EVALUATION OF ANTI-LEISHMANIAL ACTIVITIES OF *Olea europaea, Kigelia africana, Terminalia mollis, Croton macrostachyus,* AND *Bridella micrantha* EXTRACTS

BY

DENNIS WAFULA MUKHWANA

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN APPLIED PARASITOLOGY AND VECTOR BIOLOGY

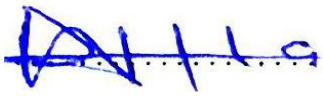
SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES

MASENO UNIVERSITY

DECLARATION

Declaration by the candidate

This thesis is my own work, and it has never been submitted for a degree or other honour at another university or college.



Sign…….....Date …….....

Dennis Wafula Mukhwana

Reg. No. PHD/SC/00045/2018

Approval by supervisors

This thesis has been submitted for examination with our approval as university supervisors

Sign…….....Date …….....

Dr. Cyrus Ayieko

Department of Zoology

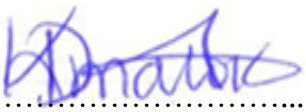
Maseno University

Sign…….....Date …….....

Dr. Mweresa Collins

Department of Biological Sciences

Jaramogi Oginga Odinga University of Science and Technology



Sign…….....Date …….....

Dr. Damaris Matoke-Muhia

Centre for Biotechnology Research and Development

Kenya Medical Research Institute

ACKNOWLEDGEMENT

My sincere appreciation goes to Dr. Cyrus Ayieko, Dr. Mweresa Collins and Dr. Damaris Matoke-Muhia for their tireless guidance and advice in the realization of this work. I wish to acknowledge Maseno University and Kenya Medical Research Institute for financing my research work. Lastly, I wish to appreciate all those who provided any form of input in this work, I sincerely thank you all.

DEDICATION

I dedicate this Thesis to the Almighty God

ABSTRACT

Leishmaniasis pose a significant medical concern worldwide which if left untreated, can be fatal. Although expensive and toxic, pentavalent antimonial drugs are the first-line treatment for leishmaniasis raising the need to find an alternative. Plants extracts contain several bioactive compounds that may offer alternative therapeutic signifcance to already developed antileismanial drugs. Based on folkloric information, a number of plants such as *Olea europaea, Kigelia africana, Terminalia mollis, Croton macrostachyus,* and *Bridella micrantha* are used to cure Leishmaniasis in the endemic regions in Baringo County. However, no studies have been done to find out the phytochemical compounds, as well as the anti-leishmanial properties. The aim of this study was to investigate the anti-leishmanial activity of *Olea europaea, Kigelia africana, Terminalia mollis, Croton macrostachyus,* and *Bridella micrantha* extracts. The plants were collected from Marigat in Baringo County (Kenya), air-dried, ground into fine particles, and extracted at the KEMRI Center for Traditional Medicine and Drug Research. Completely randomized design (CRD) was used in the in vivo studies. In vitro investigations were carried on *L. major* promastigotes to determine inhibitory concentrations (IC_{50}) and Minimum Inhibition Concentrations (MIC) and toxicity on Vero cells. A total of 35 mice, 6 in each experimental group were used. For in vivo bioassays, the mice were injected intradermally on the left posterior footpad with 1×10^6 stationary phase flagellate forms of cultured *L. major,* and then housed for 4 weeks to allow disease manifestation. Differences between treatment groups exposed to different drugs were examined using logistic regression for each parameter studied. Phytochemical components of bark of the plants contain glycoside, terpenoids, tannins, flavonoids, steroids, and saponins. *T. mollis* contaned higher concentration of tannins, phenols, alkaloids, and anthraquinone. The crude extracts from the plants significantly inhibited promastogote and amastigote growth ($P < 0.05$) after 24 hours of exposure, with the standard drug (Amphotericin B) being the most effective, while *T. mollis* was highly potent on amastigote among the plant extracts, then, *C. macrostachyus,* while *O. europeae* was least effective. Among the plant extracts, *T. mollis* had the highest efficacy (IC_{50} = 85.5 mg/l) in promastigote, while the least effective was *O. europaea*. In amastigotes, *T. mollis* exhibited the highest anti-amastigote activity (IC_{50} = 96.5 mg/l). The Leishman Donovan Units (LDU) of the *L. major* infection was lowest (0.12×10^6) in *L. major*-infected mice treated with *T. mollis,* while that of *O. europeae* was highest (4.12×10^6). After four weeks, mice administered with *T. mollis* demonstrated the greatest decrease in lesion diameter (0.68 ± 0.07 mm). Therapy with *T. mollis,* resulted in a considerably greater lesion reduction in mice. After 4 weeks of treatment, mice administered with *O. europeae* had the smallest reduction in lesion diameters of all the drugs examined. After 24 hours of treatment, the different drugs significantly influenced mammalian cell survival ($P < 0.05$), with percentage cell viability of herbal medications, *B. microstachyus,* and *O. africana* causing the most toxic effects. It is concluded that among the extracts from the back of the five test plants, *T. mollis* at concentraton 85.5 to 96 mg/l was the most effective against *Leishmania* infection. Over all, the results obtained from the crude extracts screening, suggest that these may be promising sources for the development of new drugs for controlling leishmaniasis. The study recommends the use of *T. mollis* as the most effective plant extract in management of leishmaniases.

TABLE OF CONTENTS

LIST OF ABBREVATIONS/ACRONYMS

ACUC	Animal Care and Use Committee
AI	Association Index
ANOVA	Analysis of Variance
AUC	Animal Care and Use Committee
CBRD	Centre for Biotechnology Research and Development
CE	Catechin Equivalent
CL	Cutaneous leishmaniasis
CTMDR	Centre for Traditional Medicine and Drug Research
DAF-2T	Diaminofluorescein-2- triazole
DCM	Dichloromethane extract
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicrylhydrazyl
DMRT	Duncans Multiple Range Test
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
IR	Infection rate
KEMRI	Kenya Medical Research Institute
LDU	Leishman Donovan Units
LHFD	Left hind footpad
LPG	Glycoconjugate lipophosphoglycan
MI	Multiplication Index
MIC	Minimal inhibitory concentration
ML	Mucocutaneous leishmaniasis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NO	Nitric oxide
NTDs	Neglected tropical diseases
PBS	Phosphate-buffered saline
PCR	Polymerase Chan Reaction
ROS	Reactive Oxygen Species
VL	Visceral Leishmaniasis
WHO	World Health Organization

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Considered as one of the neglected tropical diseases (NTDs), Leishmaniasis is a major medical issue, with almost 340 million individuals in 88 nations at risk and roughly 2 million infections and over 70,000 deaths yearly (World Health Organization, 2020). Female sand flies harbour the *Leishmania* species that transmit the disease (Mahdy *et al.*, 2016; Cotton, 2017; Salah *et al.*, 2020). Promastigotes, transmitted by sand flies via a blood meal, invade and multiply as amastigotes within mammalian host macrophages (Abdelhaleem *et al.*, 2019) and finally accumulate in the liver and spleen (Liévin-Le Moal and Loiseau, 2016; Thakur *et al.*, 2020a).

More than 20 flagellate parasites of the genus *Leishmania* subgenera *Leishmania* and *Viannia* are the causative agents of Leishmaniasis (Mahdy *et al.*, 2016; Cotton, 2017; Salah *et al.*, 2020). The Eurocentric worldview groups *Leishmania* parasites into Old World species: *L. major*, *L. aethiopica*, *L. infantum*, *L. donovani* and *L. tropica* (Maurício, 2018; Feres, 2019) and New World species such as *L. amazonensis*, *L. chagasi*, *L. mexicana*, *L. naiffi*, *L. braziliensis*, and *L. guyanensis* (Mitropoulos *et al.*, 2010; Kevric *et al.*, 2015). In Eastern Africa Region, the more prevalent visceral leishmaniasis (VL) is usually caused by *L. donovani* and *L. infantum* (Dulacha *et al.*, 2019; Kühne *et al.*, 2019; Bhunia and Shit, 2020).

Three general types of clinical manifeststion occur: cutaneous, muco-cutaneous and visceral leishmaniasis (Alemayehu and Alemayehu, 2017; Rojas-Jaimes *et al.*, 2019). Cutaneous leishmaniasis (CL) is the most prevalent form worldwide responsible for between 600,000 to 1 million new cases annually, with 90% occuring in the Middle East and North Africa (Aronson and Joya, 2019). Infection with CL causes skin lesions, mainly ulcers, on exposed

parts of the body, leaving life-long scars and serious disability (Bettaieb *et al.*, 2020). In Kenya, cutaneous leishmaniasis, caused by *L. tropica* is endemic in Nakuru and Laikipia counties while that due to *L. major* is endemic in Baringo County. Mucocutaneous leishmaniasis (ML) has restricted distribution in Ethiopia in the old world while in the New world it occurs in Central and Latin America (Giovanny *et al.*, 2020). The disease present in the form of lesions development on the skin which destroy the mucous membranes of the nose, mouth and throat cavities (Garrido-Jareño *et al.*, 2020). Visceral leishmaniasis (VL) is mostly distributed in Sub Saharan Africa, South Asia, as well as South and Central America (Bi *et al.*, 2018). The disease is characterized by swelling of the spleen and liver, and anaemia and is the most severe form of leishmaniasis, often fatal if not treated (Chakravarty *et al.*, 2019; van Griensven and Diro, 2019). High-burden countries such as India, Bangladesh, Ethiopia, and Brazil, account for 90% of VL patients while lower rates occur in South Europe, Central Asia, Kenya and Middle East (Alemayehu and Alemayehu, 2017). Without proper treatment, fatality rates are often high in VL (Ibarra-Meneses *et al.*, 2020; Tekalign *et al.*, 2020).

Since *Leishmania* parasites have started exhibiting resistance to conventional first line antimonial drugs (Capela *et al.*, 2019; Sundar *et al.*, 2019a), alternative cheaper drugs are required (Nassif *et al.*, 2017; Sundar *et al.*, 2019a). For many years crude plant extracts have been used in conventional folklore medicine (Tariq *et al.*, 2016; Mutoro *et al.*, 2018; Simoben *et al.*, 2018). Plants containing metabolites such as sesquiterpenes, lactones, aurones, cannabinoids, chalcones, chromenes, coumarins, flavonoids, isoflavonoids, lignans, quinones, stilbenoids, sesquiterpenes, terpenes, xanthones, and phenolics have been found to be suitable as effective control against leishmaniasis (Sosa *et al.*, 2016; Gutiérrez-Rebolledo *et al.*, 2017; da Silva *et al.*, 2019; Moreira *et al.*, 2019).

Phytochemical studies have reported bioactive triterpene acids including oleanolic acid in *Olea europaea* (Family: Oleaceae) (El and Karakaya, 2009). *Kigelia africana* (Family: Bignoniaceae), found in sub-Saharan Africa and traditionally used to treat stomach-ache and diarrhoea as well as abdominal pain (Costa *et al.*, 2017; Nabatanzi *et al.*, 2020a), is cited for the presence of tannins, steroids, flavonoids, terpenes, tannins, steroids, coumarins iridoids, naphthoquinones, saponins and caffeic acid (Bello *et al.*, 2016; Nabatanzi *et al.*, 2020b). *Terminalia mollis* (family Combretaceae) a tropical and subtropical plant (Fahmy *et al.*, 2015; Dimas *et al.*, 2020) has bioactivity attributed to triterpenes, phenolic acids, triterpenoidal glycosides, tannins, flavonoids, and lignan in its content, probably explaining its renowned antioxidant activities (Adeeyo *et al.*, 2018). *Croton macrostachyus* (family: Euphorbiaceae) - a drought-deciduous tree – has various medicinal uses (Obey *et al.*, 2016; Habtom and Gebrehiwot, 2019). *Bridella micrantha* (Hochst.) (family Phyllanthaceae (formerly Euphorbiaceae), has anti-helminthic, anti-amoebic, anti-anaemic and antibacterial properties (Ngane, 2019) and has been reported to have bioactive compounds such as saponins, steroids, flavonoids, phenolic compounds, and tannins (Adefuye and Ndip, 2013; Maroyi, 2017). In Kenya, several plants that have been evaluated in the past and contain some active compounds such as flavonoids, isoflavonoids, lignans, quinones, sesquiterpenes, terpenes and phenolics which are active against leishmania parasites (Mutoro *et al.*, 2018; Siangu *et al.*, 2019; Kamau *et al.*, 2020). However the current plants under investigation have rarely been evaluated for anti-leishmanial properties. Considering the meager information on viability of the plant based medication, the goal of this investigation was to assess the anti-leishmanial action of *T. mollis, C. macrostachyus, O. europaea, K. africana,* and *B. micrantha* both in vitro and in vivo. Because the composition of metabolites depends on the local environment, it is necessary to investigate the anti-leishmanial phytoconstituents of these plants from Baringo, to provide scientific basis for their use in treatment.

Diverse bioactivity has been reported for *Terminalia mollis, Croton macrostachyus Olea europaea, Bridella micrantha and Kigelia africana* (Atawodi and Olowoniyi, 2015; Guinda *et al.*, 2015; Hashmi *et al.*, 2015; Douglas and Gitonga, 2016; Cheurfa *et al.*, 2019). Several studies have examined these plants for efficacy of their extracts against disease causing microorganisms (Bereksi *et al.*, 2018; Gonelimali *et al.*, 2018), antiprotozoan (da Silveira Regueira-Neto *et al.*, 2018; Ohashi *et al.*, 2018) and anti-plasmodial activity (Djouwoug *et al.*, 2020; Tchatat Tali *et al.*, 2020; Waiganjo *et al.*, 2020). These botanicals have also been utilised as a traditional remedy to manage leishmaniasis (Wafula, 2020). However, in order to assess the utility of these plants as alternative treatment for Leishmanaisis, there is need to establishment of parameters such as the minimal inhibitory concentration (MIC) and rates of infections.

Despite the curative benefits of botanicals, improper dosing of herbal drugs put patients at risk of toxicity, especially as plant parts used have a mixture of metabolites (Seremet *et al.*, 2018). Toxicity of medicinal plant extracts have been reported ranging from nephrotoxicity to hepatotoxicity (Fanoudi *et al.*, 2017; Sioud *et al.*, 2020). From traditional standoff, the use of traditional medicine has been commonly associated with severe side effects. For instance, high rates of dehydration, vomiting, diarrhoea, and altered mental status have been observed in patients who reported recent use some plant derived medicines (Luyckx *et al.*, 2005). Similarly, herbal drugs have been associated with dysfunctions of the liver and kidneys resulting in high patient mortality (Luyckx and Naicker, 2008). Regardless of how effective the drug is against the parasite, the toxicity of these plants must also be known before a decision on their adoption is made (Karaali *et al.*, 2018). Despite being used by communities as herbal remedies, there have been no investigations on the toxicity profiling of these plant extracts.

In vivo studies are very important, since these studies may show that a compound is active against a certain microorganism or parasite in vitro. Things that can affect potency like physiochemical characteristics, bioavailability, metabolic stability etc It cannot be assured, therefore, that an anti-leishmanial agent that kills or prevents an organism from growing *in vitro* will be effective in treatment *in vivo* (Chen *et al.*, 2019; Prpa *et al.*, 2020). This is on the grounds that, the anti-leishmanial active ingredients of plant concentrates might be metabolised in vivo to yield inactive metabolites, which bring about their reduced efficacy in vivo (Nicolaus *et al.*, 2017). Thus, the anti-leishmanial action of the plant extracts in this study was done in vivo to assess their consistency in terms of efficacy.

1.2 Statement of the problem

Leishmaniasis is a major disease in several parts of Kenya. Most form of leishmaniasis are seldom deadly in the short term but can be fatal if left untreated (Inceboz, 2019). Current conventional anti-leishmanial drugs for treatment of leishmniasis are expensive, toxic and when used for long term, are subject to parasite resistance (Tiwari *et al.*, 2018; Barbosa Gomes de Carvalho *et al.*, 2020) requiring alternative treatment. To reduce treatment cost, there is increasing recognition for the use of herbal drugs (Eskandari *et al.*, 2020; Singh *et al.*, 2020). The major merits of herbal medicine include their low cost, low incidence of serious adverse effects and good efficacy (de Menezes *et al.*, 2015). Moreover, critical information such as active ingredients, antiparasitic potency, and toxicity of plant in relation to leishmania still lag behind. Many plant products including *T. mollis, C. macrostachyus, O. europaea, K. africana, and B. micrantha* crude extracts have been reported in several literature to contain antiparatic properties. Unfortunately, the efficacy of most of these compounds has been shown only in *in vitro* studies and hence they are still in their initial stages of preclinical trials to be applicable in clinical practice and translation of their results into clinical practice is a

neglected field. Furthermore, efficacies of phytochemical constituents are reported to be influenced by a range of contexts, including local environment, a measure of dynamic mixtures in plants, as well as the types of living organisms under investigation, making it undeniably not easy to generalise the anti-leishmanial properties of many plants. In Kenya, the use of botanical agents in treating leishmaniasis has been limited due to insufficiency of such knowledge.

1.3 Justification of the study

Leishmaniasis a neglected tropical diseases that is responsible for 2 million infections and over 70,000 deaths across the globe very year (World Health Organization, 2020). Antimonial drugs are first line of treatment against *Leishmania* parasites but they are expensive and have high toxicity (Capela *et al.*, 2019; Sundar *et al.*, 2019a), There have been reports of drug resistance thus threatening the efficiency and efficacy of the existing chemotherapeutic regimes, hence alternative cheaper drugs are required.. There are several plants with bioactive compounds that have been used for treatment of leishmania and other protozoan diseases. However, most of the plants that are candidates are based on folklore methods of identification with lack of further scientific studies on such plants (Prasad *et al.*, 2008). The plant biocides have known activities when utilized independently both target the kinetoplastid parasites hence making them proper agents for examination in vitro and in vivo frameworks as an option to the synthetic drugs. One of the objective ways to deal with treatment involves the evaluation of bioactive agents that are known to have antiparasitic activities inside the *Leishmania* species.

Herbal drugs have been evaluated against protozoan parasites with different outcomes. Plant families that contain biocidal agents that are effective against other protozoan infections could be useful in treating leishmaniasis. Choice of plant species from these families for the

present study was guided by the fact that they have antiprotozoan properties (Beshbishy *et al.*, 2019; Falode *et al.*, 2020; Pereira *et al.*, 2020). Therefore, the anti-leishmanial activities of *K. africana, T. mollis, C. macrostachyus O. europaea,* and *B. micrantha* was assessed in vitro and in vivo in this investigation. Despite folkloric applications and phytochemical investigations in other parts of the world, the plants in this study have rarely been tested for anti-leishmanial active ingredients.

