-). This could therefore suggest some relation between the side chain and the activity of the compounds as had previously been demonstrated by (Ali et al., 2011) regarding the structure/activity relationships of naphthoquinones. In their study, by introducing a methyl or methoxyl group at C-2 position on 1,4-naphthoquinone, the anti-leishmanial activity was slightly increased whereas the presence of a hydroxyl group in the same position considerably decreased its effectiveness.

5.1.2 Anti-amastigote activity of tetracyclic iridoids

In the host stages of infection and development, the matured metacyclic promastigotes phagocytosed by host are taken up by host macrophage, where they transform into amastigote forms. They adapt to the environment of the macrophage via their immune evasion machinery and multiply within these immune cells. In further investigations, mimicking the infection of resident macrophages by promastigotes, RAW264.7 macrophages were infected and the anti-proliferative effect of the active compounds, ML-F52 and Molucidin on the intracellular amastigote forms was assessed. ML-F52 and Molucidin were found to inhibit promastigote replication in macrophages. The IC_{50} values determined based on the average number of intracellular amastigotes per macrophage was found to be 1.00 μM , 0.63 μM and 0.18 μM for Molucidin, ML-F52 and Amphotericin B, respectively. *In vitro* studies by (Castillo et al., 2007) using plumericin and its isomer isoplumericin (iridoids) isolated from *Himatanthus sucuuba* have shown the toxicity of

isoplumericin against infected macrophages preventing its further evaluation against these developmental stages of *Leishmania* though plumericin was shown in this same study to have anti-amastigote activity with IC₅₀ values of 0.90 µM and an IC₅₀ of 1.00 µM for Amphotericin B. There was however, no observable effect on intracellular amastigotes with regards to ML-2-3 treatment. The effect of ML-F52 and Molucidin was profound on intracellular amastigotes, suggesting that these compounds played an inhibitory role that altered cellular events important to parasites survival in macrophages.

5.1.3 Cytotoxicity of tetracyclic iridoids on cell lines

On many instances, in other studies that sought to identify novel compounds, the potential lead compounds tend to be non-selective and can therefore induce death of normal dividing cells. Thus it is a matter of critical evaluation to determine the dosage of the compound that when applied would not necessarily or adversely affect the survival of normal cells *in vitro* as a result of the disruption of normal physiological processes that eventually results in cell death. The identification of active and specific anti-leishmanial agents remains a thrust area in drug development via drug screening or discovery mechanisms (Benedeković et al., 2015, Lavrard et al., 2015). The cytotoxic effect of these compounds against normal cell lines as published in previous works (Kwofie et al., 2016) as well as RAW 264.7 macrophages was determined. ML-F52 was found to be more selective against *Leishmania* parasites than Molucidin.

5.1.4 Tetracyclic iridoids induce apoptotic-like effect in promastigotes

Having demonstrated the anti-leishmanial efficacy of ML-F52 and Molucidin on the two main developmental stages of the parasite, the mode of cell death induced by the iridoid compounds was

also determined. Both Molucidin and ML-F52 induced apoptotic-like features in *L. donovani* promastigotes as shown by increased staining of phosphatidylserine with annexin V. During apoptosis, physiological changes cause plasma membrane blebbing/shrinkage as well as condensation of cell nucleus and cytoplasm occur (Hingorani et al., 2011). And just as in metazoans, the onset of apoptosis is marked by externalization of phosphatidylserine as a result of translocation from the inner membrane to the outer leaflet of the plasma membrane which precedes loss of membrane integrity reference (Hingorani et al., 2011). This principle was the basis for tracking the mechanism of cell death induced by active tetracyclic iridoid compounds, ML-F52 and Molucidin. As was expected, less cells were observed to be undergoing apoptosis in the untreated group.

5.1.5 Immunohistochemistry analysis of effect of compounds on *Leishmania donovani*

Treatment with ML-F52 and Molucidin induced late apoptosis in *L. donovani* promastigotes suggesting that both compounds may have induced cell death as a result of a loss in parasite membrane integrity as demonstrated by an increased integration of annexin V and PI binding. Like many controlled processes of metabolism in eukaryotes, apoptosis is a regulated self-obliteration mechanism and facilitated by energy-dependent process. These processes are characterized by some specific morphological and biochemical traits including cell shrinkage, blebbing of plasma membrane, deficiencies in the mitochondrial membrane potential as well as nuclear fragmentation (Kerr et al., 1972). The findings from this study support similar findings which have shown that apoptotic-like programmed cell destruction pathways are indeed functional in trypanosomatids (Kaczanowski et al., 2011). However, despite the findings from increasing experimental works in the area of programmed cell death, there have been conclusions that *Leishmania* parasites share

apoptosis biochemical markers similar to metazoan. However, these vary considerably in the molecular machinery of this process and hence they are not well understood (Lee et al., 2002, Paris et al., 2004, Das et al., 2001, Sen et al., 2004, Raina and Kaur, 2006).

In this current study, the cell cycle and cytokinesis of promastigotes were also monitored. *Leishmania* like most trypanosomatida are defined by distinct single morphological markers namely the nucleus, kinetoplast and flagellum with each dividing once during the cell cycle.

The expected morphologies 1K1N1F, 1K1N2F, 1K2N2F and 2K2N2F were observed in control cells. Since the organelles as stated above replicate once during the cell cycle, the flagellum growth is initiated earliest, succeeded by mitosis and then the duplication of the kinetoplast that commences after the inception of nuclear anaphase. The replication cycle and duplication of DNA content of the parasite in the two daughter cells is a dominant feature of the process of cell cycle. Hence an investigation by cellular events in this study is critical to identifying the mechanical effect and possible phenotypic effect of tetracyclic iridoids on these cellular processes within *Leishmania*. It was observed from the study that Molucidin induced loss of kinetoplastid DNA as well as a rounded morphological structure of the parasites as opposed to the normally long slender morphology shown in the control group. This observation therefore could have resulted in the decreased cell proliferation or delayed cytokinesis in the treated group. The mitochondrion plays a vital role in the coordinating apoptosis, and therefore since *Leishmania* spp. have just a single large ramified mitochondrion which is responsible for the bulk of their energy, its irreparable impairment and dysfunction would have devastating effects on their survival of the parasite. Therefore, this observation within the treated groups as shown in the study could provide desired leads in the quest to identify drugs against *Leishmania*. Also the disruption of mitochondrion

structural integrity by damage could cause loosening of the texture of chromatin of the kinetoplast (de Souza and Rodrigues, 2009).