1.4 Objectives of the study

1.4.1 General objective

This study evaluated the anti-leishmanial activity of *Terminalia mollis, Croton macrostachyus, O. europaea, Kigelia africana* and *Bridella micrantha* extracts both *in vitro* and *in vivo.*

1.4.1 Specific objectives

1. To analyze the anti-leishmanial phytochemical components of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha extracts*
2. To establish the minimal inhibitory concentration, parasite proliferation, and rates of infection of *L. major* in culture after treatment with extracts from *O. europaea, K. africana, T. mollis, C. macrostachyus,* and *B. micrantha.*
3. To determine the toxicity of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* extracts on vero cells.
4. To determine the efficacy of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* extract on *L. major* wound growth, parasite burdens in the liver and spleen in BALB/c mice.

1.5 Hypotheses

H_{01}: The Chemical composition of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* do not contain anti-leishmania active ingredients.

H_{02}: Extracts of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* are not effective on promastigotes and amastigotes of *L. major*

H_{03}: Extracts of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* are toxic to vero cells.

H_{04}: Treatment with extracts of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* does not affect on *L. major* wound growth, parasite burdens in the liver and spleen in vivo in mice.

1.6 Significance of the study

Despite good knowledge on the epidemiology of leishmaniasis, it still pose significant health risk to population especially in developing countries. There is therefore continued advocacy to continue research focusing on controlling the disease, through a suitable approach to control vectors, as well as safe, non-invasive, short and cheap treatment. Therefore this study will provide. This study aimed at providing an affordable, safe and effective anti-leishmanial herbal extract from a locally growing medicinal plants (*O. europaea, K. africana, T. mollis, C. macrostachyus,* and *B. micrantha*).

This study further increases the potential for utilization of *O. europaea, K. africana, T. mollis, C. macrostachyus,* and *B. micrantha* by local communities creating a source of income. The innovate in addition provides low cost therapy to communities in endemic areas and alleviate toxic side effects attributed to leishmanicidal drugs such as pentavalent antimonials, Amphotericin B, Paromomycin, miltefosin and liposomal-amphotericin B. Data

from this study contributes to development of drugs treatment for treatment of clinical leishmaniasis.

1.7 Limitation of the study

The research lacked funding to undertake detailed chromatographic identification of bioactive compounds, as well as isolation and purification of the plant extracts. This limited the ability to evaluate the bioactive compounds that were responsible the observed responses. Thus the study generalized the bioactivity of the compounds based on literature information.

CHAPTER TWO

LITERATURE REVIEW

2.1 Scale of leishmaniasis problem

The obligatory intramacrophage protozoan parasite of the genus *Leishmania* causes leishmaniasis, a serious vector-borne infection (Torres-Guerrero *et al*., 2017). A bite by phlebotomine sand flies causes the transmission of leishmaniasis (Giraud *et al.*, 2019). The disease infect numerous mammalian species, including humans (Cunze *et al.*, 2019; Ghatee *et al.*, 2020). As a result, the disease's range is determined by the sand fly vector's distribution (Alemayehu and Alemayehu, 2017).

The endemicity of leishmaniasis is seen in southern Europe, the tropics and subtropics (Hailu *et al.*, 2016). Leishmaniasis, on the other hand, primarily affect countries in Asia, Europe, South America, and Africa (Casulli, 2021). Leishmaniasis is localised in 88 nations as well as the only illness caused by a vector in the tropical region which has long remained prevalent in the south of Europe (Flaih, 2022). Leishmaniasis now has a wider geographical spread than it did previously, and it is considered a significant public health risk in some nations (Steverding, 2017; Wamai *et al.*, 2020). Urbanization, immunodeficiency, poverty, migration of people, destruction of forests, and poor prognosis are all factors contributing to a rise in global leishmaniasis incidence (Ruiz-Postigo *et al.*, 2021). Changes in the environment caused by humans, as well as population shifts, may modify the distribution and intensity of transmitters and hosts, increasing people's exposure to infection.

Tropical and subtropical environments, including rainforests, have higher rates of leishmaniasis infection (Jagadesh *et al.*, 2021). These countries include India, Sudan, Bangladesh, Brazil, and Nepal which account for over 90% of all Visceral leishmaniasis cases worldwide (Hao *et al.*, 2021). When a sand fly vector of the genera *Lutzomyia* or

Phlebotomus sucks blood blood from an infected blood the disease may be transmitted to humans through Leishmania species (Bates, 2018; Bettaieb *et al.*, 2020). After feeding on blood, the sandfly consumes macrophages, and the amastigotes are discharged into the fly's stomach (Inceboz, 2019). Amastigotes change into motile, flagellated, elongated promastigotes, almost instantly. The flagellate form enter the fly's gut, in which they dwell inside the cell and reproduce by binary fission (Martínez-Valencia *et al.*, 2017).

Metacyclic *Leishmania* promastigotes, as well as saliva, are transferred to a mammalian host via the second blood feeding (Giraud *et al.*, 2019). The promastigotes are picked up by macrophages once inside the host, where they swiftly convert to the amastigotes, survive, and reproduce, eventually causing the macrophages to lyse (Serafim *et al.*, 2018). Additional macrophages take up the discharged amastigotes, resulting in the next cycle. All organs that contain macrophages and phagocytes, including the liver, spleen, and bone marrow, eventually get infected (Marshall *et al.*, 2018).

2.2 Clinical manifestation and diagnosis of leishmaniases

Kala azar, dermal and mucocutaneous leishmaniasis are the major pathological categories caused by different species (Hernández-Bojorge *et al.*, 2020; Vandeputte *et al.*, 2020). Visceral leishmaniasi symptoms include intermittent fever, weakness, cough, and diarrhea, as well as chills and sweating that are similar to malaria symptoms (Lima *et al.*, 2017; Cavalcante *et al.*, 2020). The reticuloendothelial system organs that are most severely affected in VL are the bone marrow, spleen, and liver (Lima *et al.*, 2018). By the third month, non-tender splenomegaly has grown rather severe. Patients typically develop pyrexia, wasting, hepatosplenomegaly as the parasites spread (Kakooei *et al.*, 2020; Mozaffari *et al.*, 2020). *Viannia* parasites cause mucocutaneous leishmaniasis by transmitting parasites from the cutaneous to the naso-oropharyngeal membrane via haematogenous or lymphatic

transmission (do Nascimento *et al.*, 2019; Vieira-Gonçalves *et al.*, 2019). It causes lesions in the nose, mouth, and throat cavities, which can lead to severe and disfiguring loss of the mucous membranes (Thakur *et al.*, 2020b).

The incubation time for cutaneous leishmaniasis is 2–6 weeks, and the initial sign of infection is usually a little redness at the region of sandfly bite, however the prepatent period vary among people (Aoun *et al.*, 2020). Over the course of 6 months, the erythema transforms into a pimple, then a lump, which forms an open wound and generates the disfigurement that is diagnostic of localized cutaneous leishmaniasis (Handler *et al.*, 2015). Some species, such as *Leishmania mexicana* and *Leishmania aethiopica*, can cause dispersed skin infection with broad non-ulcerating nodules (Amro and Hamarsheh, 2021). Cutenous leishmaniasis lesions in non human species tend to self-cure after 2–15 months (Farash *et al.*, 2020).

Analysis of infected tissue, lymphoid tissue, spleen, or bone marrow fluids is the gold standard diagnostic method for parasite diagnosis, with positive rates of over 80% for the latter (Thakur *et al.*, 2020b). A direct agglutination test and the rK39 urine antigen test, both of which have reported sensitivities of up to 95% and 87 %, respectively, are the most promising tests (Kiros and Regassa, 2017). Indirect fluorescent antibody test has also been developed (Moreno *et al.*, 2014). Serological tests, on the other hand, can be expensive and impractical in the field, since they have a high rate of false negatives in the immunodeficient, may persist after cure, and are less useful in CL (da Silva *et al.*, 2013). The PCR-based approaches as well as other rapid tests are now being deployed more recently (Cruz *et al.*, 2013; Galluzzi *et al.*, 2018).

2.3 Current treatment of the leishmaniases

Across many regions of the globe, pentavalent antimonials, which were first developed in 1940s and are currently the initial treatment for both cutaneous and visceral leishmaniasis, are still in use (Kotb Elmahallawy and Agil, 2015; Aronson *et al.*, 2016). Sodium stibogluconate (Pentostam) and megluminate antimonite (Glucantine) are the most common examples (Lagat *et al.*, 2014). Second-line anti-leishmanial medicines include amphotericin B and pentamidine, both of which need lengthy parenteral administration (Kaur and Rajput, 2014). With the amount time taken for it's active substance in the body to reduce by half of around 2 hours and a remaining half-life of around 76 hours, pentavalent antimonials are rapidly taken up and excreted (Faucher *et al.*, 2016; Chakravarty and Sundar, 2019). Amphotericin B, Ketoconazole, Miltefosine, and Paramomycin are newer anti-leishmanial medicines that have been introduced in response to a great rise in the occurrence of tolerance to sodium stibogluconate or meglumine antimoniate in recent years (Salari *et al.*, 2022).

Despite the wide range of *Leishmania* species and associated manifestations, the antimonial therapy regimen remains rather consistent: For 21-28 days, one takes 15-20 mg SbV/kg every day, but for 40 days when resistance is detected (Sundar and Chakravarty, 2015; Sundar *et al.*, 2019a). Chemotherapy can be given intramuscularly or intravenously and treatment regimens vary based on the species, the patient's condition, and the patients access to healthcare facilities and infrastructure (Brito *et al.*, 2017). Whereas sudden healing is common, the recovery rate depends on the *Leishmania* pathogen, and complete recovery might take months or years (Aronson and Joya, 2019). These drugs have a number of disadvantages, including a lengthy treatment period, and non-reversible adverse outcomes (Olías-Molero *et al.*, 2021).

Despite having *Leishmania peruviana* infection, 21 of 103 CL patients in Peru did not respond to pentavalent antimonial therapy (22.3%) (Hashiguchi *et al.,* 2018). The protracted course of treatment permits the drug's anti-leishmanial levels to build up in visceral organs, particularly the spleen and liver. The lengthy dose schedule of SbV/kg therapy, on the other hand, frequently leads to complications like muscle pain, inflammation of the pancreas, irregular heartbeats, and , resulting to treatment reductions or termination (Valencia *et al.*, 2013). However, these compounds are currently being reintroduced with changes to formulations or dose regimens for use in specific situations, such as those where antimonials are resistant (Noli and Saridomichelakis, 2014; Bekhit *et al.*, 2018). It is difficult to completely treat and eradicate leishmaniasis due to the parasite's enhanced chemoresistance (Ponte-Sucre *et al.*, 2017; Ghorbani and Farhoudi, 2018).

Since therapy is becoming more difficult, new formulation drugs that can replace or supplement currently available therapeutic options are required (Chávez-Fumagalli *et al.*, 2015; de Menezes *et al.*, 2015; Roatt *et al.*, 2020). After the introduction of triglyceride versions of amphotericin B, a far barely toxic medication for mycotic diseases, anti-leishmanial pharmacotherapy has advanced and been used to treat leishmaniasis (Lanza *et al.*, 2019). The high expense of these amphotericin B formulations, however, prevents them from being widely used in developing nations (Guery *et al.*, 2017; Rebouças-Silva *et al.*, 2020).

Miltefosine originally created to treat skin cancer is currently used to treat leishmaniasis (Sunyoto *et al.,* 2018). Miltefosine's potency in the chemotherapy of Leishmania significantly contributed to the development of the novel class of anti-leishmanial drugs. It is now the first oral leishmaniasis treatment in a number of countries (Ostyn *et al.*, 2014; Carnielli *et al.*, 2018). For all cases of leishmaniasis, however, this medicine may not be superior to parenteral therapy (Ponte-Sucre *et al.,* 2017). The necessity for a lengthy treatment time may

accelerate the development of more appropriate use techniques to avert sick people from acquiring tolerance to the therapy.

Other alkylphospholipids, such as edelfosine, ilmofosine, and perifosine, have shown to have substantial antiparasitic activity in vitro (Tadele *et al.*, 2020). In a study using perifosine and edelfosine improved effects were found on *Leishmania amazonensis*-infected Balb/c mice (Cabrera-Serra *et al.*, 2008). In the pre-clinical study, perifosine showed greater activity in the animal model, suggesting that it could be a feasible therapy for cutaneous leishmaniasis. For visceral leishmaniasis, sitamaquine seems to be a viable oral therapy (Jain and Jain, 2013). A period of therapy at an experimental dosage of 2.0 mg per day was successful and highly resisted in 61 Kenyans infected with *L. donovani* (Mbui *et al.*, 2013); however, additional research was recommended to determine the best dose.

The only aminoglycoside with clinically significant anti-leishmanial action is paramomycin (Seifert and Croft, 2006). Although this antibiotic can be used to treat both visceral and cutaneous infections, injectable and dermal medications for internal organs and skin infections, accordingly, have been created due to poor oral absorption. In individuals with visceral leishmaniasis in India, it was found that paromomycin delivered via sub-abdominal route was equally effective as amphotericin B (Sundar and Singh, 2106). Novel paromomycin dermal medication, on the other hand, have demonstrated encouraging results (Sousa-Batista *et al.*, 2019b). In a randomized, controlled study, the treatment potency of two paromomycin skin creams with meglumine antimoniate was compared (Neves *et al.*, 2011). Dermal paromomycin could be a viable treatment for cutaneous leishmaniasis, according to the findings, but clinical recovery takes longer. Meanwhile, paromomycin gel was substantially more successful than the antimony therapy against *L. amazonensis*, while both drugs were similarly efficient against *L. braziliensis* (Aguiar *et al.*, 2009).

2.4 Plants in treatment of leishmaniasis

In the pasts, refined phytoconstituents and synthetic larvicides were commonly used as natural insecticides against a range of protozoan parasites until biological laboratory-synthesized insecticides emerged in the 1940s (Sarwar, 2015). Plant bioactive constituents such as pyrethrin, nicotine, and rotenone were some of the first insecticides to be used to control both medically and agriculturally important insects (Kamaraj *et al.*, 2010). There is continued search for new molecules to cure leishmaniasis, and various screenings have been done with synthetic chemicals and their derivatives. There is an estimated 250,000 medical plant species in the world (Dekebo, 2019). However, the biological activities of only about 6% of them have been screened. Furthermore, only approximately 0.75% medical herbal compounds have been studied in clinical trials (Akbari *et al.*, 2022). Thus novel therapies and utilization of plant-derived products as a source of natural bioactive molecules against leishmaniasis or specific leishmania parasites with increased potency and lesser adverse consequences have sparked increased attention lately (Gouri *et al.*, 2022).

Phytoconstituents, and several other complementary drug therapies, have been increasingly popular in recent years all around the world. Given the current state of affairs, it is critical to investigate new extracts obtained from medicinal plants for leishmaniasis treatment (Jha and Sit, 2021). The number of recent *in vitro* and *in vivo* animal studies regarding the effectiveness of medicinal plants and plant-derived compounds such as the crude extracts against CL and VL caused by different *Leishmania* species has been summarized elsewhere (Mathew and Negi, 2019). Plant contain bioactive possessing anti-leishmanial activities (Ivanović *et al.*, 2020). Nevertheless such studies are rare in Kenya (Tonui, 2006). Natural herbal preparations have already been proven to offer therapeutic potential in plant varieties including: *Warburgia ugandensis* Sprague (Canellanaceae) (Ngure *et al.*, 2009); *Maytenous senegalensis* Lam. (Celastraceae) and *Eucalyptus globules* (Tahir *et al.*, 1998); *Aloe vera*

Burman and Miller (lilliaceae). *Tanacetum parthenium* (Asteraceae), is useful for treating many disorders, including leishmainasis (Moraes Neto *et al.*, 2019). Previous studied have reported anti-leshmanial properties of *Anacetum parthenium* against *Leishmania amazonensis* (Tiuman *et al.*, 2005). A progressive increase in the anti-leishmanial effect was observed in the course of the purification process. The plant powder (PTP) had a 50% inhibitory concentration (IC_{50}) at 490 µg/ml, whereas the dichloromethane fraction (DF) showed an IC_{50} of 3.6 µg/ml against the growth of promastigote forms after 48 h of culturing. Biological profile of *Croton cajucara* essential oil against *L. chagasi* promastigotes and amastigotes have also been reported (Rodrigues *et al.*, 2013). The results indicated that the 7-hydroxycalamenene-rich essential oil from *C. cajucara* were promising source of leishmanicidal compounds.

There are also studies involving anti-leishmanial properties of several plants. Extracts from five plants: *Tibouchina semidecandra, Tinospora cordifolia, Alstonia scholaris, Swertia chirata,* and *Nyctanthes arbortristis* exhibited a 75 % reduction of parasite multiplication (at 1g/kg/day orally) and elevated viable time (Rocha *et al.*, 2005). By inhibiting $^{14}CO_2$ catabolism from a variety of 14C-substrates in vitro, promastigotes were used to evaluate 11 plants used in Nigeria for probable anti-leishmanial action (Ebiloma *et al.*, 2017). Five extracts from *picralima nitida, Cola altiensis, Gongronema latifolia, Dorstenia multiradiata,* and *Desmodium* sp. were among the 13 methanol extracts studied contaning good anti-leishmanial properties. In Columbian research, *Annona muricata* was found to be highly efficacious than meglumine antimoniate (Glucantime) against *L. braziliensis* and *L. panamenesis* (Vila-Nova *et al.*, 2013). The findings highlight the usefulness of the herbs with treatment efficiency against leishmaniases.

2.5 Phytochemical in plant extracts for treatment of leishmaniasis

Plants are certainly a major source of drugs against leishmania (Rocha *et al.,* 2005). Phenylpropanoids, steroids, alkaloids, flavonoids, and terpenoids are some of the chemical groupings that have been attributed to the biological action of plant extracts against Leishmania parasites (Tiuman *et al.,* 2011; Karen, 2022). Evaluation of traditional use, chemical composition, plant toxicity, or a combination of various criteria is useful to obtain a herbal medication or fractions of active ingredients. Various plant components and solvents have been utilized in the extraction operations in the past. Since a number of procedures can be utilized to prepare extracts, there is certainly potential for advancement in the methods of extraction when screening for biological activity (Soosaraei *et al.,* 2017). To purify and isolate the bioactive components of the plant, they are separated, and each portion and/or pure product can be tested for efficacy and safety (Dzah *et al.,* 2020). Several research have indicated that purified plant extracts have more biological activities in against many protozoans (Da Silva *et al.,* 2018; Sridhar *et al.,* 2021). Have there is still less research outputs on efficiacy of purified plant extracts on *Leishmania* species. However, phytopharmaceuticals and phytotherapy may only be fully accepted and integrated into the idea of classical medicine if they have similar quality standards as conventional drugs. Furthermore, knowledge of the principal toxicological and pharmacological assays are required for the optimal processes for phytopharmaceutical standardization.