Earlier studies have described similar findings, with regards to loss of kinetoplast-DNA, when *Leishmania amazonensis* promastigotes were treated with a squalene synthase inhibitor, BPQ-OH (Rodrigues et al., 2005), or putrescine analogue (Vannier-Santos et al., 2008).

Cells undergoing apoptosis have been shown to feature active endonucleases that preferentially catalyze the degradation of DNA. The degraded DNA is captured in a flow cytometry plot as an increased displacement of cell population detected adjacent to the left side of the G₀-G₁ peak on a DNA frequency histogram, sub-G₁ (Sen et al., 2007). Evident in this study is a relatively higher percentage of cells in treated group with a significantly higher peak at the G₀-G₁ phase ($p < 0.05$) induced by Molucidin, ML-F52 and Amphotericin B compared to the untreated group (1.71 %). This observed phenomenon reaffirms the apoptotic effect induced by the compounds on the cultures. Also this observation is in line with accessions in reports of other compounds used in previous studies (Dutta et al., 2008, Sen et al., 2007, Li et al., 2008, Mukherjee et al., 2009, Das et al., 2001) when cultures *Leishmania* promastigotes were challenged with hydrogen peroxide, *Aloe vera* leaf extracts and artemisinin. However, in this study, there was cell cycle arrest observed at G₂-M in the treated groups, with ML-F52 inducing a significantly higher peak at G₂-M (54.5 %, $p < 0.05$). It has been established that *Leishmania* [cdc2-related protein kinase 3 (*L* CRK3)], which is a homologue of Cyclin-Dependent Kinase1 (CDK1), and the glycogen synthase kinase-3 (*L* GSK-3), is associated with the *Leishmania* viability as well as being a central regulator of cell-cycle progression in the parasite (Xingi et al., 2009, Naula et al., 2005, Grant et al., 2004, Ojo et al., 2008, Walker et al., 2011). *LCRK3* when inhibited resulted in a G₂-M cell-cycle arrest and a

subsequent apoptosis-like death of the parasites (Xingi et al., 2009, Hassan et al., 2001). The data showed ML-F52 treatment to induce an accumulation of polynucleated cells (DNA content) which could therefore explain the effect observed in the treated group linked to cell-cycle arrest at G₂-M. There was also displacement of cells adjacent the G₁-phase (the sub-G₁ phase) indicating an increased population of cells with less DNA content or damaged DNA relative to the untreated group substantiating the accession that tetracyclic iridoids induced apoptotic effect as shown in Nexin assay.

In kinetoplastid organisms, such as trypanosomes and *Leishmania*, apoptosis induction can be triggered by certain antimicrobial agents, the mechanism of which presents the disposition of Phosphatidylserine in the plasma bilayer. Some studies have reported an association of the kinetoplastid membrane protein (KMP-11) with lipid bilayers of *Leishmania spp.* via carboxyfluorescein release from liposomes suggesting KMP may to an extent increase bilayer pressure, stabilizing molecules such as lipophosphoglycan (Jardim et al., 1995). It has also been established *in vitro* (Li and Wang, 2008), that silencing of KMP-11 expression by RNA-interference inhibited parasites' basal body segregation and cytokinesis. Based on the apoptotic effect of our compounds, we fluorescently tagged treated and untreated parasites with anti-KMP-11 to determine the effect of compounds on KMP-expression and monitoring of phenotypic changes. Although no change in anti-KMP-11 fluorescence was observed in tagged-treated group relative to the controls, there was evidence of changes in parasite morphology with increasing concentrations of compounds (Molucidin: 5.88 μ M and ML-F52: 1.82 μ M). Morphological changes during the cell cycle of trypanosomatids has been extensively studied. In *Trypanosoma brucei*, *T. cruzi*, and *Leishmania tarentolae*, the first onset of division is the appearance of the new flagellum, then kinetoplast segregation with accompanied nuclear replication and finally with

cytokinesis (Elias et al., 2007, SIMPSON and BRALY, 1970, Shapiro and Englund, 1995, McKean, 2003). However, recent studies have shown contrary reports in cell cycle events among various species of *Leishmania* promastigotes (Minocha et al., 2011, Wheeler et al., 2011, Ambit et al., 2011).

Molucidin at 1.47 μM (half- IC_{50}), induced a significant accumulation of slightly elongated *L. donovani* promastigotes (''nectomonad-likes''). In the development of *Leishmania* vector stage forms, the procyclic promastigotes differentiate into the nectomonad promastigotes (12-20 μm body length), which are non-dividing migratory forms Lawyer et al. (1990) have shown however, that the accumulation of these forms in the sand fly does not mean properly established parasites as some parasites may not progress beyond the blood-meal phase depending on the *Leishmania* spp. or vector ('unnatural' vector). These trait changes observed in the current study can also be associated with the diminishing of source of nutrients *in vitro* at the late exponential phase (Bates, 2007, Gossage et al., 2003).

5.2 Limitations of the study

This study assessed the anti-leishmanial activity of tetracyclic iridoids on laboratory strains of *Leishmania* species, however findings from this study does not compromise the quality of the study as the species used in this study are clinal relevant strains of *Leishmania* (*Leishmania donovani*: causes visceral leishmaniasis and *L. major*: causes cutaneous leishmaniasis).

5.3 CONCLUSIONS

The study reports for the first time the activity of two tetracyclic iridoid compounds, Molucidin and ML-F52 against both the promastigote and intracellular amastigote forms of *L. donovani* and *L. major*. ML-F52 had the highest activity with significantly a lower cytotoxic effect on normal humanized cell lines. Also both Molucidin and ML-F52 induced apoptosis as well as cytokinesis in *Leishmania* parasites. These findings, together with the different presentations of apoptotic markers validated, supports the apoptosis inducing potential of Molucidin and ML-F52. The findings also demonstrated the different effects of the compounds on the morphology, and cellular events suggesting different mechanism of action against *Leishmania* parasites. This study therefore shows *Morinda lucida* as a potential beneficial source of candidates as chemotherapy against *Leishmania* species.

5.4 RECOMMENDATIONS

- (I) Active tetracyclic iridoids should be screened against field isolates or clinically relevant strains to determine their anti-*leishmania* effect.
- (II) Chemical structure of compounds could potentially be modified to improve toxicity levels.
- (III) Also more invasive analysis using methods such as Electron and Scanning Electron Microscopy techniques need to be employed to understand the mechanism or targets sites of compound activity.
- (III) *In vivo* efficacy study of compounds using closely modeled natural host of *Leishmania spp* should be performed and the pharmacokinetics and Pharmacodynamics properties of compounds assessed.

REFERENCES

- Alexander, J., Satoskar, A. R., & Russell, D. G. (1999). *Leishmania* species: models of intracellular parasitism. *J Cell Sci*, 112(18), 2993-3002.
- Ali, A., Assimopoulou, A. N., Papageorgiou, V. P., & Kolodziej, H. (2011). Structure/antileishmanial activity relationship study of naphthoquinones and dependency of the mode of action on the substitution patterns. *Planta medica*, 77(18), 2003-2012.
- Ambit, A., Woods, K. L., Cull, B., Coombs, G. H., & Mottram, J. C. (2011). Morphological events during the cell cycle of *Leishmania major*. *Eukaryotic cell*, EC. 05118-05111.
- Amoa-Bosompem, M. (2016). In vitro screening of extracts from selected medicinal plants in Ghana for anti-leishmania properties (Master's thesis), Retrieved Kwame Nkrumah University of Science and Technology, Kumasi.
- Antwi, A., Owusu, K., Amoa-Bosompem, M., Williams, N., Ayertey, F., Akyeh, L., Agyapong, J., Tetteh, T., Djameh, G. I., Botchie, S. K., Azerigyik, F., Ablordey, A., Tung, N. H., Uto, T., Appiah, A. A., Iwanaga, S., Shoyama, Y., Ohta, N., Egyir, B., and Ohashi, M. (2017). Anti-microbial Activities of Selected Ghanaian Medicinal Plants and Four Structurally Similar Anti-protozoan Compounds against Susceptible and Multi-drug Resistant Bacteria. *European Journal of Medicinal Plants*, ISSN: 2231-0894, Vol.: 20, Issue.: 2.
- Ashford, R. (2000). The leishmaniasis as emerging and reemerging zoonoses. *International journal for parasitology*, 30(12-13), 1269-1281.
- Barrett, M. P., & Croft, S. L. (2012). Management of trypanosomiasis and leishmaniasis. *British medical bulletin*, 104(1), 175-196.
- Bates, P. A. (2007). Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. *International journal for parasitology*, 37(10), 1097-1106.
- Benedeković, G., Kovačević, I., Popsavin, M., Francuz, J., Kojić, V., Bogdanović, G., & Popsavin, V. (2015). Divergent total synthesis of crassalactones B and C and evaluation of their antiproliferative activity. *Tetrahedron*, 71(28), 4581-4589.
- Bensoussan, E., Nasereddin, A., Jonas, F., Schnur, L. F., & Jaffe, C. L. (2006). Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *Journal of clinical microbiology*, 44(4), 1435-1439.
- Boakye, D., Wilson, M., & Kweku, M. (2005). A review of leishmaniasis in West Africa. *Ghana medical journal*, 39(3), 94.
- Boelaert, M., Bhattacharya, S., Chappuis, F., El Safi, S. H., Hailu, A., Mondal, D., . . . Peeling, R. W. (2007). Evaluation of rapid diagnostic tests: visceral leishmaniasis. *Nature reviews microbiology*, 5, S30-S39.
- Busari, M., Ogbadoyi, E., Kabiru, A., & Sani, S. (2014). Trypanocidal Properties of Aqueous Leaf Extract of *Morinda lucida*. *Asian Journal of Biological Sciences*, 7, 262-269.
- Camacho, M., Phillipson, J., Croft, S., Solis, P., Marshall, S., & Ghazanfar, S. (2003). Screening of plant extracts for antiprotozoal and cytotoxic activities. *Journal of Ethnopharmacology*, 89(2-3), 185-191.
- Castillo, D., Arevalo, J., Herrera, F., Ruiz, C., Rojas, R., Rengifo, E., Vaisberg, A., Lock, O., Lemesre, J.L., Gornitzka, H., Sauvain, M. (2007). Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional remedy made with *Himatanthus sucuuba* (Apocynaceae). *Journal of Ethnopharmacology*, 112(2), 410-414.

- Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J., Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature reviews microbiology*, 5(11supp), S7.
- Chatterjee, K. D. (1964). Parasitology (protozoology and helminthology) in relation to clinical medicine. *Parasitology (protozoology and helminthology) in relation to clinical medicine*. (Edn 5).
- Chattopadhyay, A., & Jafurulla, M. (2011). A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis. *Biochemical and biophysical research communications*, 416(1), 7-12.
- Choi, C. M., & Lerner, E. A. (2001). *Leishmaniasis as an emerging infection*. Paper presented at the *Journal of Investigative Dermatology Symposium Proceedings*.
- Christensen, S. B., Ming, C., Andersen, L., Hjørne, U., Olsen, C. E., Cornett, C., Theander, T.G., Kharazmi, A. (1994). An antileishmanial chalcone from Chinese licorice roots. *Planta medica*, 60(02), 121-123.
- Chukwujekwu, J., Van Staden, J., Smith, P., & Meyer, J. (2005). Antibacterial, anti-inflammatory and antimalarial activities of some Nigerian medicinal plants. *South African Journal of Botany*, 71(3-4), 316-325.
- Consortium, M. (2010). Leishmaniasis control in eastern Africa: Past and present efforts and future needs. *Situation and gap analysis*, 86.
- Control, C. f. D., & Prevention. (2013). *CDC health information for international travel 2014: The yellow book*: Oxford University Press.
- Cordell, G. A. (1995). Changing strategies in natural products chemistry. *Phytochemistry*, 40(6), 1585-1612.
- Coria-Tellez, A. V., Montalvo-González, E., Yahia, E. M., & Obledo-Vázquez, E. N. (2018). *Annona muricata*: A comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arabian Journal of Chemistry*, 11(5), 662-691.
- Das, M., Mukherjee, S. B., & Shaha, C. (2001). Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes. *Journal of Cell Science*, 114(13), 2461-2469.
- Dawit, G., Girma, Z., & Simenew, K. (2013). A review on biology. *Epidemiology and public health significance of leishmaniasis. J Bacteriol Parasitol*, 4(166), 2.
- De Medeiros, M. d. G. F., Da Silva, A. C., Citó, A. M. d. G. L., Borges, A. R., De Lima, S. G., Lopes, J. A. D., & Figueiredo, R. C. B. Q. (2011). In vitro antileishmanial activity and cytotoxicity of essential oil from *Lippia sidoides* Cham. *Parasitology international*, 60(3), 237-241.
- de Menezes, J. P. B., Guedes, C. E. S., Petersen, A. L. d. O. A., Fraga, D. B. M., & Veras, P. S. T. (2015). Advances in development of new treatment for leishmaniasis. *BioMed research international*, 2015.
- De Queiroz, A. C., Dias, T. d. L. M. F., Da Matta, C. B. B., Silva, C., Agra, L. H., de Araújo-Júnior, J. X., de Araújo, G.B., Moura, Fde B., Alexandre-Moreira, M. S. (2014). Antileishmanial activity of medicinal plants used in endemic areas in northeastern Brazil. *Evidence-Based Complementary and Alternative Medicine*, 2014.
- de Souza, W., & Rodrigues, J. C. F. (2009). Sterol biosynthesis pathway as target for anti-trypanosomatid drugs. *Interdisciplinary perspectives on infectious diseases*, 2009.
- Delmas, F., Di Giorgio, C., Elias, R., Gasquet, M., Azas, N., Mshvildadze, V., . . . Timon-David, P. (2000). Antileishmanial activity of three saponins isolated from ivy, α -hederin, β -

- hederin and hederacolchiside A1, as compared to their action on mammalian cells cultured in vitro. *Planta medica*, 66(04), 343-347.
- Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative immunology, microbiology and infectious diseases*, 27(5), 305-318.
- Dorlo, T. P., Balasegaram, M., Beijnen, J. H., & de Vries, P. J. (2012). Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *Journal of Antimicrobial Chemotherapy*, 67(11), 2576-2597.
- Dougall, A. M., Alexander, B., Holt, D. C., Harris, T., Sultan, A. H., Bates, P. A., Rose, K., Walton, S. F. (2011). Evidence incriminating midges (Diptera: Ceratopogonidae) as potential vectors of *Leishmania* in Australia. *International journal for parasitology*, 41(5), 571-579.
- Dutta, A., Sarkar, D., Gurib-Fakim, A., Mandal, C., & Chatterjee, M. (2008). In vitro and in vivo activity of Aloe vera leaf exudate in experimental visceral leishmaniasis. *Parasitology research*, 102(6), 1235-1242.
- Elias, M. C., da Cunha, J. P., de Faria, F. P., Mortara, R. A., Freymüller, E., & Schenkman, S. (2007). Morphological events during the Trypanosoma cruzi cell cycle. *Protist*, 158(2), 147-157.
- Elmahallawy, E. K., Martínez, A. S., Rodriguez-Granger, J., Hoyos-Mallecot, Y., Agil, A., Mari, J. M. N., & Fernández, J. G. (2014). Diagnosis of leishmaniasis. *The Journal of Infection in Developing Countries*, 8(08), 961-972.
- Fakoya, A., Owojuyigbe, O., Fakoya, S., & Adeoye, S. (2014). Possible antimicrobial activity of Morinda lucida stem bark, leaf and root extracts. *African Journal of Biotechnology*, 13(3).
- Forester, J., & Hartmut, T. (2006). MetaCyc Pathway: saponin biosynthesis I. *Advances in Experimental Medicine and Biology*, 405, 377-385.
- Fryauff, D. J., Hanafi, H. A., Klena, J. D., Hoel, D. F., Appawu, M., Rogers, W., . . . Koram, K. (2006). ITS-1 DNA sequence confirmation of *Leishmania major* as a cause of cutaneous leishmaniasis from an outbreak focus in the Ho District, southeastern Ghana. *The American journal of tropical medicine and hygiene*, 75(3), 502-504.
- Geu-Flores, F., Sherden, N. H., Courdavault, V., Burlat, V., Glenn, W. S., Wu, C., . . . O'connor, S. E. (2012). An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. *Nature*, 492(7427), 138.
- Gossage, S. M., Rogers, M. E., & Bates, P. A. (2003). Two separate growth phases during the development of *Leishmania* in sand flies: implications for understanding the life cycle. *International journal for parasitology*, 33(10), 1027-1034.
- Grant, K. M., Dunion, M. H., Yardley, V., Skaltsounis, A.-L., Marko, D., Eisenbrand, G., Croft, S.L., Meijer, L., and Mottram, J. C. (2004). Inhibitors of *Leishmania mexicana* CRK3 cyclin-dependent kinase: chemical library screen and antileishmanial activity. *Antimicrobial agents and chemotherapy*, 48(8), 3033-3042.
- Halder, A. K., Sen, P., & Roy, S. (2011). Use of antimony in the treatment of leishmaniasis: current status and future directions. *Molecular biology international*, 2011.
- Hassan, P., Fergusson, D., Grant, K. M., & Mottram, J. C. (2001). The CRK3 protein kinase is essential for cell cycle progression of *Leishmania mexicana*. *Molecular and biochemical parasitology*, 113(2), 189-198.
- Hingorani, R., Deng, J., Elia, J., McIntyre, C., & Mittar, D. (2011). Detection of apoptosis using the BD Annexin V FITC Assay on the BD FACSVerser™ System. *BD Biosciences*, 1-12.