Antibiotics are becoming less effective, as protozoans are developing resistance to treatment (Barrett *et al.,* 2019). As a result, conventional medicine is becoming more appreciative of antimicrobials and other plant-derived treatments. Therefore more attention is continuously shifting towards plants. Plant derivatives are among the most effective anti-protozoan therepeutics (Antwi *et al.,* 2017). Nerolidol appears to block the production of ergosterol and dolicol, both of which are required for parasite membrane formation. Nerolidol suppressed

the parasites' synthesis of ergosterol and dolicol in culture and in animal model versus *L. chagasi*, *L. amazonensis,* and *L. braziliensis* amastigote and promastigote forms (Soosaraei *et al.*, 2017). When injected intraperitoneally into infected mice, the chemical was also effective. Topical treatments, on the other hand, did not produce sufficient results, and other lesion recurrences occurred after the treatment ended, indicating the necessity for a longer treatment duration.

Other medications work in a similar way to nerolidol, although they target the last stages of ergsterol production. Because some leishmania species employ host sterols, these are ineffective (Zhang, 2021). *Moringa oleifera* Lam. extracts and phytoconstituents were studied for their anti-leishmanial action. The ethyl acetate portion was reported to be the highest in terms of efficacy. Among the isolated compounds from the ethyl acetate fraction, niazinin, a thiocarbamate glycoside, was shown to be extremely potent (IC_{50} 5.25 M) (Kaur and Rajput, 2014).

Quinones have been shown to have anti-leishmanial properties. Cordiachromes (quinone derivative product) derived from *Cordia fragrantissima* show anti-leishmanial potential (Maroyi, 2017). Cordiachrom A and cordiachrom B were found in all of the examined samples. As fresh shoots were administered to the ulcer, nephthoquinones like plumbagin are commonly used in Bolivian traditionally to treat CL caused by *L. braziliensis* (Da Silva *et al.*, 2018). Lichochalcone, from Lichorice tree, was already proven to hinder *L. major* and *L. donovani* from growing (Dinesh *et al.*, 2017). Anti-leishmanial activity was also demonstrated in iridoid glycosides, lignin, diospyrin, saponin, sequiterpenes, anthocynadins, alkylamines, and aromatic polysulphur compounds (Singh *et al.*, 2022).

Until recently, thousands of phytochemicals have been discovered throughout the world that have inhibitory effects in animal model on a variety of protozoans (Koley *et al.*, 2019). Ethnobotanical-based animal model screening programs are critical for confirming traditional herbal medicines and offering guides in the quest for latest bioactive compounds. While in vitro activity does not always imply that a plant extract is a useful treatment or a promising option for new therapeutics, it could indicate that it has potential, and it does give one a good idea of how effective it is and, in some situations, how poisonous it is (Bereksi *et al.*, 2018). More of these chemicals, on the other hand, must be submitted to medical research to find out their potency in the organism settings.

Numerous plants and associated volatile compounds have therapeutic potentials that can help eliminate or alleviate disease symptoms, and scientific evidence is increasing that several botanicals and associated volatile oils can help alleviate or relieve disease symptoms (Ullah *et al.*, 2016). Many essential oils are recognized to have antibacterial properties. In reality, the essential oils biological action should take into account the stimulatory activity of their diverse main and secondary components. As a result, infusions, tinctures, and syrups of basic plant extracts have often been utilized to heal various ailments associsated with leishmania infection (Gutiérrez-Rebolledo *et al.*, 2017; van Griensven and Diro, 2019).

Flavonoids and phenolic compounds both have high anti-protozoan action (Vemula *et al.*, 2022). The complicated active ingredients of herbal isolates are a major stumbling block to active component extraction. However, using a bioautography agar overlay bioassay to detect antimicrobial components in a crude plant extract is possible (Podolsky and Lie, 2016). Any prospective large-scale utilization of plants discovered to have significant activity, on the other hand, must consider not only biological attributes such as recommended standards of safety, but their mode of acton on target organisms (Gurnani *et al.*, 2016). Despite the fact

that oregano essential oil and its constituent carvacrol raises the risk of cell apoptosis by a little amount, even at low doses, they showed significant bactericidal effect (Tomiotto-Pellissier *et al.*, 2022).

Alkaloids and steroidal sapogenins have been the main compounds of interest in these investigations (Yang *et al.*, 2018; El Aziz *et al.*, 2019), but unsaturated sterols, triterpenoids (Ahmed *et al.*, 2018), and other chemicals have also been reported. Alkaloids, terpenes, flavonoids, benzopyrans, phenolics, and sesquiterpene lactones are the frequently extracted phytochemicals found in plant species that have confirmed potency against *Leishmania* (Kanhar and Sahoo, 2021; Pengelly, 2021).

The search for new anti-leishmanial chemicals is still in progress. At doses ranging from 2.5 to 10.0 g/mL, dehydrozaluzanin C, a sesquiterpene lactone a derivative of benzene of *Munnozia maronii* leaf extract, prevented the proliferation of eleven leishmania flagellate forms (Felix-Cuencas *et al.*, 2022). In addition to certain recognized chemicals, two novel sesquiterpenes, fnarthexone and fnarthexol, derivatives have also been confirmed to have anti-elishmanial activity (Bashir *et al.*, 2014). Beside there are novel extracts such as conferol that has been determined to contan highly active chemical capable of lomiting growth of leshmanai parasites (Gonçalves *et al.*, 2020). Some plant extracts contain quercitrin (quercetin 3-O—L-rhamnopyranoside), which also show potential anti-leishmanial potency and low toxicity (Muzitano *et al.*, 2006; Milad *et al.*, 2014). In some cases some plants have been shown to have combination of luteolin, quercetin, fisetin, and 3-hydroxyflavone, which subsequently affected the activity of the extracts against leishmania (Fonseca-Silva *et al.*, 2013).

Pharmacological investigations on the properties of heterocyclic derivatives against the promastigotes of *Leishmania chagasi* have also been found to be effective. Among these is 1,3,5-triazines and 2,4,6-trisubstituted pyrimidines were also examined for action against leishmania *L. donovani* (Tiuman *et al.*, 2011; Singh *et al.*, 2021). Also production of 2-pyridyl pyrimidines had significant anti-leishmanial activity in vitro (Musonda *et al.*, 2009). Hetorcyclic compounds derived from nitroimidazolyl-1,3,4-thiadiazole also showed significant anti-leishmanial activity in vitro (Matysiak, 2015). At less cell-toxic concentrations, the majority of the components demonstrated anti-leishmanial action towards the *Leishmania major* promastigotes. The extracts were active on internal *L. major* and lowered pathogenic potential greatly. There are also azoles derived from plant extracts that have shown to posses efficacious anti-leishmanial action (Beach *et al.*, 1988; Rangel *et al.*, 1996). There is less information available concerning these derivates for many plants especially in Africa. These investigations are the basis of a pressing need to produce new medications that are both affordable and effective, as well as to find the potent compounds with significant anti-leishmanial action and better treatment properties.

2.6 Parasite growth inhibition and infection

Restricting growth of parasites through growth inhibition are critical for innovative drug development for control the proliferation of parasitic agents. As a result, there are several investingations on the potential of therapeutic options for the growth inhibition experiments against *Leishmania* parasites (Kotb Elmahallawy and Agil, 2015). Several culture growth proliferation suppression techniques are documented, though differ slightly in terms of organism selection, culture and macrophage host cell, invading phase, degree of infections, test length, and ultimate final characteristics (Tadele *et al.*, 2020).

In a study investing the growth inhibition of *Peschiera australis* extract against *L. amazonensis* flagellate as well as amastigote forms in culture medium, it was clearly evident that the phytoconstituents completely inhibited promastigote proliferation (Delorenzi *et al.*, 2001; Luize *et al.*, 2005). In the same experiment the chloroform fraction of the *P. australis* extract showed great growth inhibition potential against amastigotes in vitro and macrophages, decreasing infection by up to 99% in single or multiple treatments. The anti-leishmanial profile of the extract is attributed to the indolic alkaloid, which has been demonstrated to be potent against amastigotes and predominantly promastigotes (Omar *et al.*, 2021). However, since these conventional medications exhibit side effects, alternative treatment agents must be sought. Whereas the amastigote-macrophage design is regarded as the best for determining the drug sensitivity profile of an anti-leishmanial compound (Hendrickx *et al.*, 2019), there is need for further studies especially in Africa.

Paraste growth inhibition has also beeen studied relative to essential oils extracted from the plant biocides. The essential oil of *Ocimum gratissimum* has abundant eugenol, gradually inhibited growth of *L. amazonensis* (Pansera *et al.*, 2021). The MIC_{50} of the essential oil was 135 g/ml and 100 g/ml for promastigotes and amastigotes, respectively, and eugenol's MIC_{50} was 80 g/ml for promastigote stages. Regardless of the parasites' developmental form, both essential oil and eugenol (at 100 g/ml) killed all *L. amazonensis* parasites after 60 minutes of treatment (promastigote or amastigote). Although the role of essential oils is well understood in controlling parasites causing several diseases (Cortes *et al.*, 2020), there is is still paucity of nformation on how essential oil derived from various plants affect leishmania growth in vivo and vitro.

Growth inhibition action of plant extracts have been shown to occur due to oxidants generated by macrophages after stimulation by interferon and tumor necrosis factor

producing nitric oxide (Trinh *et al.*, 2020). For both promastigotes and amastigotes, the *O. gratissimum*'s minimum inhibitory concentration of essential oils was 150 g/ml (Afolabi *et al.*, 2007). After treatment of mice abdominal macrophages using 100 and 150 g/ml active ingredients, the association indicators amongst promastigotes and macrophages were reduced, accompanied by production of more nitric oxide by parasitized macrophages. The findings imply that the essential oil of *O. gratissimun* and its constituents could be exploited to develop new anti-leishmanial agents. Despite the fact that nitric oxide allows *Leishmania* killing by macrophages being well understood, no knowledge is available about the influence of crude extracts on the observed macrophage response.

Leishmanicidal growth inhibition was also shown for essential oil abundant with linalool extracted from *Croton cajucara* on promastigotes of *L. amazonensis* injected with 15ng/ml of essential oil in the culture medium (Rodrigues *et al.*, 2013). The kinetoplast and nuclear chromosomes of leishmania were destroyed within one hour, followed by cell disruption. The interaction between mouse abdominal monocytes and *L. amazonensis* was inhibited by 50% after pretreatment with volatile oil amounting to 15 µg/ml, but NO production by infected macrophages increased by 220%. The interaction between pre-infected macrophages and parasites was reduced by 50% after treatment with 15 ng/ml essential oil, as a result, the quantity of NO generated by pre-parasitized macrophages increased by 60%. Simiilar studies showing that essential oil abundant in linalool being a powerful anti-leishmanial phytochemical that reduced the proliferation of several species of *Leshmania* promastiotes has also been reported elsewhere (Da Silva *et al.*, 2018; Macêdo *et al.*, 2020; Maaroufi *et al.*, 2021).

Among the plants species that have been investigated to have growth inhibition on Leishmania is the species from the Lamiaceae family (Kheiri Manjili *et al.,* 2012; Eddaikra *et al.*, 2019). Several extracts from Laminaceae family have been on growth inhibition of amastigote and promastigote of *Leishmania tropica* and *Leishmania infantum* (Zeouk *et al.*, 2020; Kosari and Khamesipour, 2022). Among the laminaceae plants, *Tridax procumbens* extracts revealed high action against promastigotes of *Leishmania mexicana* in vitro, with little harm to cell cultures (Ahmed *et al.*, 2019; Ingole *et al.*, 2021). There are also reports of potential anti-leishmanial growth inhibition among Apocynaceae including *Urechites andrieuxii* and *Salvia verbenaca* in vitro (Soni *et al.*, 2019).

There are some plants whose parts are consumed by humans but they have provided evidence of possessing anti-leishmanial properties. A polyphenolic preparation from the husk fiber of *Cocos nucifera* Linn. (Palmae) prevent growth of *Leishmania* at hgher concentration (Felix-Cuencas *et al.*, 2022). Microscopic analysis of promastigote cells after injection of 10 or 20 g/ml of *C. nucifera* polyphenolic-rich preparation for 60 minutes corroborated this. The similar profile and morphological alterations were observed in the amastigote forms. Pretreatment of peritoneal mouse macrophages with 10 g/ml of *C. nucifera* polyphenolic-rich isolate reduced the association index between these macrophages and *L. amazonensis* promastigotes by 44%. At 10 g/ml, this *C. nucifera* preparation is a highly efficient leishmanicidal agent in experimental animal models, without animal model allergenic or in vitro cytotoxic effects.

To evaluate the anti-leishmanial or leishmanicidal activity of a compound, several criteria are considered including MIC and IC_{50} (Mahmoudvand *et al.*, 2014). MIC refers to the minimum inhibitory concentration of a compound or drug against the parasite. IC_{50} is the concentration of drug that causes 50% growth inhibition of amastigote or promastigote forms

of *Leishmania* (Monzote *et al.*, 2014). IC_{50} is used for macrophages of the host, too. CC_{50} is the cytotoxicity concentration of drug that results in 50% of mortality of macrophage from usually BALB/c mice (Islamuddin *et al.*, 2012).

From the foregoing it is clear that, lack of an efficient drugs and resistance to drugs administered for the treatment of leishmaniasis, coupled with their high cost, parenteral route of administration and toxicity, have been regarded as a great concern especially in endemic areas of developing countries. Hence, there could be no doubt that the search for novel botanical agents having anti-leishmanial or leishmanicidal potency is one of the critical challenges in the field of the current drug discovery program. Much information about their potential growth inhibition of the leishmania parasites are needed to evaluate their efficacy. Such information is lacking for so many potentially promising plants and their exctracts.

2.7 Efficacy of herbal leishmaniasis treatments

The effectiveness of herbal treatment is based on their ability to heal lesions in infected murine models (Ghodsian *et al.*, 2020; Parvizi *et al.*, 2020). As a result, various studies on the potential of plant extracts in stimulating wound healing in animals and humans have both been done (Chouhan *et al.*, 2014; Bahmani *et al.*, 2015; Tajbakhsh *et al.*, 2021). Some phytoconstituents have consistently been found to be beneficial against *Leishmania major* in in vivo tests (Bahmani *et al.*, 2015; Andrade *et al.*, 2016). Wound healing was used to demonstrate the anti-leishmanial properties of *Pistacia khinjuk* ethanolic isolate on *Leishmania tropica* and *Leishmania major* in vivo (Ezatpour *et al.*, 2015). Furthermore, gum derived from *P. atlantica* was shown to reduce dermal leishmaniasis in a *L. major* parasitized mice in a similar investigation (Taran *et al.*, 2010). In another investigation, *Artemisia annua's* potential usefulness against *Leishmania* in vivo was reported (Mesa *et al.*, 2017). The methanolic extract of *Maesa balansae* leaves demonstrated good wound healing

and in vivo action against *Leishmania infantum*, reducing liver amastigote burden by 90% (Taran *et al.*, 2010). Triterpenoid, saponin isolated from *Maesa balansae* shows remarkable anti-leishmanial efficacy in vivo against visceral *Leishmania* parasite in a mouse model (Heidari-Kharaji *et al.*, 2016). Another study found that phyto-ointments were efficient in diminishing lesion size in *Leishmania major*-infected inbred mice and could enhance NO release, which is required to inhibit parasite propagation (Asthana *et al.*, 2013).

Achillea millefolium, Thymus vulgaris, and *propolis* extracts, on the other hand, were beneficial in treating cutaneous leishmaniasis infected mice, and the compounds were also potent in reducing lesion size (Iqbal *et al.*, 2016; Tajbakhsh *et al.*, 2021). It has been found that the alcoholic extracts of *Berberis vulgaris* improved skin leshmaniasis infection in mice and reduced the lesion sizes (de Melo *et al.*, 2013). *Artemisia essence* was found to be beneficial on *Leishmania major* promastigote infected mice, and reduced the size of ulcers (Heidari-Kharaji *et al.*, 2016). *Tephrosia vogelii* extracts have been shown to exhibit anti-leishmanial efficacy against *L. major* parasitized mice, by a significant reduction in wound size and parasite density reduction (Marango *et al.*, 2017). Allicin has also been shown to have anti-leishmanial properties in *Leishmania major* experimentally infected mice (Khademvatan *et al.*, 2011; Corral-Caridad *et al.*, 2012).

Several in vivo tests have also revealed that crude extracts of plant species exhibit significant anti-leishmanial action in *L. major* at concentrations of less than 0.5 mg/ml (Oskuee *et al.*, 2012; Khan *et al.*, 2015; Mahmoudvand *et al.*, 2016; Keshav *et al.*, 2021). The IC_{50} values of *Maytenus senegalensis, Azadirachta indica,* and *Eucalyptus globulus,* respectively, were 55, 11.5, and 78 mg/ml (Liaqat and Asgar, 2021). Indian plants, were tested for activity against leishmaniasis in *L. donavani* infected hamsters (Singh *et al.*, 2022). On day 7 and/or 28 after treatment, phytochemicals from five plants (*Tibouchina semidecandra, Tinospora cordifolia,*

Alstonia scholaris, Swertia chirata, and *Nyctanthes arbortristis*) revealed inhibition of greater than 75% (at 1 g/kg/day 5 orally) of parasite growth, with a longer survival time (Nigatu *et al.*, 2021; Padilha Ferreira *et al.*, 2021; Pospíšil *et al.*, 2021).

Although there is reach plethora of studies on the wound healing effect of plant extracts on murine models, there is still much research needed on the potential efficiacy, dosage, duration of treatment that result in complete healing of the animals from leishmaniasis. Such information is still insufficient to make conclusion concerning which plant extracts would qualify as alternatives to the first and second line drugs being used to day for leishmania thereapy.

2.8 Toxicity of herbal treatments of leishmaniasis

There has been increasing interest in the plast decade over the use of of natural compounds for treatment of leishmaniasis, often from plants as a source of medicine, as adjuvants and even as replacement of chemical treatment (Sundar and Singh, 2106). While prescription medicines have well-defined chemical composition and are supported by evidence-based published studies, in terms of their efficacy and toxicity, this has not been the case with botanical medicines (Kundishora *et al.*, 2020). Indeed, the popular misconception that all ‘natural or plant’ products are safe has tended to discourage investigation into their potential toxicity (Heidari-Kharaji *et al.*, 2016). Chemical compounds from plants such as flavonoids, terpenes, alkaloids, anthraquinones, saponins, tannins, steroids, lactones, and volatile oils have received considerable attention in recent years due to their varied pharmacological properties, including cytotoxic and chemopreventive effects (Ismail *et al.*, 2017). Differences in Leishmania species' medication responsiveness, the patient's immunological status, and other factors all play a role or the drug's pharmacokinetic qualities can all affect the safety of pharmaceuticals used to treat leishmaniasis (Sharwan *et al.*, 2015; Mensah *et al.*, 2019).