- Hostettmann, K., & Marston, A. (1995). Saponins (Chemistry and pharmacology of natural products). *Journal of Natural Products* 1996 59 (1), 96-96: University Press, Cambridge.
- Islamuddin, M., Sahal, D., & Afrin, F. (2014). Apoptosis-like death in *Leishmania donovani* promastigotes induced by eugenol-rich oil of *Syzygium aromaticum*. *Journal of medical microbiology*, 63(1), 74-85.
- IUPAC, M. A., & Wilkinson, A. (1997). IUPAC Compendium of Chemical Terminology: the Gold Book: Oxford: Blackwell.
- Jardim, A., Hanson, S., Ullman, B., McCubbin, W., Kay, C., & Olafson, R. (1995). Cloning and structure-function analysis of the *Leishmania donovani* kinetoplastid membrane protein-11. *Biochemical journal*, 305(1), 315-320.
- Kaczanowski, S., Sajid, M., & Reece, S. E. (2011). Evolution of apoptosis-like programmed cell death in unicellular protozoan parasites. *Parasites & vectors*, 4(1), 44.
- Kam, T.-S., Subramaniam, G., Sim, K.-M., Yoganathan, K., Koyano, T., Toyoshima, M., Munchual R., Hayashi, M., Komiyama, K. (1998). Reversal of multidrug resistance (MDR) by aspidofractinine-type indole alkaloids. *Bioorganic & medicinal chemistry letters*, 8(19), 2769-2772.
- Kamhawi, S., Ramalho-Ortigao, M., Pham, V. M., Kumar, S., Lawyer, P. G., Turco, S. J., . . . Valenzuela, J. G. (2004). A role for insect galectins in parasite survival. *Cell*, 119(3), 329-341.
- Kato, H., Gomez, E., Cáceres, A., Uezato, H., Mimori, T., & Hashiguchi, Y. (2010). Molecular epidemiology for vector research on leishmaniasis. *International journal of environmental research and public health*, 7(3), 814-826.
- Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. *British journal of cancer*, 26(4), 239.
- Killick-Kendrick, R. (1999). The biology and control of phlebotomine sand flies. *Clinics in dermatology*, 17(3), 279-289.
- Kimutai, A., Tonui, W. K., Gicheru, M. M., Ngure, P. K., Ingonga, J., Kepha, S., Njerua, L.I., Wachira, D., Muhia, R.K., Mwangi, M., Nyamwamua, L.B. (2009). Evaluation of the adjuvanticity of artemisinin with soluble *Leishmania major* antigens in BALB/c mice. *Journal of Nanjing Medical University*, 23(6), 359-372.
- Kittakoop, P., Mahidol, C., & Ruchirawat, S. (2014). Alkaloids as important scaffolds in therapeutic drugs for the treatments of cancer, tuberculosis, and smoking cessation. *Current topics in medicinal chemistry*, 14(2), 239-252.
- Kwakyee-Nuako, G., Mosore, M.-T., Duplessis, C., Bates, M. D., Puplampu, N., Mensah-Attipoe, I., Desewu, K., Afegbe, G., Asmah, R.H., Jamjoom, M.B., Ayeh-Kumi, P.F., Boakye, D.A., Bates, P.A. (2015). First isolation of a new species of *Leishmania* responsible for human cutaneous leishmaniasis in Ghana and classification in the *Leishmania enriettii* complex. *International journal for parasitology*, 45(11), 679-684.
- Kweku, M. A., Odoom, S., Puplampu, N., Desewu, K., Nuako, G. K., Gyan, B., Raczniak, G., Kronmann, K.C., Koram, K., Botero, S., Boakye, D., and Akuffo, H. (2011). An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and preparation for future outbreaks. *Global health action*, 4(1), 5527.
- Kwofie, K. D., Tung, N. H., Suzuki-Ohashi, M., Amoa-Bosompem, M., Adegle, R., Sakyiamah, M. M., Ayertey, F., Owusu, K.B., Tuffour, I., Atchoglo, P., Frempong, K.K., Anyan, W.K., Uto, T., Morinaga, O., Yamashita, T., Aboagye, F., Appiah, A.A., Appiah-Opong, R., Nyarko, A.K., Yamaguchi, Y., Edoh, D., Koram, K.A., Yamaoka, S., Boakye, D.A.,