Some effects associated with use of medicinal plants include allergic reactions, irritation of the gastrointestinal tract, destruction of red blood cells, and injury to vital body organs such as the heart, liver, kidney, and carcinogenicity (Ekor, 2014). It has been reported that 35% of all cases of acute renal failure in Africa have been associated with the use of herbal remedies (Naicker *et al.*, 2008). Moreover, this figure is thought to be an underestimate because of the secrecy surrounding traditional health practices and the use of traditional remedies (Luyckx *et al.*, 2005; Luyckx and Naicker, 2008). These cases point out that medicinal plants may be rich source of beneficial chemical molecules of diverse structures with different pharmacological properties on biological systems, but some plants may be toxic to humans.

Plant extracts exert their cytotoxic effects through common mechanisms including cell cycle arrest and cell death by apoptosis (Kwan *et al.*, 2016). Most important causes of medicinal herb toxicity are the presence of toxic phytochemicals in the medicinal plant (Kharchoufa *et al.*, 2021; Oloya *et al.*, 2022), improper identification or verification of herbals (Gouws and Hamman, 2020), and inappropriate or mislabelling of the plant material (Zhou, 2019). Other causes are contamination of herbals with microorganisms, fungal toxins such as aflatoxins, and interactions with standard drugs upon simultaneous ingestion (Kahraman *et al.*, 2020). Majority of toxicological studies indicate that the toxic effects in herbal medicines are associated with hepatotoxicity (Amadi and Orisakwe, 2018; Parvez and Rishi, 2019). Other harmful effects on the nervous system, kidneys, blood and cardiovascular system, as well as mutagenicity and carcinogenicity, have been reported (Guldiken *et al.*, 2018; Zhao *et al.*, 2020).

Studies to assess the potentially harmful effects of herbal medicines and other chemical compounds can be carried out both in vitro and in vivo (Saeidnia *et al.*, 2015). In vitro tests are just the initial stage in demonstrating the efficacy of phytochemicals to be used in the therapy of leishmaniasis.

It is important to establish their safety. It is necessary to be aware of the toxicity of these plants in order to advocate for phytochemical use in leishmaniasis therapy. Cytotoxicity tests are useful to screen chemicals for their intrinsic and relative toxicities, which allows for the determination of potential toxic or harmful effect of such compounds to human health that may occur inadvertently during use (Schulz *et al.*, 2021). Many herbs contain active compounds (Oryan, 2015). Only a few of them have been evaluated for efficacy and safety, even when various reports have implicated many of them in toxicity. Therefore the identities of toxic substances contained in herbal medicines, their toxicology and pathogenesis are largely unknown (Anywar *et al.*, 2021).

Efforts are still being made to find an effective treatment for CL that has minimal adverse effects on the cell (Coria-Téllez *et al.*, 2018). Although plant extracts from *Maytenus putterlickoides* (Celastracea), *Acasis melliflera* (leguminoseae), *Aloe kedogensis* (Liliaceae), *Albizia coriaria* (Febaceae), and *Warbugia ugandenisis* have antleishmanial properties with low toxicity, according to in vitro experiments in Kenya utilizing crude extracts (Tonui, 2006), there is still large gap in knowledge concerning the toxicity of thousands of plants that may be considered as herbal drugs.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The research was conducted at the Centre for Biotechnology Research and Development (CBRD), at Nairobi's Kenya Medical Research Institute (KEMRI). When handling experimental materials and samples in the center, biosafety level 2 (BSL-2) facilities and techniques were used.

3.2 Plant Collection

Mature *Terminalia mollis, Croton macrostachyus, Olea europaea, Kigelia africana,* and *Bridella micrantha* were collected from Marigat in Baringo County, Kenya's and kept in refrigerated boxes to protect the sample's integrity. For authenticity, the voucher specimen was sent to the National Museums of Kenya's herbarium for identification by a trained plant taxonomist. The plant species were then delivered to the CTMDR, in KEMRI Nairobi, for sample preparation.

Plant Collection sites

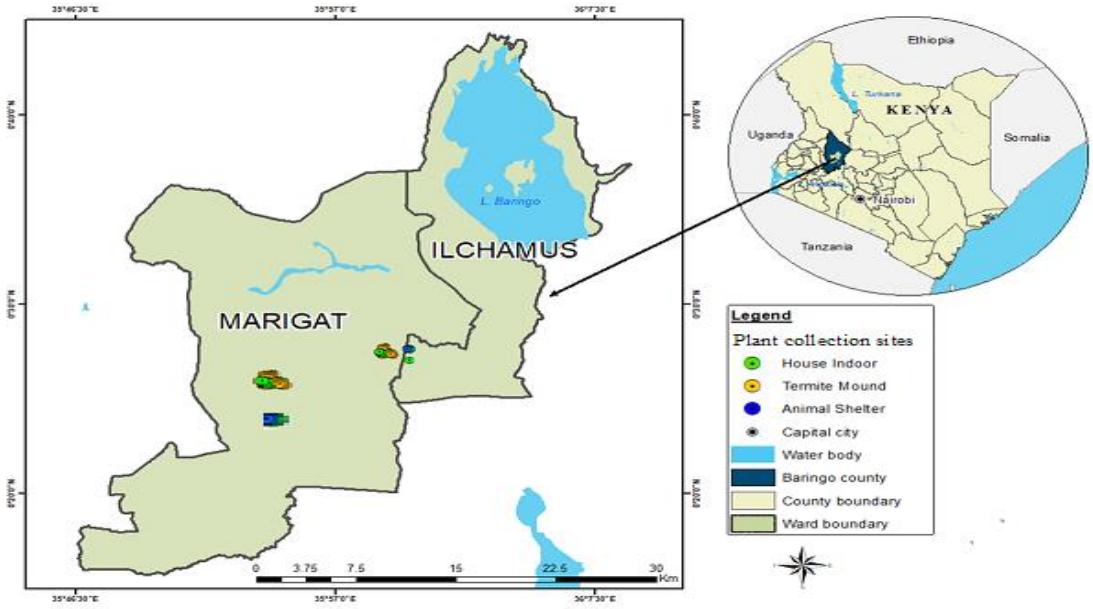


Figure 3.1: The map showing plants collection sites in Marigat, Baringo County

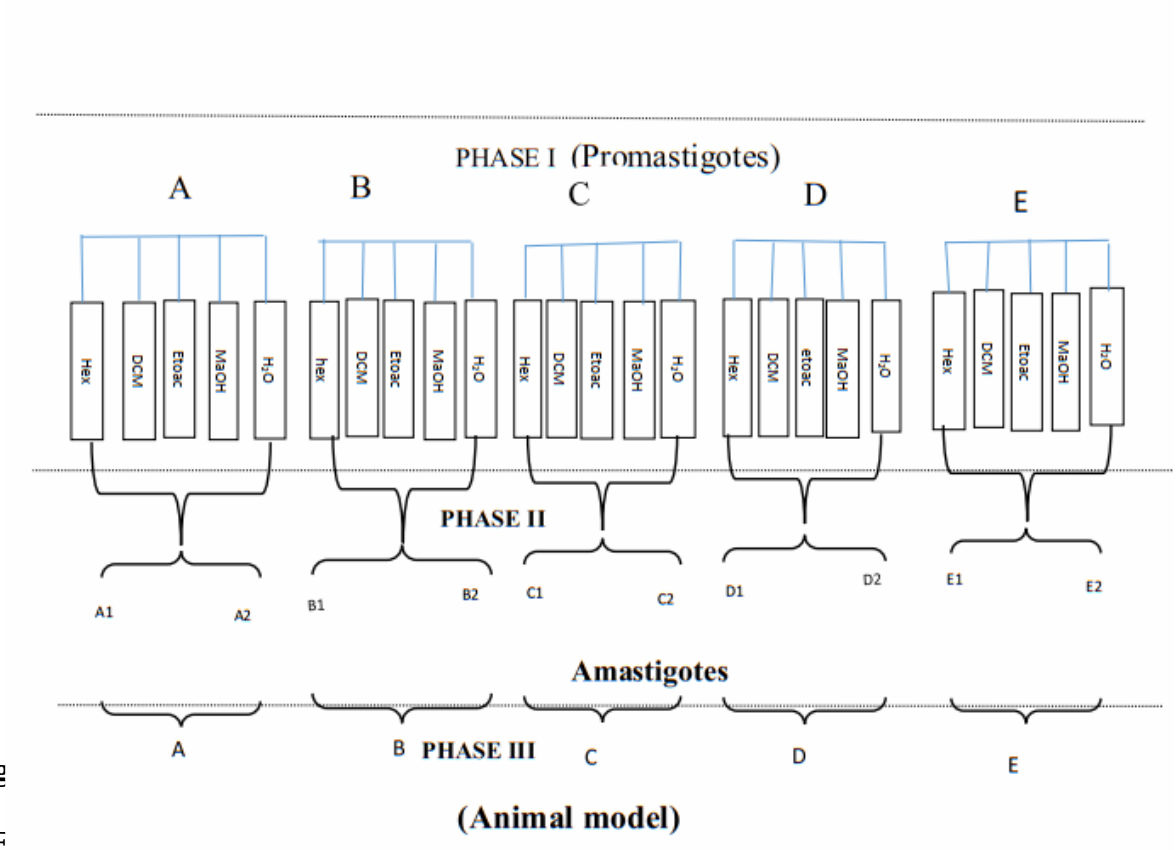
3.3. Extract Preparation

Briefly, the barks were cut into small pieces and air-dried for 14 days under a shed. The dried specimens were ground using an electrical mill in readiness for extraction. Cold sequential extraction was carried out on each plant materials (*Olea europaea, Kigelia africana, Terminalia mollis, Croton macrostachyus,* and *Bridella micrantha)* with distilled organic solvents of increasing polarity (petroleum ether > dichloromethane > ethyl acetate > aqueous > methanol). One hundred grams (100 g) of the fine powders of every plant sample were macerated in 1000 ml methanol at room temperature. The supernant were concentrated by rotary evaporation at 30 - 35°C in a vacuum (Azmir *et al.*, 2013; Sahne *et al.*, 2017). The extracts were filtered using whatman Filter paper No.1 and concentrated using a rotary evaporator (BUCHI R 200, Labortechnik Switzerland) at (40ºC). The filtrate were freeze-dried, weighed and stored at -20ºC until required for use. A stock solution was prepared by measuring the required mass of the plant extracts and dissolving in water. The concentration of the stock solution prepared were 25, 50, 100, 250, 500, and 1000 mg/l by dissolving respective grams of the plant extracts in 1000 ml distilled water.

The *in vitro* and *in vivo* testing was done at the CBRD's leishmania laboratory as per the study framework (Figure 3.2).

In phase I, the hexane, dichloromethane, ethy acetate, methanolic, and aqueous extracts of each of the five plants were subjected to the promastigote assay. This was done using MTT assay, where two drugs with best IC_{50} activity against promastigotes were chosen to enter phase II. The methanolic and aqueous extracts from each plant showed the best promastigote activity. During phase II, the two drugs per plant (methanolic and aqueous extracts) from phase I were subjected to macrophage assay, where one drug with the best MI (multiplication index) was chosen for enter phase III. Lastly, in phase III the methanolic extract, which

exhibited the best multiplication index in the macrophage assay in each of the five plants was selected to be used in the animal model for the in vivo studies.



Fig

KE

Hex- Hexane extract

DCM – Dichloromethane extract

Etoac – Ethy acetate extract

MeOH- Methanolic extract

H_2O – Aqueous extract

A- *Olea europaea,*

B- *Kigelia africana*

C- *Terminalia mollis*

D- *Croton macrostachyus*

E- *Bridella micrantha*

Phase I and II were done *in vitro* while phase III were done *in vivo.* The best extract were progressively chosen to the next phase.

3.4 Phytochemical analysis of plant extracts

Using previously described phytoconstituent procedures, five extracts (0.05 g/ml) were subjected to testing for bioactive compound identification (Angelova *et al.*, 2008; Yadav and Agarwala, 2011). Adding a chemical agent to a test tube preparation is required to detect the presence or absence of phytochemical substances. The mixture was vortexed then, the compounds presence or absence was determined. The samples were analyzed three times and if they consistently showed certain concentration ranges they were flagged as high, moderate and trace, as follows:

3.3.1 Steroids

About 2g of the solvent extract was put in a test tube and 10ml of chloroform added and filtered. 2ml of the filtrate was mixed with 2ml of a mixture of acetic acid and concentrated sulphuric acid. Blue green ring indicated the presence of steroids.

3.3.2 Terpenoids:

5ml of aqueous extract of each plant sample is mixed with 2ml of chloroform ($CHCl_3$) in a test tube. 3ml of concentrated sulphuric acid (H_2SO4) is carefully added to the mixture to form a layer. An interface with a reddish brown colouration is formed if terpenoids constituent is present.

3.3.4 Anthraquinones

About 5gm sample of the extract was put in a test tube and 10ml of benzene added. The mixture was shaken and filtered. 5ml of ammonia solution was added to the filtrate and the mixture shaken. Presence of violet color in the ammonical phase (lower phase) indicated the presence of anthraquinones.

3.3.5 Tannins

0.5g of powdered sample of each plant is boiled in 20ml of distilled water in a test tube and filtered. 0.1% ferric chloride ($FeCl_3$) is added to the filtered samples and observed for brownish green or a blue black colouration which shows the presence of tannins.

3.3.6 Saponins

The crude solvent extract was mixed with 5ml of water and vigorously shaken. The formation of stable foam indicated the presence of saponins.

3.3.7 Flavonoids

A few drops of 1% ammonia (NH_3) solution were added to the aqueous extract of plant sample in a test tube. A yellow coloration was observed if flavonoid compounds are present.

3.3.8 Glycosides

Salkowsks' test was used to investigate the presense of glycosides in the exracts: The extract of the plant material was mixed with 2ml of chloroform and 2ml of concentrated sulphuric acid which were carefully added and shaken gently, then the observations were made. A red brown colour indicated the presence of steroidal ring (glycone portion of glycoside).

3.3.9 Alkaloids

100mg of powdered sample was dissolved in 5 ml of methanol and then filtered. Then 2ml of filtrate was mixed with 5ml of 1% aqueous HCl. One milliliter of mixture was taken separately in two test tubes. Few drops of Dragendorff's reagent were added in one tube and occurrence of orange-red precipitate was taken as positive. To the second tube Mayer's reagent was added and appearance of buff-colored precipitate was taken as positive test for the presence of alkaloids.

3.3.10 Phenolic compounds

The extract (500mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

3.3.11 Phlobatannins

Sample was boiled with 1% aqueous hydrochloric acid to produce red precipitate indicating the presence of phlobatannins

3.3.12 Cardiac Glycosides (Keller-Killani Test).

In a test tube, 5 ml of plant extract, 2 ml of glacial acetic acid, and few drops of ferric chloride solution were added. 2 ml of concentrated H_2SO4 was added along the side of test tube. Formation of a brown ring at the interface indicated the presence of glycosides.

Total flavonoid compounds (TFC) were determined by a colorimetric assay (Chen and Chen, 2011). 250 µL of sample were added to a volumetric flask containing 1 mL of distilled water. At 0 min, 75 µL of sodium nitrite ($NaNO_2$, 5 %) were added to the flask. After 5 min, 75 µL of aluminum chloride ($AlCl_3$, 10 %) were added. After 6 min, 500 µL of sodium hydroxide (NaOH, 1 N) were added to the mixture. At this time the mixture was diluted with 2.5 mL of distilled water, then the vortexed samples were kept at room temperature. The absorbance was directly measured at 510 nm. For use as calibration curve, diluted solutions of catechin were used and total flavonoids were expressed as mg of catechin equivalent per 100 g of dry matter (mg CE/100 g DM). As for TPCs, absorbance measurements were performed with UV–visible spectrophotometer Cary 50 Scan (Agilent, Santa Clara, California, USA).

3.5 The DPPH free radical scavenging assay and Ferric Reducing Antioxidant Potential (FRAP) Assay

The capacity of the selected plant isolates to search for DPPH free radicals was determined by decrease in color response between DPPH solution and test preparations. In a nutshell, 2 mL of 0.12 mM DPPH solution in methanol were combined with 1 mL of various dosages of each test sample (50 - 1000 g/mL). A spectrophotometer was used to measure the absorbance of the reaction mixture at 517 nm for 30 minutes at room temperature. Positive controls included Vitamin C (2–20 g/mL).

I % = (A Control-A Sample)/A Control) × 100 was used to quantify the scavenger activity. Where A Sample is the absorbance of the test extract or standard after 30 minutes, and A Control is the absorbance of the blank sample after 0 minutes. The tests were performed three times. Using Probit techniques, the IC_{50} values were calculated using the inhibitory percentage versus plotted concentration. The information was expressed in the form of mean values ± standard deviations (n = 3).

Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700 nm. In this study, Ferric reducing power of plant extracts were determined using FRAP assay. This method is based on the reduction of colourless ferric complex (Fe^{3+} tripyridyltriazine) to blue-colored ferrous complex (Fe^{2+} tripyridyltriazine) by the action of electron donating antioxidants at low pH. The reduction was monitored by measuring the change of absorbance at 593 nm. The working FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer, pH 3.6, with 1 volume of 10 mM TPTZ (2,4,6-tri(2-pyridyl)-*s*-triazine) in 40 mM HCl and with 1 volume of 20 mM ferric chloride. All solutions

were freshly prepared before their uses. 100 μL of samples were added to 3 mL of prepared FRAP reagent. The reaction mixture was incubated in a water bath for 30 min at 37°C. Then, the absorbance of the samples was measured at 593 nm. The difference between absorbance of sample and the absorbance of blank was calculated and used to calculate the FRAP value. FRAP value was expressed in terms of mmol Fe^{2+}/g of sample using ferric chloride standard curve prepared from the stock solution All measurements were calculated from the value obtained from triplicate assays.

3.6 Leishmania parasite culture

A female *P. dubosci* was taken from Marigat, Baringo County, Kenya, and was used to generate *Leishmania major* (strain IDUB/KE/83=NLB-144) following standard methods (Hide *et al.,* 2007). The parasites were grown in Schneider's Insect Culture, which contained 20% high – temperature activated foetal cow sera, 100 g/ml penicillin, 50 g/ml streptomycin, and 250 g/ml 5 fluorocytosine arabinioside. Fresh culture was prepared for each experiment and the culture kept for 7 days.