- Ohta, N., Shoyama, Y., Ayi, I. (2016). In-vitro Anti-trypanosomal Activities and Mechanisms of Action of Novel Tetracyclic Iridoids from *Morinda lucida* Benth. *Antimicrobial agents and chemotherapy*, AAC. 01916-01915.
- Lachaud, L., Dereure, J., Chabbert, E., Reynes, J., Mauboussin, J.-M., Oziol, E., . . . Bastien, P. (2000). Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. *Journal of clinical microbiology*, 38(1), 236-240.
- Lartey, M., Adusei, L., Hanson-Nortey, L., & Addy, J. (2006). Coinfection of cutaneous leishmaniasis and HIV infection. *Ghana medical journal*, 40(3).
- Lavrard, H., Salvetti, B., Mathieu, V., Rodriguez, F., Kiss, R., & Delfourne, E. (2015). Synthesis and in vitro antiproliferative activity of amido and amino analogues of the marine alkaloid isogranulatimide. *ChemMedChem*, 10(4), 607-609.
- Lawal, H., Etatuvie, S., & Fawehinmi, A. (2012). Ethnomedicinal and pharmacological properties of *Morinda lucida*. *Journal of Natural Products*, 5, 93-99.
- Lawyer, P. G., Ngumbi, P. M., Anjili, C. O., Odongo, S. O., Mebrahtu, Y. B., Githure, J. I., . . . Roberts, C. R. (1990). Development of *Leishmania major* in *Phlebotomus duboscqi* and *Sergentomyia schwetzi* (Diptera: Psychodidae). *The American journal of tropical medicine and hygiene*, 43(1), 31-43.
- Le Ven, J., Schmitz-Afonso, I., Touboul, D., Buisson, D., Akagah, B., Cresteil, T., . . . Champy, P. (2011). Annonaceae fruits and parkinsonism risk: Metabolisation study of annonacin, a model neurotoxin; evaluation of human exposure. *Toxicology Letters*(205), S50-S51.
- Lee, M. (2002). Plants against malaria. Part 1: Cinchona or the Peruvian bark. *The journal of the Royal College of Physicians of Edinburgh*, 32(3), 189-196.
- Lee, N., Bertholet, S., Debrabant, A., Muller, J., Duncan, R., & Nakhasi, H. (2002). Programmed cell death in the unicellular protozoan parasite *Leishmania*. *Cell death and differentiation*, 9(1), 53.
- Levine, R. A., Richards, K. M., Tran, K., Luo, R., Thomas, A. L., & Smith, R. E. (2015). Determination of neurotoxic acetogenins in pawpaw (*Asimina triloba*) fruit by LC-HRMS. *Journal of agricultural and food chemistry*, 63(4), 1053-1056.
- Lewis, D., & Dyce, A. (1982). The subgenus *Australophlebotomus* Theodor of *Phlebotomus* Rondani and Berté (Diptera: Psychodidae). *Australian Journal of Entomology*, 21(1), 37-54.
- Li, N., Shi, Z., Tang, Y., Chen, J., & Li, X. (2008). Recent progress on the total synthesis of acetogenins from Annonaceae. *Beilstein Journal of Organic Chemistry*, 4(1), 48.
- Li, Z., & Wang, C. C. (2008). KMP-11, a basal body and flagellar protein, is required for cell division in *Trypanosoma brucei*. *Eukaryotic cell*, 7(11), 1941-1950.
- Liu, H. W. (2011). Extraction and isolation of compounds from herbal medicines. *Traditional Herbal Medicine Research Methods: Identification, Analysis, Bioassay, and Pharmaceutical and Clinical Studies*, 81-138.
- Long, S. S., Pickering, L. K., & Prober, C. G. (2012). Principles and practice of pediatric infectious disease: *Elsevier Health Sciences*.
- Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., . . . Ahn, S. Y. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet*, 380(9859), 2095-2128.

- Mahapatra, D. K., Bharti, S. K., & Asati, V. (2017). Chalcone Derivatives: Anti-inflammatory Potential and Molecular Targets Perspectives. *Current topics in medicinal chemistry*, 17(28), 3146-3169.
- Mahiou, V., Roblot, F., Hocquemiller, R., Cavé, A., Barrios, A. A., Fournet, A., & Ducrot, P.-H. (1995). Piperogalin, a new prenylated diphenol from *Peperomia galioides*. *Journal of Natural Products*, 58(2), 324-328.
- Mahiou, V., Roblot, F., Hocquemiller, R., Cavé, A., De Arias, A. R., Inchausti, A., . . . Angelo, A. (1994). New aporphine alkaloids from *Gutteria foliosa*. *Journal of Natural Products*, 57(7), 890-895.
- Majester-Savornin, B., Elias, R., Diaz-Lanza, A., Balansard, G., Gasquet, M., & Delmas, F. (1991). Saponins of the ivy plant, *Hedera helix*, and their leishmanicidal activity. *Planta medica*, 57(03), 260-262.
- Mangal, M., Imran Khan, M., & Mohan Agarwal, S. (2016). Acetogenins as potential anticancer agents. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, 16(2), 138-159.
- Manske, R. H. F., & Holmes, H. L. (2014). *The alkaloids: chemistry and physiology*: Elsevier. Volume 1, Pages iii-v, 1-525 (1950)
- Mba-tihssommah, M. (2016). *Leishmania* infection in sand flies in a Cutaneous Leishmaniasis focus in Ghana, (Master's thesis). Retrieved Kwame Nkrumah University of Science and technology, Kumasi.
- McKean, P. G. (2003). Coordination of cell cycle and cytokinesis in *Trypanosoma brucei*. *Current opinion in microbiology*, 6(6), 600-607.
- McNaught, A. D., & McNaught, A. D. (1997). *Compendium of chemical terminology* (Vol. 1669): Blackwell Science Oxford.
- Miller, L. H., & Su, X. (2011). Artemisinin: discovery from the Chinese herbal garden. *Cell*, 146(6), 855-858.
- Minocha, N., Kumar, D., Rajanala, K., & Saha, S. (2011). Kinetoplast Morphology and Segregation Pattern as a Marker for Cell Cycle Progression in *Leishmania donovani* 1. *Journal of Eukaryotic Microbiology*, 58(3), 249-253.
- Mondragon-Shem, K., & Acosta-Serrano, A. (2016). Cutaneous leishmaniasis: the truth about the 'flesh-eating disease' in Syria. *TRENDS in Parasitology*, 32(6), 432-435.
- Moretti, C., Sauvain, M., Lavaud, C., Massiot, G., Bravo, J.-A., & Munoz, V. (1998). A novel antiprotozoal aminosteroid from *Saracha punctata*. *Journal of Natural Products*, 61(11), 1390-1393.
- Mukherjee, S., LaFave, M. C., & Sekelsky, J. (2009). DNA damage responses in *Drosophila* nbs mutants with reduced or altered NBS function. *DNA repair*, 8(7), 803-812.
- Muñozb, A. F. A. A. V., Roblot, F., Hocquemiller, R., & Cavé, A. (1992). Biological and chemical studies of *Pera benensis*, a Bolivian plant used in folk medicine as a treatment of cutaneous leishmaniasis. *Journal of Ethnopharmacology*, 37, 159-164.
- Naula, C., Parsons, M., & Mottram, J. C. (2005). Protein kinases as drug targets in trypanosomes and *Leishmania*. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1754(1-2), 151-159.
- Ofori, D., Gyimah, A., Obiri-Darko, B., Adam, K., Addae, A., & Jimoh, S. (2011). *Ethnobotany of Some Selected Medicinal Plants*: Forestry Research Institute of Ghana (Forig).