3.7 Experimental animals (mice) and sample size

A total of 6 female inbred BALB/c mice from the KEMRI animal house were used per experiment. All *in vivo* tests were performed eight weeks old mice that weighed 20 ± 2.1 g. Before beginning investigations, the mice were transferred into the experimental room for a week of acclimation. The mice were kept in translucent plastic cages that were 15 cm by 21 cm by 29 cm and were filled with wood shavings. They were fed pellets and water *adlibitum*. Every two days, the wood shaving dressings in the cages were changed. The animal house's experimental room was locked and every cage was assigned experimental details such as: assigned treatment group numbers, procedure dates, and protocol SSC number. The tests were performed as per the KEMRI's Animal Care and Use Committee (AUC)

recommendations. The CBRD Leishmania laboratory has standard operating procedure for vaccinating the mice with recommended 21G needles, anaesthetizing them, and killing as well as disposing them after killing them in a humane way allowed by ACUC (100l of Sagata).

Sample size determination was by the Mead's resource equation (Kirkwood & James, 2010).

n = N – B – T

Where:

- N is the total number of individuals or units in the study (minus 1)
- B is the blocking component, representing environmental effects allowed for in the design (minus 1)
- T is the treatment component, corresponding to the number of treatment groups (including control groups)
- P is the sample size

In this study, 49 mice were to be used with 7 treatment groups. Therefore actual animals used in the study = 48-0-6=42

3.8 Promastigote and antimastigote action

Cellular density of the promastigotes was assesed by microscopy before the anti-leishmanial activity was tested. The promastigotes of *L. major* were rinsed two times in phosphate-buffered saline (PBS) and spun for 10 minutes at 2500 rpm. Then, 100 mL of parasite culture were resuspended in fresh schneider's Insect Medium supplemented with 20% heat inactivated foetal bovine serum, 100 µg/ml penicillin and 50 µg/ml streptomycin and 250 µg/ml 5-fluorocytosine arabinioside in a 96-well tissue culture plate to investigate antipromastigote activity (Et-Touys *et al.*, 2016). Different doses (25, 50, 100, 250, 500, and 1000 mg/l) of extract (mg/mL) were mixed in 1 % DMSO, parasites were grown at 23°C for 72 hours with 2.5 106 cells per well. Negative controls included sterile PBS and 1% DMSO

at concentrations greater of 1%, which are not poisonous to parasites (Essid *et al*., 2015; Oliveira *et al.,* 2011). The positive control was amphotericin B.

The anti-amasigote test was carried out according to the directions provided by Delorenzi *et al*. (2001). Pentobarbital sodium 80 mg/kg was used to anesthetize mice. After that, 70% ethanol was used to sterilize the mice body surface. To expose the peritoneum, the abdominal skin was ripped from the top downwards. The peritoneum was injected with 10 ml of pure chilled phosphate-buffered saline using a sterile syringe and needle. The abdominal macrophages were collected after agitating the mice and removing phosphate-buffered saline. The tube's contents were transferred to a clean microcentrifuge tube. After centrifugal washing at 2000 rpm for 10 minutes, each pellet was resuspended in complete RPMI 1640 media. Tissue monocytes were coated in 24-well plates and left to attach for 4 hours in 5 % carbon (IV) oxide at 37°C. Before being cultivated in RPMI overnight, non-attached cells were rinsed in chilly PBS. After that, sticky macrophages were inoculated at a 6:1 parasite/macrophage ratio and cultured at 37°C in 5% Carbon (IV) oxide for 4 hours. Before being grown in RPMI for 24 hours, the cultures were thoroughly washed with PBS to remove any free macrophages. The samples were used only once to treat infected macrophages. To compare parasite inhibition, amphotericin B was utilized as a positive control medication. Each three days, the culture and test drugs were refilled everyday. The single layers were rinsed with phosphate-buffered saline at 37°C after 5 days, preserved in methanol, and Giemsa- stained. The quantity of amastigotes was assessed by counting about 100 macrophages in triplicate media. The data was expressed as infection rate (IR) and multiplication index (MI) (Berman and Lee, 1984).

The number of infected macrophages per 100 macrophages is denoted IR.

$$MI = \frac{\text{Number of macrophages in test medium/100 macrophages} \times 100}{\text{Number of macrophages in 100 control media /1000 macrophages}}$$

3.9 Test for cell viability

The MTT colorimetric test was used to determine the vitality of *Leishmania* pathogens, as explained by Essid *et al* (2015). In each micro-well, 10 mL of MTT (10 mg/mL) was applied and incubated for 3 hours at 30°C. After that, each well received 100 L of a 50% (v/v) isopropanol-10% (w/v) sodium dodecyl sulfate combination to dissolve the insoluble formazan. An ELISA plate reader (Model Model: ABER-2) was used to assess the extent of absorption at 560 nm following 30 minutes of culturing at 25ºC. Tests were done thrice and contrasted with the negative and positive controls. Viability of the cells was determined by plotting percentage of inhibition versus isolate concentration using Original Program and calculating extract concentrations that inhibited 50% of the cell population (IC_{50}).

Cell viability (percentage) =

$$\frac{\text{Duplicate drug wells' average absorbance - blank wells average}}{\text{Control wells' average absorbance}} \times 100$$

3.10 The Minimum Inhibitory Concentration (MIC) determination

The flagellate forms of *L. major* were cultured for 12 hours at 26°C in fresh media (Schneiders Insect Media) enriched with 20% FBS in the exclusion or addition of various doses of the extracts (1mg/ml to mg/ml). Microscopically, cell growth was measured using a compound microscope. The following formula (Essid *et al.,* 2015) was used to obtain the Minimum inhibition Concentration in percentage (MIC):

$$MIC(\%) = \frac{100 \times (Absorbance \text{ of untreated cells} - \text{Absorbance of treated cells})}{\text{Absorbance of untreated cells}}$$

3.11 Vero cell cytotoxicity test

Trypan blue stain assay has initially been developed in 1975 to measure viable cell count and is still used as a confirmatory test for measuring changes in viable cell number caused by a drug or toxin Trypan blue stain, a large negatively charged molecule, is one of the simplest

assays that are used to determine the number of viable cells in a cell suspension (Stone, Johnston, & Schins, 2009). The principle of this assay is that living cells have intact cell membranes that exclude the trypan blue stain, whereas dead cells do not. Cell suspension is mixed with the trypan blue stain and examined visually under light microscopy to determine whether cells include or exclude the stain. A viable cell will have a clear cytoplasm, whereas a nonviable cell will have a blue cytoplasm (Strober, 2015).

To perform the trypan blue stain assay, 0.4% trypan blue stain and phosphate-buffered saline (PBS) or serum-free medium are obtained. Trypan blue stain should be stored in dark and filtered after prolonged storage. As trypan blue stain binds to serum proteins and causing misleading results, serum-free medium should be used to obtain reliable results. The cell suspension to be tested is centrifuged at 100 × g for 5 min. The supernatant is discarded and the pellet is resuspended in 1-ml PBS solution or serum-free medium. Then, one portion of this cell suspension is mixed with one portion of trypan blue stain. The mixture is allowed to stay at room temperature for 3 min. It is important to note that the cells should be counted within 3–5 min of mixing with trypan blue, as longer incubation periods will lead to cell death and hence reduced viability counts. Following the incubation, a drop of the mixture is applied to a hemocytometer, which is placed on the stage of a binocular microscope. Viable, that is, unstained, and nonviable, that is, stained, cells in the hemocytometer are counted separately.

After counting viable and nonviable cells, the total number of viable cells per milliliter of aliquot is determined by multiplying the total number of viable cells by 2, which is the dilution factor for trypan blue. Similarly, total number of cells per milliliter of aliquot is determined by addition of number of viable and nonviable cells and multiplying it by 2.

The test therapies and vero cells were then cultured for a further 48 hours at 37°C in a humidified 5 % CO_2 environment. Vero cells and medium were used in the control wells, while media was used alone in the blank wells. Each well was filled with 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reagent, which was incubated for another 3 hours till Formazan was seen using the microscope. After carefully sucking out the culture and MTT reagent, 100 µl of DMSO was poured and forcefully agitated for 5 minutes to make formazan soluble. A micro-titer plate reader (Corning 3367, UK) was used to measure the optical density of every well plate at a wavelength of 562 nm. Then, the percentage of viable cells is calculated using the following equation:

$$\%\ \text{viable cells} = \frac{\text{Total number of viable cells per ml aliquot}}{\text{Total number of cells per ml aliquot}} \times 100$$

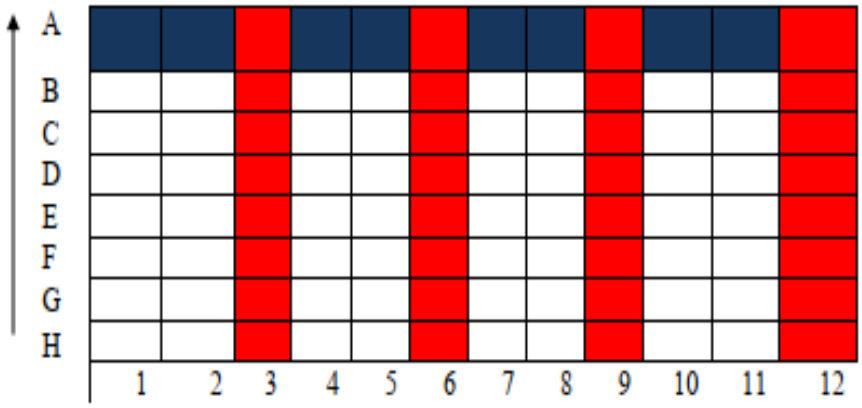


Figure 3.3: The cytotoxicity analysis of the extracts using MTT and Vero cells was performed in a 96-well plate

Key

Only maintenance media and plant extracts were used in Key Blank wells (Negative control)

These pits included cell lines, sustaining medium, and MMT

Cell lines, sustaining medium, and phytoconstituents were used in the wells, with concentrations decreasing from H to B.

In that trend, the dilution of phytocostituents diminished upwards from H to B. The concentrations of drugs ranged from 1000 to 333.33, 111.11, 37.04, 12.35, 4.12, and 1.37g/ml, respectively. A did not have any drug (Positive control).

3.12 Experimental animals and parasite inoculation

The mice were injected subcutaneously with parasites. Various groups were infected intradermally with 10^6 stationary phase flagellate forms of cultured *L. major* on the left hind footpad (LHFD). Promastigotes in the media were counted using an optical microscope and an improved double Neubauer hemocytometer.

The mice were separated into seven groups, each group with six mice each. Group 1,2,3,4,5 were treated with plant extracts; *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha* respectively, while group 6 and 7 was treated with amphotericin B and PBS respectively. The Nolan and Farrell (1987) method was used to measure the lesions utilizing a high precision vernier scale.

The wound diameter was calculated as follows:

Infected footpad size - uninfected control footpad size = lesion size

The World Health Organization's standard procedure was then used to count the parasites (WHO, 2002)

Any dead mouse was immediately dissected and parasites examined. For mice infected with *L. major* impression smears of both spleen and liver were made.

3.13 Lesion progression

Mice were left for 3 weeks for lesion to develop. To follow lesion growth, the width of the diseased footpad was measured weekly with a Vernier calliper (in mm). The wound diameters were determined as the variation in width between the exposed and unexposed opposite footpads to compare pharmacological action.

3.14 Quantification of *Leishmania major* parasite burden

Before quantifying the *Leishmania major* parasites, weight of the mice were taken. Impression smears were made from the spleen. The quantity of amastigotes in 1000 host nuclei was counted using a compound microscope after the slides were Giemsa-stained and preserved in methanol.

The total and relative numbers of parasites (Leishman-Donovan Units LDU) and total Leishman-Donovan Units (total LDU) were computed using the Bradley and Kirkley (1977) formula:

a) LDU = No. of parasites/1000 host nuclei

b) $\text{LDU} = \frac{\text{nucleated cells amastigotes} \times \text{organ weight (g)} \times 2\times10^5}{1000}$

3.15 Ethical Considerations

The permissions was granted by the Kenya Medical Research Institute's Scientific and Ethics Review Unit (SERU) (Review number KEMRI/SERU/CBRD/205/3968) and Animal Care and Use Committee (ACUC). All animals involved the infection investigation were euthanized in accordance with KEMRI's Animal Use and Care Committee (ACUC) rules.

3.16 Analysis of data

EXCEL spreadsheet for Windows was used to enter, organize, and manage all of the data obtained. STATISTICA 10.0 Statistical package was used to conduct all statistical analyses. The skewness and kurtosis of data distributions were examined for normality (Mishra *et al.*, 2019). To test if there were differences in concentration of the flavonoids among plants, One Way ANOVA test was performed. Differences in means were analyzed using Duncans Multiple Range Test.

The bioassays performed on effectiveness of the plant extracts on promastigotes growth inhibition, amastigotes growth inhibition as well as scavanging activities and reducing power assay were all considered as response variables against varying concentrations of plant

extracts. All the response variables are binary in nature and follow binomial distribution (Agresti, 2018). Therefore the relationship between the response variables and factors were modeled using logistic regression Probit analysis. Maximum likelihood was used to estimate the regression coefficient (R square) because it gives more precise estimation of necessary parameters for correct evaluation of the results (Bewick *et al.*, 2005). During logistic regression, Probit analysis (MacKinnon *et al.*, 2007), all data were transformed to Log Base 10 to linearize the relationship between the response variables and factors. For each analysis, the response frequency was observed as response variables from a total observation of 10 Lesihmania parasite responses. Meanwhile the plant extract concentrations was the covariate.

The resulting probability outcomes were multiplied by 100 to determine the expected % of the response frequency. To test for the significance of the Probit plots, Z statistics was calculated; the lager the Z statistics the larger the differences from the smaller sized Z. Nevertheless the differences was verified using P-value. The modeled fit was confirmed using chi-square goodness of fit test between the observed response values and predicted probability of response values. The resultant graph plotted consisted of Probits of response variables in Y-axis and Log_{10} concentration in X-axis.

The LC_{50} and LC_{90} were determined by projecting the Y axis for a probit of 5.00 and 9.00 and taking the inverse $Log_{10}(X)$ of the concentration associated with plant extracts. The LC_{50} and LC_{90} were then compared using One Way Analysis of variance (One-Way ANOVA) to test which plant extracts was more efficacious. Where significant differences existed following ANOVA, means were separated using Duncans Multiple Range Test (DMRT).

Repeated measure ANOVA was used to examine the effect of extracts on parasite burdens in spleens and livers. Proportion data were arcsine transformed prior to statistical ANOVA test. Duncans Multiple Range test was employed to compare means where substantial variations were found. $P < 0.05$ was used to consider all of the results significant.

CHAPTER FOUR

RESULTS

4.1 Chemical composition of the *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* plant extracts

Durng qualitative analysis glycoside, terpenoids, tannins, flavonoids, steroids, and saponins were found in the extracts after phytochemical examination (Table 4.1). The most phytochemical substances were found *in T. mollis* and *B. micrantha*. *T. mollis* contaned higher concentration of tannins, phenols, saponins, alkaloids, anthraquinone and terpenoids. *Kigelia africana* had higher concentraton of phenols, terpenoids and glycosides while *C. macrostachyus* showed higher abundance of alkaloids and saponins

Table 4.1: Phytochemical components of plant extracts

Phytochemical compound	*O. europa*	*K. Africana*	*T. mollis*	*C. macrostachyus*	*B. micrantha*
Tannins	++	++	+++	++	++
Phenols	-	+++	+++	+	+++
Flavonoids	+	++	++	++	++
Steroids	-	+	+	+	+++
Alkaloids	+	++	+++	+++	-
Saponins	+	++	+++	+++	-
Phlobatannin	-	+	+	-	-
Anthraquinone	+	++	+++	-	+
Cardiac glycoside	++	++	++	-	+
Terpenoids	-	+++	+++	-	++
Anthocyanins	+	-	++	++	-
Glycosides	-	+++	-	+	-

+++ = high amount; ++ = moderate amount; + = trace amount; - = Not detected

This study determined total flavonoid contents (TFC QE/mg of extract) and Total Phenolic Content (TPC mg g^{-1} DW) (Figure 4.1). The flavonoid content concentration was significantly ($P < 0.05$) highest in *Olea europaea* bark extracts (12.4 ± 1.54 mg QE/mg of dry weight extract) followed by *K. africana* (9.2 ± 0.95 mg QE/mg of dry weight extract). The

total phenolic composition in the plant extracts was highest for *K. africana* (9.4 ± 0.46 mg/mg DW), followed by *T. mollis* (7.4 ± 0.53 mg/mg DW) while the range of Total Phenolic Content in the remaining plant extracts were similar (0.6-1.8 mg/mg DW) but higher than control (0.23 ± 0.02 mg/mg DW). Antioxidant natural compounds found in therapeutic plants include polyphenols and flavonoids. In comparison to the control (0.59 0.04 gCE/mg dry extract), the results are statistically significant.

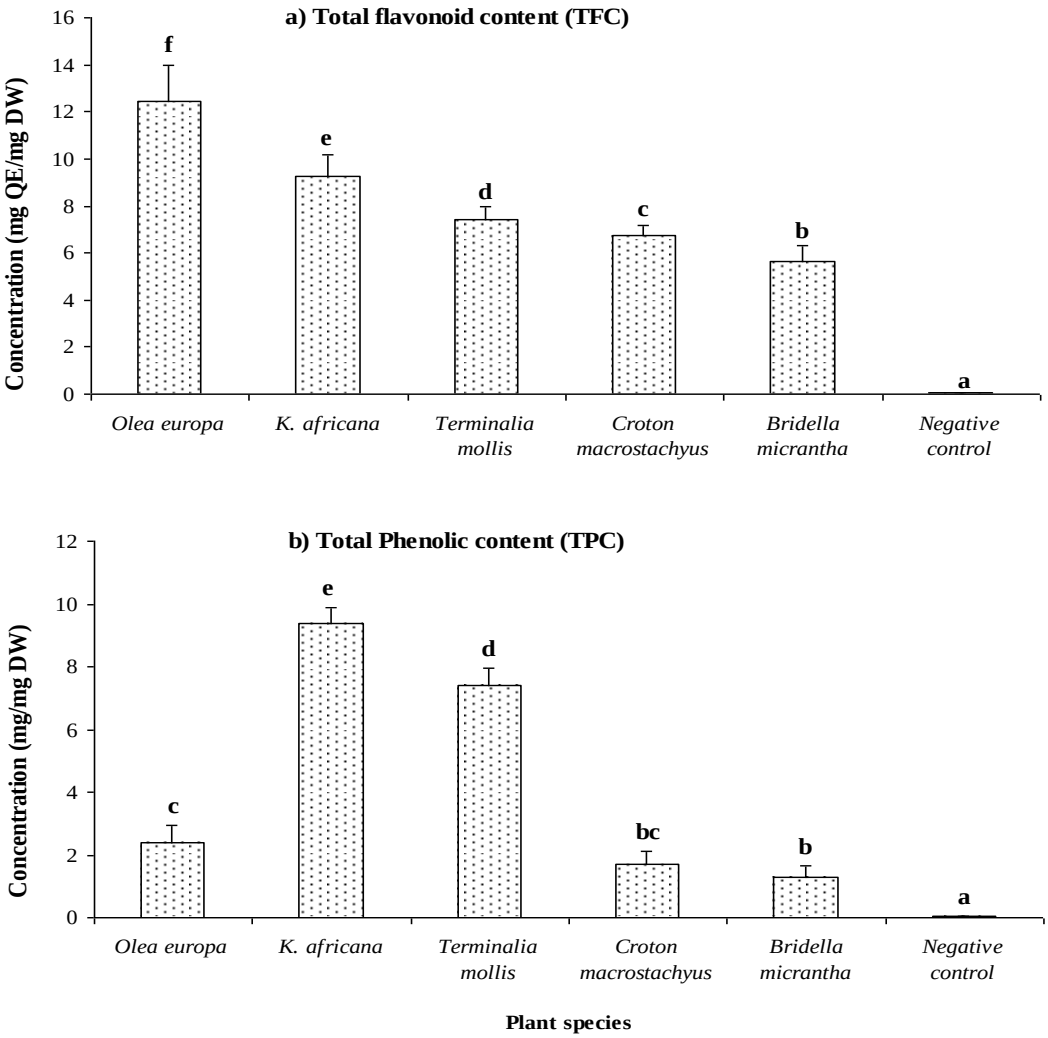


Figure 4.1: Total flavonoid Content (TFC) and Total phenolic contents (TPC) analyzed in the plant isolates

Data are shown as mean ± SD, N = 3 experiments. Values with different letters differ significantly ($p < 0.05$)

Figure 4.2 depict the potential of various plant extracts to scavenge the DPPH+ free radical under specific conditions. The antioxidant activity increased in the following order according to the DPPH test: *T. mollis* > *C. macrostachyus* > *K. africana* > *M. micrantha* > *O. europaea*. The *T. mollis* root extracts showed the highest DPPH radical scavenging activity. Negative control posted 0% inhibition throughout the experiment (data not shown).

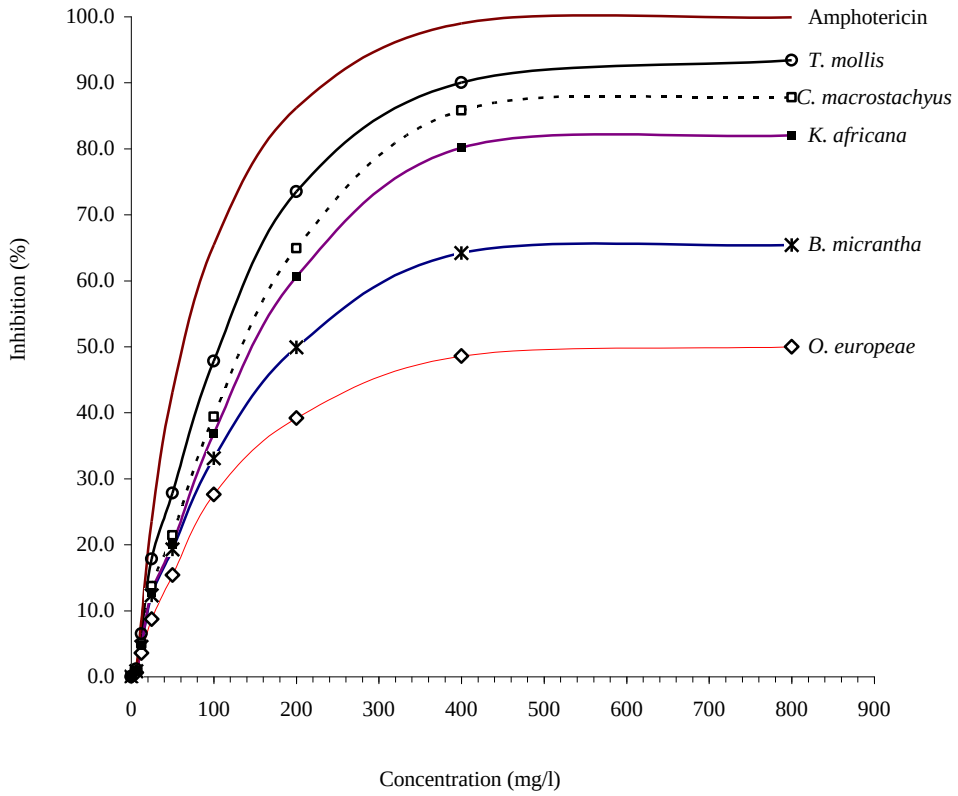


Figure 4.2: The ability of five plant extracts to scavenge DPPH radicals

Data are presented as mean SD, N = 3 experiments.

The % inhibition, IC_{50}, and IC90 of the plant extracts on promastigotes that were evaluated. There were significant differences in the test medicines' optimal effectiveness ($P < 0.05$). The most effective DPPH radical scavenging activity of five plant extracts was *T. mollis*, followed

by *C. macrostachyus*, and *O. europaea* was the least effective. Negative control posted 0% inhibition throught the experiment (data not shown).

The findings of the reducing power assay (RPA) of the analyzed plant extracts are shown in Figure 4.3. Our extracts exhibited a potency decreasing power, according to the results. *T. mollis* extract had the highest absorbance, followed by *C. macrostachyus*, while *O. europeae* extract had the lowest absorbance. *T. mollis*, *C. macrostachyus*, *K. africana*, and *B. micrantha*'s reducing power was dosage dependant and increased with increasing volumes of extracts. After 200 mg/l, however, the lowering power of O. europaea showed a lower level of rise. Negative control posted 0% absorbance throughout the experiment (data not shown).

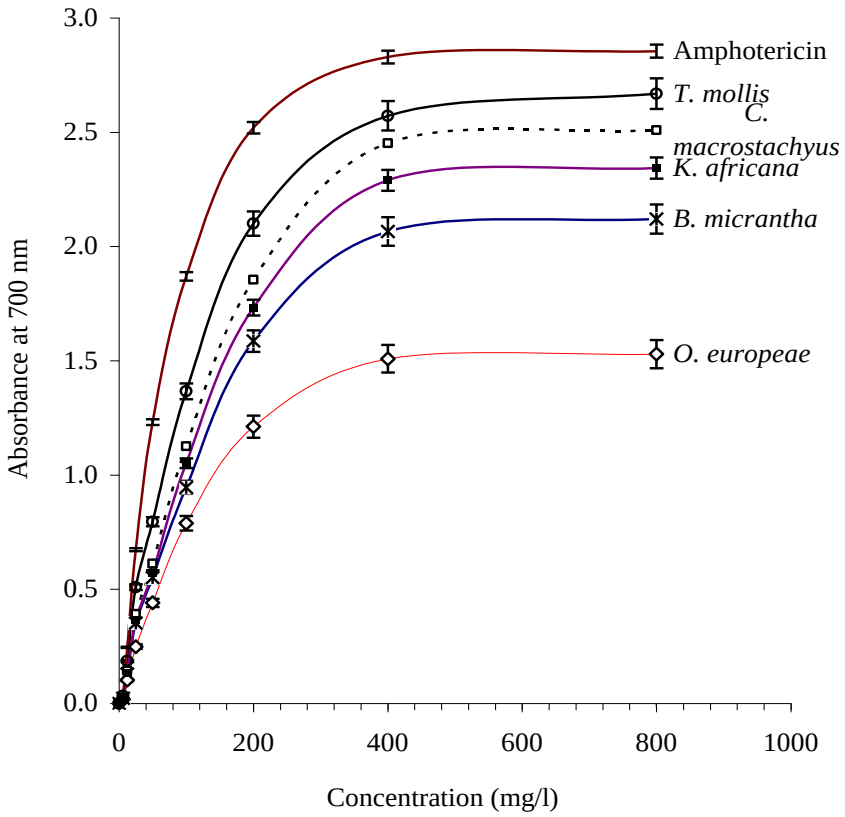


Figure 4.3: Ferric Reducing power assay (FRPA) of extracts from five plants

Data are shown as mean ± SD, N = 3 experiments.

4.2 Efficacy of crude extracts of medicinal plants on parasite growth inhibition, parasite loads and infection rates *in-vitro*

Figure 4.4 depicts the effectiveness of different amounts of extracts from *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha*, and a standard medicine (Amphotericin B) on promastigotes of *L. major*. After 24 hours of treatment, the crude extracts from the plants significantly inhibited promastogote growth ($P < 0.05$). Negative control posted 0% promastigote growth inhibition throughout the experiment (data not shown).

The logistic regression model was fully fitted by the projected percentage growth suppression for the parasite promastigote (Table 4.2). The standard medicine (Amphotericin B) was the most effective against promastigotes, according to the model parameter coefficients of concentration (C). The most efficacious anti amastigote was *T. mollis*, then, *C. macrostachyus*, and the lowest was *O. europaea* in terms of optimal efficacy, according to the gradient analysis of logistic regression of the crude extracts of the herbal medications. Negative control posted 0% promatigote growth inhibition throughout the experiment (data not shown).

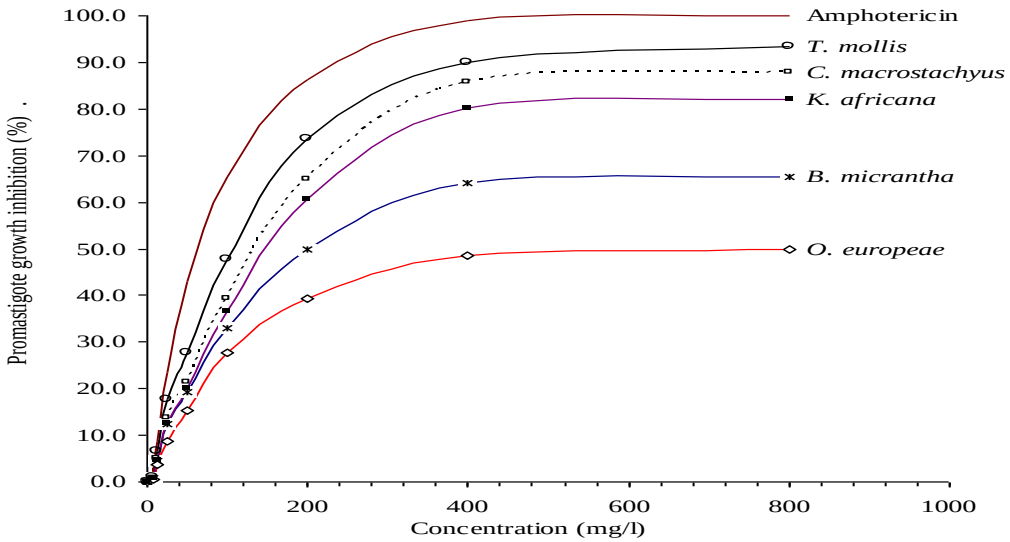


Figure 4.4: Inhibition of promastigote proliferation after treatment with several test drugs.

Table 4.2: The regression analysis of the five different drugs against *L. major* flagellate forms

Test drug	Model	Parameter significance
Amphotericin	$\text{Log}\ (\rho/1-\rho) = 0.013 + 0.784*C - 0.0008*C^2 + 0.0004*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0003)\ \beta_3(0.0011)$
T. mollis	$\text{Log}\ (\rho/1-\rho) = 0.012 + 0.724*C - 0.0008*C^2 - 0.0014*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0001)\ \beta_3(0.0003)$
C. micrantha	$\text{Log}\ (\rho/1-\rho) = 0.025 + 0.674*C - 0.0008*C^2 + 0.0008*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0001)\ \beta_3(0.0123)$
K. Africana	$\text{Log}\ (\rho/1-\rho) = 0.034 + 0.648*C - 0.0008*C^2 + 0.0001*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0001)\ \beta_3(0.0021)$
B. micrantha	$\text{Log}\ (\rho/1-\rho) = 0.014 + 0.484*C - 0.0008*C^2 + 0.0001*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0001)\ \beta_3(0.0022)$
O. Africana	$\text{Log}\ (\rho/1-\rho) = 0.012 + 0.414*C - 0.0008*C^2 + 0.0011*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0005)\ \beta_3(0.0016)$

The ideal efficacy, concentration at optimum efficacy, IC_{50}, and IC_{90} of the test agents against flagellate forms of parasites are shown in Table 4.3. The test therapies' optimum effectiveness vary significantly ($P < 0.05$). The positive control or standard treatments had the best efficacy. In terms of ideal efficacy among the plant extracts, *T. mollis* had the highest anti-amastigote activity, then *C. macrostachyus*, while the least effective was *O. europaea*. Except for *T. mollis*, none of the plant extracts attained IC_{90}.

Table 4.3: Ideal effectiveness, LC50, and LC90 of experimental drugs against the leishmania promastigote type over 24-hours

	Experimental drugs						Parameter and statistics	
Concentration (μg/ml)	Amphotericin B	*T. mollis*	*C. macrostachyus*	*K. africana*	*B. micrantha*	*O. europaea*	F-value	P-value
Optimal efficacy (%)	100	92	84.5	81.5	64.5	46.5	17.311	0.000
Concentration at optimal efficacy (mg/l)	425	545	565	596	625	645	39.212	0.000
Concentration at IC_{90}	260	525	-	-	-	-	89.221	0.000
Concentration at IC_{50}	45	85.5	118	145	200	-	112.489	0.000

Figure 4.5 shows the effectiveness of crude extracts from *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha,* and a standard medicine (Amphotericin B) against *L. major* amastigotes. After 24 hours of treatment, the various test drugs significantly inhibited amastogote development ($P < 0.05$).

The logistic regression model was fully fitted by the projected percentage proliferation suppression for leishmania amastigotes (Table 4.4). Amphotericin B was the most efficacious medication, which was a typical drug, according to the model parameter coefficients of C (Table 4.4). *T. mollis* was determined to be the most effective herbal test therapies in the current investigation, followed by *C. macrostachyus,* while *O. eauropeae* showed the lowest efficacy. Negative control posted 0% amastigote growth inhibition throughout the experiment (data not shown).

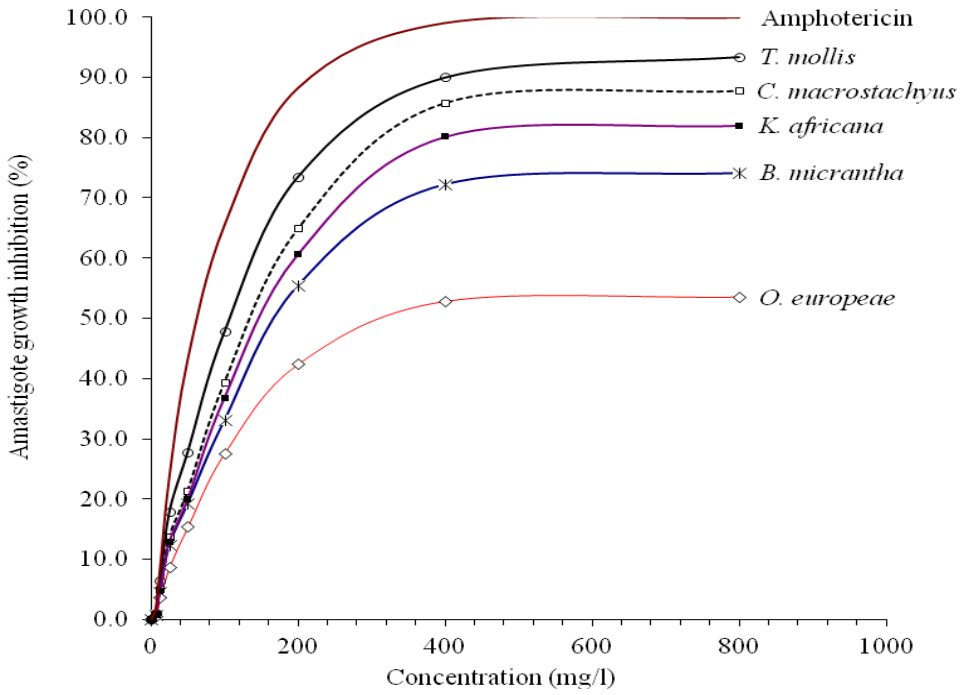


Figure 4.5: Inhibition of amastigote multiplication after treatment with several test drugs.

Table 4.4: Parametric statistics from the five experimental drugs' logistic regressions against *L. major* amastigotes

Experimental drug	Model	Parameter significance
Amphotericin B	$\text{Log}\ (\rho/1-\rho) = 0.011 + 0.8948*C - 0.0568*C^2 + 0.0004*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0004)\ \beta_3(0.0021)$
T. mollis	$\text{Log}\ (\rho/1-\rho) = 0.013 + 0.7544*C - 0.0312*C^2 + 0.0001*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0001)\ \beta_3(0.0026)$
C. macrostachyus	$\text{Log}\ (\rho/1-\rho) = 0.007 + 0.5544*C - 0.0342*C^2 + 0.0011*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0003)\ \beta_3(0.0016)$
K. Africana	$\text{Log}\ (\rho/1-\rho) = 0.005 + 0.4543*C - 0.0342*C^2 + 0.0011*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0003)\ \beta_3(0.0016)$
B. micrantha	$\text{Log}\ (\rho/1-\rho) = 0.012 + 0.3142*C - 0.0342*C^2 + 0.0011*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0003)\ \beta_3(0.0016)$
O. europaea	$\text{Log}\ (\rho/1-\rho) = 0.011 + 0.\ 2412*C - 0.0118*C^2 + 0.0008*C^3$	$B_0(0.0000)\ \beta_1(0.0000)\ \beta_2(0.0002)\ \beta_3(0.0123)$

The ideal efficacy, LC_{50}, and LC_{90} of the experimental medicines against amatigotes (Table 4.5). Significant differences were observed in the test medicines' optimal effectiveness ($P < 0.05$). Even though the test medications did not attain this efficacy threshold, the standard treatments had an ideal efficacy of 99.8%. *T. mollis* exhibited the highest anti-amastigote activity, followed by *C. macrostachyus,* while *O. europeae* was the lowest in terms of optimal efficacy among the herbal test medications. There was a significant difference ($P < 0.05$) in the LC50, with the standard medication having the lowest LC_{50}, followed by crude extracts from *T. mollis, and O. europaea*. Except for the standard drug, crude extracts of *T. mollis* and *C. macrostachyus,* none of the non-standard test compounds achieved LC_{90}.