- Ojo, K. K., Gillespie, J. R., Riechers, A. J., Napuli, A. J., Verlinde, C. L., Buckner, F. S., . . . Scheer, A. (2008). Glycogen synthase kinase 3 is a potential drug target for African trypanosomiasis therapy. *Antimicrobial agents and chemotherapy*, 52(10), 3710-3717.
- Okpekon, T., Yolou, S., Gleye, C., Roblot, F., Loiseau, P., Bories, C., . . . Hocquemiller, R. (2004). Antiparasitic activities of medicinal plants used in Ivory Coast. *Journal of Ethnopharmacology*, 90(1), 91-97.
- Organization, W. H. (2009). Leishmaniasis: magnitude of the problem. *World Health Organization, Geneva*.
- Organization, W. H. (2013). Leishmaniasis Fact sheet N 375. 2014. Retrived from http://www.who.int/topics/tropical_diseases/factsheets/neglected/en/. Accessed, 24/05/2018.
- Osman, O. F., Oskam, L., Zijlstra, E. E., Kroon, N., Schoone, G. J., Khalil, E., . . . Kager, P. A. (1997). Evaluation of PCR for diagnosis of visceral leishmaniasis. *Journal of clinical microbiology*, 35(10), 2454-2457.
- Ozbilgin, A., Durmuskahya, C., Kayalar, H., Ertabaklar, H., Gunduz, C., Ural, I. O., . . . Toz, S. (2014). Antileishmanial activity of selected turkish medicinal plants. *Tropical Journal of Pharmaceutical Research*, 13(12), 2047-2055.
- Paris, C., Loiseau, P. M., Bories, C., & Bréard, J. (2004). Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrobial agents and chemotherapy*, 48(3), 852-859.
- Peraza-Sánchez, S., Cen-Pacheco, F., Noh-Chimal, A., May-Pat, F., Simá-Polanco, P., Dumonteil, E., . . . Mut-Martín, M. (2007). Leishmanicidal evaluation of extracts from native plants of the Yucatan peninsula. *Fitoterapia*, 78(4), 315-318.
- Potts, L. F., Luzzio, F. A., Smith, S. C., Hetman, M., Champy, P., & Litvan, I. (2012). Annonacin in *Asimina triloba* fruit: implication for neurotoxicity. *Neurotoxicology*, 33(1), 53-58.
- Preedy, V. R., Watson, R. R., & Patel, V. B. (2011). *Nuts and seeds in health and disease prevention*: Academic press. vol. 2011, p. 1226.
- Pucadyil, T. J., & Chattopadhyay, A. (2007). Cholesterol: a potential therapeutic target in *Leishmania* infection? *TRENDS in Parasitology*, 23(2), 49-53.
- Raina, P., & Kaur, S. (2006). Chronic heat-shock treatment driven differentiation induces apoptosis in *Leishmania donovani*. *Molecular and cellular biochemistry*, 289(1-2), 83-90.
- Riguera, R. (1997). Isolating bioactive compounds from marine organisms. *Journal of Marine Biotechnology*, 5, 187-193.
- Rodrigues, J. C., Urbina, J. A., & de Souza, W. (2005). Antiproliferative and ultrastructural effects of BPQ-OH, a specific inhibitor of squalene synthase, on *Leishmania amazonensis*. *Experimental parasitology*, 111(4), 230-238.
- Rogers, M. E. (2012). The role of *Leishmania* proteophosphoglycans in sand fly transmission and infection of the mammalian host. *Frontiers in microbiology*, 3, 223.
- Sacks, D. L. (2001). *Leishmania*-sand fly interactions controlling species-specific vector competence. *Cellular microbiology*, 3(4), 189-196.
- Sadeghi-Nejad, B., Saki, J., Khademvatan, S., & Nanaei, S. (2011). In vitro antileishmanial activity of the medicinal plant-Satureja khuzestanica Jamzad. *Journal of Medicinal Plants Research*, 5(24), 5912-5915.
- Sahpaz, S., Bories, C., Loiseau, P., Cortes, D., Hocquemiller, R., Laurens, A., & Cave, A. (1994). Cytotoxic and antiparasitic activity from *Annona senegalensis* seeds. *Planta medica*, 60(6), 538-540.