Table 4.5: Ideal efficacy, LC_{50} and LC_{90} of experimental drugs against the amastigotes in 24-hours.

	Experimental drugs						Parameter and statistics	
Concentration (μg/ml)	Amphotericin	*T. mollis*	*C. macrostachyus*	*K. africana*	*B. micrantha*	*O. europaea*	F-value	P-value
Optimal efficacy (%)	99.9	93.4	87.8	82.1	74.2	53.2	117.311	0.000
Minimum concentration at optimal efficacy	412.3	542.8	584.2	672.6	874.5	854.8	139.212	0.000
Concentration at IC_{50}	52.8	96.4	138.2	158.5	175.2	360.2	112.489	0.000
Concentration at IC_{90}	223.4	401.2	554.2	-	-	-	89.221	0.000

Table 4.6 shows the body weights, spleen weights, and spleen-somatic index in mice innoculated with *L. major* and treated with *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha*, and standard drug. The spleen weight and the spleno-somatic index differed significantly between tests (ANOVA, $P < 0.05$). The splenic and spleno-somatic index in mice infected with *L. major,* were significantly larger when administered with conventional drug followed by *T. mollis,* whereas the spleen and spleno-somatic index in mice treated with *C. macrochystus* exhibited the least. The untreated controls had the greatest spleen weight. *O. europaea* had the highest number of parasites, followed by those treated with *Croton macrostachyus* and *T. mollis* had the lowest number of parasites. Despite this, the treatment with Amphotericin and *T. mollis* resulted in the lowest parasite levels.

Table 4.6: The body weight, spleen weight, and spleeno-somatic index in mice after different tests

Test	Body weight	Spleen weight	Spleeno-somatic index
Amphotericin B	22.11 ± 0.54	0.23 ± 0.021^{d}	0.70 ± 0.02^{a}
T. mollis	21.78 ± 0.89	0.18 ± 0.021^{c}	0.75 ± 0.05^{b}
C. macrostachyus	20.50 ± 0.45	0.15 ± 0.006^{b}	0.79 ± 0.02^{b}
K. Africana	21.00 ± 1.00	0.15 ± 0.005^{b}	0.83 ± 0.06^{c}
B. micrantha	21.00 ± 0.58	0.14 ± 0.010^{b}	0.85 ± 0.07^{c}
O. europaea	20.00 ± 0.58	0.11 ± 0.010^{a}	1.02 ± 0.04^{d}
ANOVA			
F	2.1332	35.255	71.214
df	5	5	5
P	0.3245	0.0001	0.0000

Values with different letters differ significantly down the column ($p < 0.05$)

Figure 4.6 shows the Leishmania Donovani Unit (LDU) of the mice innoculated with *L. major* and treated with *O. europaea, K. africana, T. mollis, C. macrostachyus,* and *B. micrantha*. The LDU differed significantly depending on the therapy (ANOVA; F = 34.55 df = 34.5 P < 0.0001). Treatment with *T. mollis* and Amphotericin B resulted in the lowest LDU. Other extracts exhibited the following order: C. *macrostachyus* > *K. africana* >*B. micrantha* >*O. europaea*

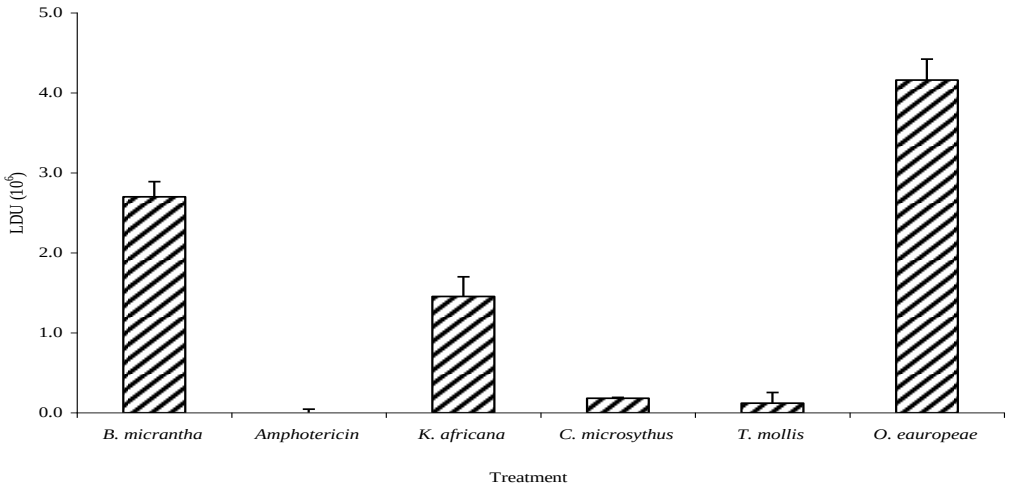
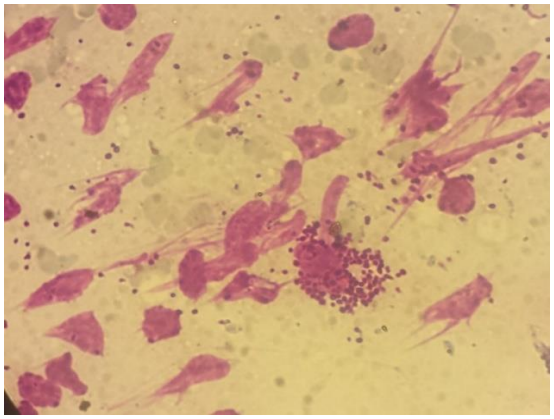
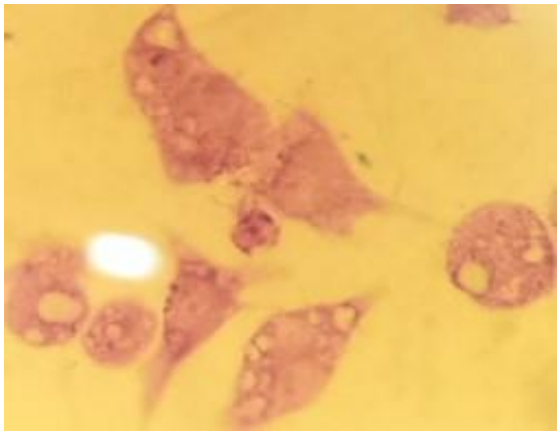


Figure 4.6: *L. major* Leishmania Donovani Unit in the mice administered with different therapies

Photographic images of the splenocytes and amastigotes in splenic impressions after treatment *in vivo* are shown in Figure 4.7.



Macrophages with no amastigotes after treatment *in vitro*



Macrophages with amastigotes after treatment *in vitro*

Figure 4.7: Photographic images of macrophages with and without amastigote following treatment in vivo

4.3 Efficacy of extracts of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* against *L. major* lesion development

Figure 4.8 shows the lesion diameters of the mice at the beginning of infection, during therapy with *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha, and amphotericin B*, as well as untreated controls. Following innoculation with *L. major*, the mice developed a consistent lesion over the first five weeks. Repeated measure ANOVA was used to examine variations in lesion sizes from 5^{th} to 8^{th} week, and it revealed that there were significant variations in lesion diameter across tests ($F = 13.44$, $df = 6$, $P < 0.0001$). After infection, the lesion sizes of the control mice grew larger till the end of the trial. Amphotericin B treated mice had highest lesion size reduction in four weeks of all the drugs studied. Therapy with *T. mollis*, on the other hand, resulted in a significantly ($P < 0.05$) greater wound reduction in mice than treatment using extracts from other plants. After 4 weeks of treatment, the mice innoculated with *O. europeae* had the least lesion diameters reduction among therapies examined.

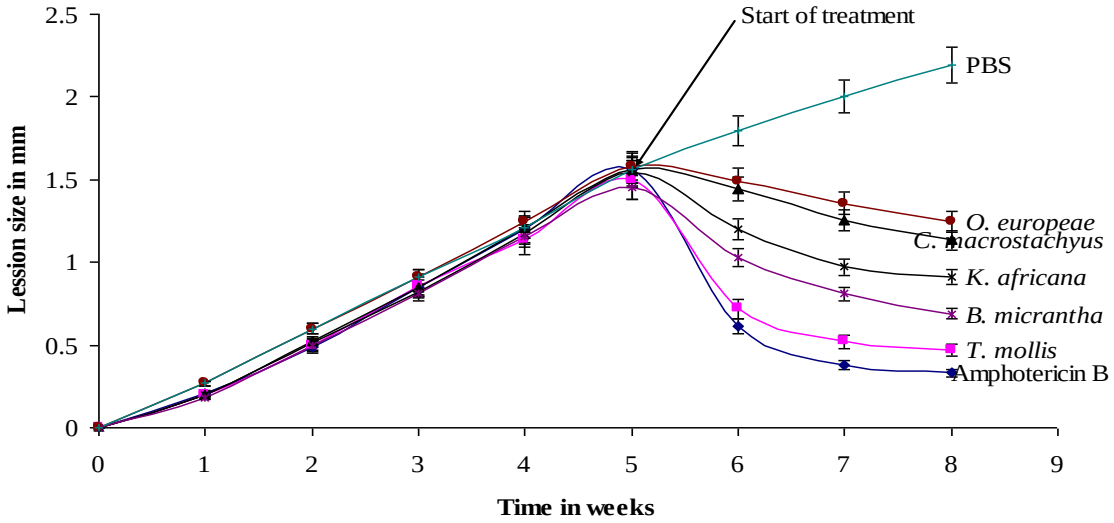


Figure 4.8: Sizes of lesions in mice during commencement of parasite injection and throughout therapy using crude phytochemicals and amphotericin B.

Table 4.7 shows the overall mean lesion diameters before treatment and reduction in mice innoculated with *O. europaea*, *K. africana*, *T. mollis*, *C. macrostachyus*, *B. micrantha*, and amphotericin B before and after treatment with *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha,* and amphotericin B. There were no notable changes in the diameters of lesions at the commencement of the experiment ($P > 0.05$). However, after the experiment, significant variations in reduction of lesion were observed ($P < 0.05$). Mice injected with the conventional drug had the largest reduction lesion sizes than those inoculated with *T. mollis*, while the smallest reduction in lesion sizes were identified in mice treated with *O. europaea* after the experiment.

Table 4.7: Mean lesion diameters before treatment and reduction of mice before and after treatment with *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha*, and amphotericin B.

			Mean lesion size (mm)	
Treatment agent	Duration	Number of mice	Pre-treatment	Lesion reduction
Amphotericin B	28	6	1.03 ± 0.17	0.21 ± 0.04^{a}
T. mollis	28	6	1.05 ± 0.09	0.25 ± 0.09^{a}
C. macrostachyus	28	6	1.05 ± 0.10	0.42 ± 0.20^{b}
K. africana	28	6	1.12 ± 0.05	0.49 ± 0.09^{b}
B. micrantha	28	6	1.09 ± 0.05	0.68 ± 0.07^{c}
O. europae	28	6	1.09 ± 0.12	2.14 ± 0.07^{d}
BPS	28	6	1.09 ± 0.12	3.51 ± 0.43^{e}
	ANOVA	F		17.3212
		df		5
		P		0.0001

4.3 Toxicity extracts *of O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* on vero cells

Microscopic lesions were found in the livers of *L. major* inoculated mice, indicating that the infection was causing harmful effects. Normal liver tissue was seen in sections in the mice inoculated with amphotericin B (Figure 4.9a). Mild granulomas were encountered more frequently in the balb/c mouse treated with *T. mollis* which was the most effective herbal drug (Figure 4.9b). However, liver granuloma from *L. major* infected mice treated with *C. macrostachyus* (Figure 4.10a) and *K. africana* (Figure 4.10b) caused severe cell damage and thus toxicity of the test drugs; also *L. major* injected mice treated using *B. micrantha*, showed same patterns (Figure 4.11a) and *O. europaea* (Figure 4.11b), at week ten.

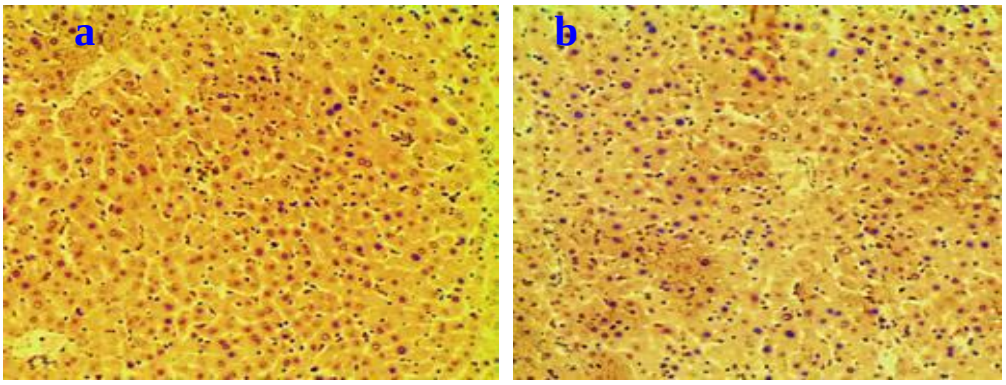


Figure 4.9: Normal hepatic histology from an *L. major*-injected mouse after ten weeks of therapy using a standard drug (a) and *Terminalia mollis* herbal extracts (b) (H&E, 200). Magnification ×1000

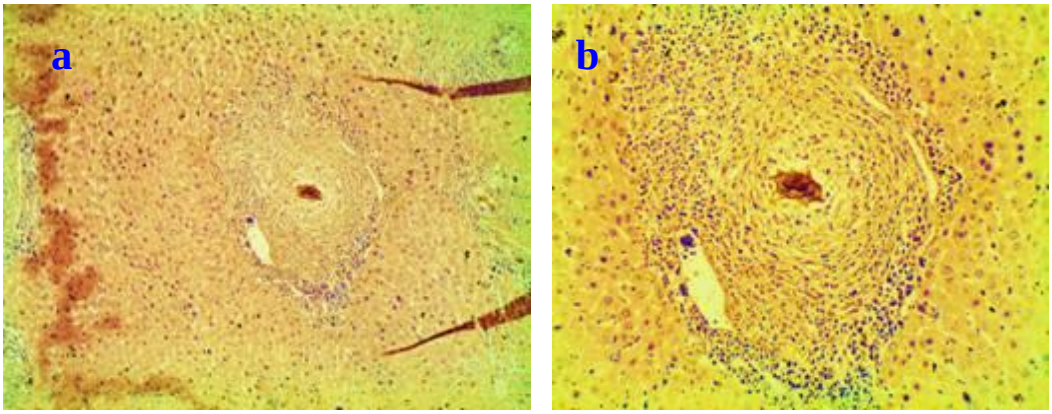


Figure 4.10: Inflammatory cells and fibrous tissues in hepatic periovular granuloma from infected mice treated with (a) *C. microstachyus* and (b) *K. africana* at week ten after infection (H&E, 200). Magnification ×1000

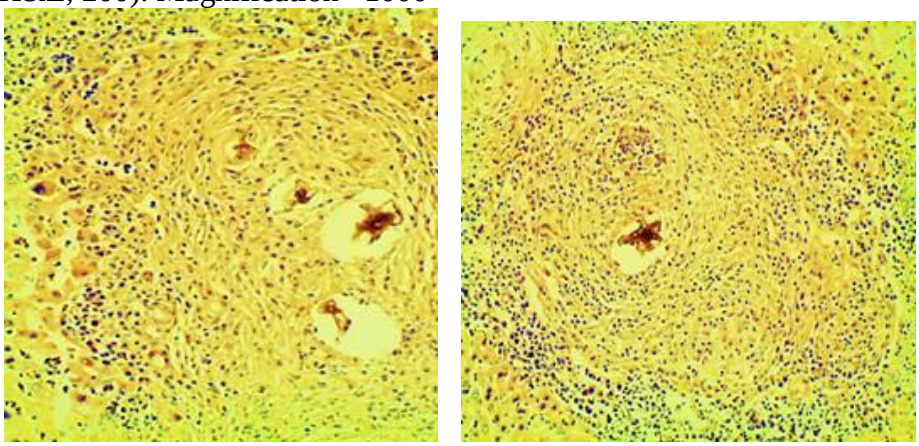


Figure 4.11: Phagocytosed fibrous structures in the hepatic periovular granuloma of mice inoculated with *L. major* and therapy using (a) *B. micrantha* and (b) *O. europaea* (H&E, 200). Magnification ×1000

After 24 hours of exposure, the diverse test substances had a substantial impact on mammalian cell viability ($P < 0.05$). Estimates of mammalian cell viability after treatment with a completely fitted logistic regression model defining a dosage dependent therapy (R^2 = 0.9756). The model equation was log (/1-) = 100 - 14.2924C – $1.0440C^2$ + $0.1445C^3$, and the parameter significance (P-value) found for each coefficient was 0 (P = 0.1351), 1 (P = 0.0001), 2 (P = 0.1857), and 3 (P = 0.0091). The % cell vitality estimations of mammalian cells administered with the standard medicine and other phytoconstituents were completely fitted to the logistic regression model explaining dosage response therapy. The logistic regression model explaining a dosage response therapy was fully fitted by the % cell vitality estimations of *T. mollis* treated mammalian cells (R^2 = 0.9961). According to the results of a gradient analysis of logistic regression of crude extracts of herbal medications, *B. microstachyus* and *O. europaea* had the most hazardous effects, reducing cell viability to less than 50%.

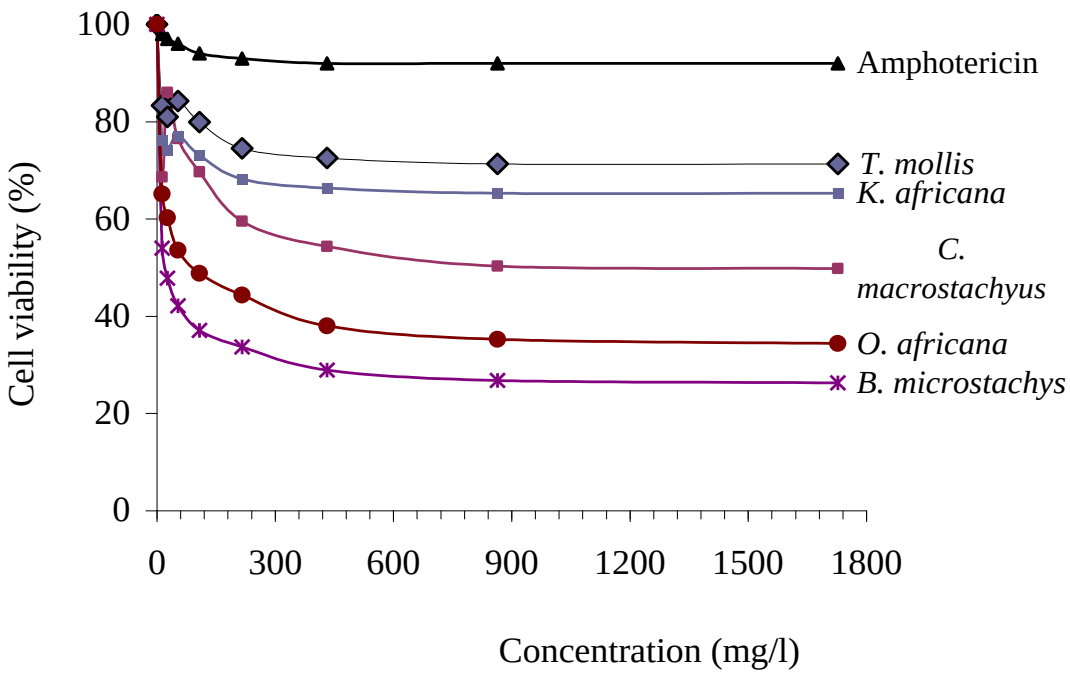


Figure 4.12: Mammalian cell mortality levels in the lab (LC_{50} and LC_{90} of the test drugs).