- Sauvain, M., Kunesch, N., Poisson, J., Gantier, J. C., Gayral, P., & Dedet, J. P. (1996). Isolation of leishmanicidal triterpenes and lignans from the Amazonian liana *Dolioscarpus dentatus* (Dilleniaceae). *Phytotherapy Research*, *10*(1), 1-4.
- Schmeda-Hirschmann, G., Razmilic, I., Sauvain, M., Moretti, C., Munoz, V., Ruiz, E., . . . Fournet, A. (1996). Antiprotozoal activity of jatrogrossidione from *Jatropha grossidentata* and jatrophone from *Jatropha isabellii*. *Phytotherapy Research*, *10*(5), 375-378.
- Seigler, D. S. (2012). *Plant secondary metabolism*: Springer Science & Business Media, *Molecular Ecology*, *18*, 23, (4974-4983).
- Sen, N., Das, B., Ganguly, A., Mukherjee, T., Tripathi, G., Bandyopadhyay, S., . . . Majumder, H. (2004). Camptothecin induced mitochondrial dysfunction leading to programmed cell death in unicellular hemoflagellate *Leishmania donovani*. *Cell death and differentiation*, *11*(8), 924.
- Sen, R., Bandyopadhyay, S., Dutta, A., Mandal, G., Ganguly, S., Saha, P., & Chatterjee, M. (2007). Artemisinin triggers induction of cell-cycle arrest and apoptosis in *Leishmania donovani* promastigotes. *Journal of medical microbiology*, *56*(9), 1213-1218.
- Shah, N. A., Khan, M. R., & Nadhman, A. (2014). Antileishmanial, toxicity, and phytochemical evaluation of medicinal plants collected from Pakistan. *BioMed research international*, *2014*.
- Shapiro, T. A., & Englund, P. T. (1995). The structure and replication of kinetoplast DNA. *Annual Reviews in Microbiology*, *49*(1), 117-143.
- Simpson, L., & Braly, P. (1970). Synchronization of *Leishmania tarentolae* by hydroxyurea. *The Journal of protozoology*, *17*(4), 511-517.
- Singh, N., Curran, M. D., Rastogil, A. K., Middleton, D., & Sundar, S. (1999). Diagnostic PCR with *Leishmania donovani* specificity using sequences from the variable region of kinetoplast minicircle DNA. *Tropical medicine & international health*, *4*(6), 448-453.
- Sundar, S., & Chakravarty, J. (2013). Leishmaniasis: an update of current pharmacotherapy. *Expert opinion on pharmacotherapy*, *14*(1), 53-63.
- Sundar, S., & Rai, M. (2002). Laboratory diagnosis of visceral leishmaniasis. *Clinical and diagnostic laboratory immunology*, *9*(5), 951-958.
- Tandon, J., Srivastava, V., & Guru, P. (1991). Iridoids: a new class of leishmanicidal agents from *Nyctanthes arbortristis*. *Journal of Natural Products*, *54*(4), 1102-1104.
- Vannier-Santos, M. A., Menezes, D., Oliveira, M. F., & de Mello, F. G. (2008). The putrescine analogue 1, 4-diamino-2-butanone affects polyamine synthesis, transport, ultrastructure and intracellular survival in *Leishmania amazonensis*. *Microbiology*, *154*(10), 3104-3111.
- Vos, T., Allen, C., Arora, M., Barber, R. M., Bhutta, Z. A., Brown, A., . . . Chen, A. Z. (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet*, *388*(10053), 1545-1602.
- Waechter, A. I., Yaluff, G., Inchausti, A., de Arias, A. R., Hocquemiller, R., Cavé, A., & Fournet, A. (1998). Leishmanicidal and trypanocidal activities of acetogenins isolated from *Annona glauca*. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*, *12*(8), 541-544.
- Walker, R. G., Thomson, G., Malone, K., Nowicki, M. W., Brown, E., Blake, D. G., . . . Mottram, J. C. (2011). High throughput screens yield small molecule inhibitors of

- Leishmania* CRK3: CYC6 cyclin-dependent kinase. *PLoS neglected tropical diseases*, 5(4), e1033.
- Wang, H., Naghavi, M., Allen, C., Barber, R. M., Bhutta, Z. A., Carter, A., . . . Coates, M. M. (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet*, 388(10053), 1459-1544.
- Weiss, F., Vogenthaler, N., Franco-Paredes, C., & Parker, S. R. (2009). *Leishmania tropica*–induced cutaneous and presumptive concomitant viscerotropic Leishmaniasis with prolonged incubation. *Archives of dermatology*, 145(9), 1023-1026.
- Wheeler, R. J., Gluenz, E., & Gull, K. (2011). The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. *Molecular microbiology*, 79(3), 647-662.
- WHO. (2010). *Control of the Leishmaniases: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010* (Vol. 949): World Health Organization.
- Wink, M. (2012). Medicinal plants: a source of anti-parasitic secondary metabolites. *Molecules*, 17(11), 12771-12791.
- Wright, C. W., & Phillipson, J. D. (1990). Natural products and the development of selective antiprotozoal drugs. *Phytotherapy Research*, 4(4), 127-139.
- Xingi, E., Smirlis, D., Myrianthopoulos, V., Magiatis, P., Grant, K. M., Meijer, L., Mikros, E., Skaltsounis, A.L., Soteriadou, K. (2009). 6-Br-5methylindirubin-3' oxime (5-Me-6-BIO) targeting the leishmanial glycogen synthase kinase-3 (GSK-3) short form affects cell-cycle progression and induces apoptosis-like death: exploitation of GSK-3 for treating leishmaniasis. *International journal for parasitology*, 39(12), 1289-1303.
- Xu, R., Fazio, G. C., & Matsuda, S. P. (2004). On the origins of triterpenoid skeletal diversity. *Phytochemistry*, 65(3), 261-291.

http://dna.kdna.ucla.edu/parasite_course-old/leish_files/subchapters/images/Slide8.JPG/15/04/2018

<https://ecdc.europa.eu/en/disease-vectors/facts/phlebotomine-sand-flies/13/07/2018>

<http://www.liberianfaunaflora.org/liberian-flora/rubiaceae/6223-morinda-lucida/13/07/2018>