CHAPTER FIVE

DISCUSSION

5.1 Screening test of medicinal plants for biologically active compounds

The current research sought to establish the phytochemical components that could be used to cure leishmaniasis in *Terminial mollis, Croton macrostachyus, Olea europaea, Kigelia Africana,* and *Bridella micrantha.* Qualitative phytochemical investigations demonstrated the presence of major documented categories of compounds such as cumarins, anthocyanins, trepenoids, polyphenols, alkaloids, flavonoids, saponins, and tannis in these extracts. The presence of polyphenols, alkaloids, flavonoids, saponins has been reported in several medicinal plants (Gutiérrez-Rebolledo *et al.*, 2017). The presence of coumarins and flavonoids, tannins and anthocyanins was also reported in some medicinal plants (fig, guava, olive and pomegranate) from Egypt (Farag *et al.*, 2020). Meanwhile phytochemical screening and qualitative estimation of 50 medicinal plants in Bihar, India, showed presence of anthocyanins, coumarins, emodins, leucoanthocyanins, tannins, terpinoids, sterods and saponins (Kumari *et al.*, 2017). Steroids and terpenoids along with tannins and saponins are found to be rich in most of the medicinal plants for the present study. The presence of bioactive compounds indicates the medicinal value of the plants.

The concentration of these most active compounds, the total phenolic contents in some of the plants such as *Olea europaea* and *Kigelia africana* were in large enough quantity and may be suitable for control of *Leishmania* parasites. These results obtained suggest that concentration of TPC is in values that may provide active bioactive ingredients for a range of parasites including leishmaniacidal properties (Zeouk *et al.*, 2019). Moreover, the TPC in all the tested plants were higher than the control suggesting that they may be active against Leishmania parasites. The most phytochemical substances were found *in K. africana* and *Olea europaea*.

Herbal medications (particularly those from the Asteraceae, Rosaceae, and Lamiaceae families) have been utilized as treatments for ailments since ancient times because they contain pharmacological and biological active components (Hajimehdipoor *et al.*, 2013; Hajimehdipoor *et al.*, 2014). Bioactivity of plant preparations against a variety of parasites are contained in the phytochemicals of these plants.

Diverse bioactivity has been reported for *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* (Atawodi and Olowoniyi, 2015; Guinda *et al.*, 2015; Hashmi *et al.*, 2015; Douglas and Gitonga, 2016; Cheurfa *et al.*, 2019). This requires determination of measures such as antioxidant and inhibitory potential (Lambert and Pearson, 2000; Van de Vel *et al.*, 2019). Furthermore, more information about the polyphenolics, flavonoids, and antioxidant capabilities of the extracts from these plants is needed. As a result, ferric reducing antioxidant power and free radical scavenging activity assays were employed to identify and analyze the bioactive components involved, including the antioxidant capability.

Antioxidant properties, can reduce lipid catabolism and give resistance to oxidative distress by neutralizing free radicals (Lugo-Huitrón *et al.*, 2011), have been linked to a variety of diseases. Using the DPPH and FRAP methodologies, to determine the antioxidant characteristics in this study. The findings of DPPH assays revealed that the extracts of the plants examined had high free radical scavanging activity, with the following being the order: *T. mollis* > *C. macrostachyus* > *K. africana* > *M. micrantha* > *O. europeae.* The findings acquired are in agreement with those earlier published (Elgorashi and McGaw, 2019).

These extracts' FRAP experiments revealed an antioxidant potency that depended on dosage, and the results were consistent with an earlier finding (Rabeta and Faraniza, 2013). These

extracts' crucial ability to scavenge free radicals like ROS, suppress oxidative degredation of lipids, and block cancerous pathways could be explained by earlier findings (Benzie and Devaki, 2018). The abundance of therapeutically potent phytochemicals like alkaloids, flavonoids and polyphenols in *T. mollis*, *C. macrostachyus*, and *K. africana* may be responsible for their great antioxidant activity, which was validated by our analysis of phytochemical substances.

5.2 Potency of test samples of medicinal plants on parasite growth inhibition, parasite loads and infection rates *in-vitro*

The conventional treatment (Amphotericin B) acting as a positive control was the most efficient in reducing promastigote growth after 24 hours of exposure in *L. major* infected mice, which agrees with several previous research (Sundar *et al.*, 2019b; Kumari *et al.*, 2022). On a similar note, *T. mollis* has much more potency than other herbal drugs and was comparable to the standard drug. Therefore it is probable that *T. mollis* has a stronger attraction for ergosterol, found in fungus and Leishmania species membranes, which explains its relative potency. Anti-promastigote potency of the conventional drug is due to its capacity to attach ergosterol in the parasite membrane or trap cholesterol in the host membrane, preventing parasite-macrophage connection, which is required for macrophage invasion (Chattopadhyay and Jafurulla, 2011). In addition, effective anti-leishmanial drugs causes: lipid peroxidation, which favours cell membrane damage; endosome-lysosome fusion inhibition and immunoadjuvant activity by triggering the release of IFN-, which aids macrophage activation (Galvis *et al.*, 2020).

The study established that among the plant extracts, *T. mollis* and *C. macrostachyus* exhibited the most potent growth inhibition of promastigotes and were comparable to the standard drug. The activity of *T. mollis* against several parasites has been previously attributed to

punicalagin, ellagic acid and their derivates (Souto *et al.*, 2019; Jambwa and Nyahangare, 2020; Wiart, 2020). These compounds also have high solubility in methanol and could therefore be in large quantitative in the present sample. *T. mollis* extract also contain other active compounds including urolithins and benzopyranones, which are cystein protease inhibitors (Chang *et al.*, 2019; Dimas *et al.*, 2020; Muganga *et al.*, 2020). The plant has been tested and showed good antioxidant activity attributed to ellagitannins, ellagic acid, as well as condensed tannins such as epicatechin, gallocatechin, catechin, and apigallocatechin and garlic acid derivates (Adeeyo *et al.*, 2018; Vilegas *et al.*, 2018).

Phytoconstituents of *C. macrostachyus* bark were the second most portent plant against *Leishmania* promatigotes. A number of pharmacological studies confirmed the anti-leishmanial activities of *C. macrostachyus* (Tariku *et al.*, 2010) due to its phytochemical constituencies. According to phytochemical studies, *Croton* extracts have a broader spectrum of bioactive compounds including alkaloids, terpenoids, flavonoids, and essential oils such as mono and sesquiterpenoids (Meresa, 2019; Moremi *et al.*, 2021). Terpenoids, primarily diterpenoids, are the most abundant secondary metabolite ingredients in the genus, which includes the skeletal types neoclerodane, clerodane, kaurane, phorbol, labdane, and trachylobane (Eksi *et al.*, 2020). Among the identified chemicals from various portions of *Croton macrostachyus* are cyclohexane diepoxides such as crotepoxide, lupeol, and betulin, cis-clerodane, crotomacrine, 3-Acetoxy tetraxer-14- en-28-oic acid, trachylina-19-oic acid, and trachylina-18-oic acid (Meresa, 2019).

The least effective plant extract inhibiting promastigote growth was *Olea europeae*. *Olea europaea* contains a variety of chemicals, including biophenols (Difonzo *et al.*, 2017). Oleuropein, a secoiridoid consisting of elenolic acid and hydroxytyrosol, is the most common biophenol and is considered the bitter component (Mkaouar *et al.*, 2018). Other bio-phenols

found in smaller amounts include luteolin-7-glucoside, apigenin-7-glucoside, verbascoside, and hydroxytyrosol (Iorizzo *et al.*, 2016). Most of these chemicals have been reported to be effective against bacteria like *Klebsiella pneumonia, E. coli, Staphylococcus species,* but not against Leishmania species (Seow, 2021). The efficacy of different doses of extracts from the test plants in *L. major* infected mice with revealed similar patterns as observed in amastigotes. *Terminalia mollis,* followed by *C. macrostachyus* and *K. africana,* was the most effective in inhibiting promastigote growth, while *O. europeae* was the least effective. The test compounds were shown to have short-term activity against a *Leishmania* infection that had been present for four weeks prior to medication delivery. The activity of these test compounds appear to act in similar manner as in the promastigotes. The protracted effect of the test chemicals on pathogen proliferation exhibits decreased degradation as well as delayed elimination, implying that the test drugs have a prolonged physiological half-life.

The splenic and spleno-somatic index were significantly higher in mice inoculated with normal medication, followed by *T. mollis* and *C. macrostachyus,* and the splenic and spleno-somatic index were significantly lower in mice treated with *O. europeae*. In a normal infection, the spleen is one of the most important sites for Leishmania multiplication (Kaye *et al.,* 2020). In contrast to the hepatic parasite load, the splenic parasite load was at the beginning less in mice, however, it continued to rise for at least three months and does not naturally reduce without therapy. Megakaryocytes are linked to *Leishmania* infection resistance, and their numbers rise in an effort to control the disease (Kumar, 2021). The parasite can cause these cells to multiply in the spleen, enhancing infection due to their immaturity (Odiwuor *et al.,* 2012; Rivera-Fernández *et al.*, 2019).

It could be compounded by the greater cell count in all infected mice, where the decrease in megakaryocytes has previously been associated to an anti-inflammatory reaction as well as

restriction of inflammatory cells recruitment onto damaged tissue (de Santana *et al.*, 2014). These effects in leishmaniasis-infected animals occur in tandem with the splenic suppression of megakaryocyte growth. The splenic potency of the test drugs need to be highlighted, as splenectomy was previously the last resort for antimony resistant leishmaniasis cases. Furthermore, the splenomegaly development and splenic load rise were shown to be considerably reduced in mice treated with Amphotericin B, *T. mollis and C. macrostachyus,* showing that parasite suppression and growth inhibition lasted for at least 7 to 8 weeks after the treatment was stopped. The findings suggested that *L. major* should be managed therapeutically to prevent adverse consequences associated with parasite disorders as previously reported (Makwali *et al.*, 2012).

This study found that in *L. major* infected mice, therapy with Amphotericin B, *T. mollis,* and *C. macrostachyus* resulted in the greatest reduction in LDU suggesting that the *Leishmania* parasite load reduction was enhanced by application of herbal drugs mainly *T. mollis* and *C. macrostachyus* as reported in other studies (Makwali *et al.*, 2012; Kaur and Kaur, 2018). Previously, A GC/MS quantitative study based on the peak area also indicated that the benzyl benzoate comprised 51.46 % of the oil of *Croton macrostachyus* followed by γ-muurolene (11.98 %), linalool (10.06%) and α-farnesene (3.21%). The oil contained phenololic esters (51.46%), sesquiterpene hydrocarbons (17.57%), oxygenated monoterpene (10.81 %), oxygenated sesqueterpenes (8.58%) and monoterpene hydrocarbons (1.09%) (Azizi *et al.*, 2016). Some identified constituent volatile oils from *C. macrostachyus* include Heptyl acetate, αTerpineol, 4-Terpineol, Isocaryophyllene, γ-Muurolene, αFarnesene, α-Cadinol and Benzyl benzoate, Alpha-pinene, βPinene, Linalool, and others (Kibret *et al.*, 2018; Abera *et al.*, 2019), which may account for some of the observed activity in reducing the parasite load.

5.3 Toxicity of extracts from *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha* on vero cells

Toxins and pharmaceuticals are excreted mostly through the liver. Because the liver has a high capacity for drug uptake, it is susceptible to poisoning.. Overall, the pathology of liver cells in infected mice treated with various test agents showed significant differences, indicating toxicity. In *L. major* parasitized mice treated with conventional drug and *T. mollis,* large sections of the liver showed normal physiology, indicating that Amphotericin B and *T. mollis* have low toxicity to macrophages and cells. Low toxicity of Amphotericin at appropriate doses has been previously observed (do Vale Morais *et al.,* 2018), mainly due to differing formulation modes. Antimonial treatment, on the other hand, has a number of negative consequences, including: pancreatitis, malaise, diarrhoea and cardiac arrhythmia (Das *et al.*, 2020) which means that even if they are of low toxicity to the cells, there is a need to search for safer alternatives.

Toxicity testing can reveal some of the risks that may be associated with the use of medicinal plants especially in sensitive populations. Several related plants from the genus Parinari have been assessed for toxicological activity in both in vitro and in vivo model systems. These plants were fairly were fairly of low toxicity. Generally, most species in the genus are nontoxic. Toxicological screening using some of the most important body cells such as red blood cells and immune cells is essential in determining the plant's safety profile.

Mild granulomas were more common in *L. major* treated with *T. mollis* that showed symptoms of mild toxicity. Exogenous toxicants that can damage cells induce cell toxicity, especially when the toxicant can cause cell death and significant organ malfunction. Cell toxicity has a wide range of mechanisms. It has long been established that toxicants can cause an excess of nitric oxide, reactive oxygen species, and oxidative stress (Sullivan and Weber,

2022). Among the most important mechanisms of severe cell toxicity or even organ malfunction has been recognized as a high amount of NO, ROS, and the following oxidative burst (Di Giulio *et al.*, 2020). Toxicity agents can also cause cell apoptosis and toxicity by inducing and releasing chemicals that directly harm DNA (Wang and Tang, 2020). Macrophages, the target cells in leishmaniasis therapy, produce cytokines and oxygen metabolites, which aid in the immune regulation of intracellular parasites (Sousa-Batista *et al.*, 2019a; Mendonça *et al.*, 2020). Treatment for leshmaniasis with *T. mollis* entails a single dose therapy, in which the metabolites are completely cleared four days after an oral dose, whereas treatment for *L. major* with Amphotericin B lasts up to 28 days as recommended, despite indicators of cytotoxicity in the inoculated and simultaneously medication administered group of mice (Makwali *et al.*, 2012).

Although similar trends were detected in *L. major* inoculated mice at the tenth week following therapy with standard drug, hepatic periovular granuloma from the one administered with *C. macrostachyus*, revealed presence of inflammatory cells and fibrous tissues. Generally, hepatic damage was moderate to severe in *L. major* inoculated mice that received Amphotericin B and *T. mollis* therapy. It's clear when you look at the histopathological changes and the worm numbers that have been achieved. On the Balb/c mice, the three herbal drugs (*T. mollis, C. microstachyus,* and *B. micrantha*) non toxic.

Inoculated mice treated with *B. micrantha* and *O. europeae* showed a number of negative consequences, indicating autophagy. This shows that bioactive compounds in extracts were more likely to affect host cells by triggering apoptosis in cells that had been stimulated to autophagy (Rahman *et al.*, 2020). Infected mice treated with Amphotericin B and *T. mollis,* on the other hand, showed mild to moderate pathological effects and no mortality, according to the assessment criteria.

5.4 Efficacy of plant extracts against *L. major* lesion development

Infection of the mice resulted in larger lesions in the therapy group in this study. Chemotherapy was used starting at week 5 after infection, and the formation of a lesion in BALB/c mice was seen. The medications utilized in this investigation were Amphotericin B, which is a standard drug. Normally, Pentostam is effective against the protozoan parasite *Leishmania major* (Khayeka–Wandabwa *et al.*, 2013), whereas first line drugs are effective against the *Leishmania major* (AbbasP, 2015). The Anti-leishmanial Pentavalent antimonial (Sb^V) is administered intramuscularly/intravenously in mammals and intraperitoneal administration is the preferred mode in murine model. With a average complete evident drug concentration of 0.22 0.057 L/kg/mass and a half-life of 2 hours, it is concentrated in the plasma, spleen, and liver (Monzote, 2009).

Antimonials have a complex mode of action that is currently unclear, various studies suggest that their anti-leishmanial effect is likely dependent on the conversion of SbV to a more lethal SbIII in animal model. As a result, only amastigotes are susceptible to the SbV, which affects the Leishmania parasite's energy metabolism by suppressing lipid catabolism, parasite glucolysis, and suppression of addition of phosphate group on ADP, according to a consensus among investigators (Bekhit *et al.*, 2018; Capela *et al.*, 2019).

Despite this, investigations in various mouse models, show that infection by *L. major* may be lethal if not treated. *L. major* has shown an increase in the extent of lesions and/or a failure to clear footpad lesions as quickly as mice, which correlates to a reduced ability to regulate parasitaemia even when other Leishmania species are present (Shibata *et al.*, 2012). Consequently, as demonstrated by murine model studies, aggravation of cutaneous leishmaniasis occurs. This study demonstrated the effectiveness of differential therapy of common anti-leishmania drugs. The drugs applied in BALB/c mice showed different

efficacies in their treatment of mice exemplified by reduced lesion sizes suggesting differential efficacies. Based on the study, *T. mollis* were considered the most effective drug in healing of *L. major* lesions. Also, when Amphotericin B was utilized to heal *L. major* disease in mice, the reduction in diameter of lesion was identical to that seen in BALB/c animals treated with *T. mollis*, implying that *T. mollis* has hiigher potency in in killing Leishmania parasites just like the pentavalent antomonials.

These drugs and *T. mollis* together with *C. microstachyus* confers better protection against lesion progression and may complements immune response in activity against the infections (Chakravarty and Sundar, 2019). Though further research is needed, having both arms of immunity functioning simultaneously is likely to account for the reported efficacies.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

First this study analyzed the phytochemical components of the of bark of *O. europaea, K. africana, T. mollis, C. macrostachyus* and *B. micrantha extracts* and established that they contain glycoside, terpenoids, tannins, flavonoids, steroids, and saponins. *T. mollis* contaned higher concentration of tannins, phenols, saponins, alkaloids, anthraquinone and terpenoids. *Kigelia africana* had higher concentraton of phenols, terpenoids and glycosides while *C. macrostachyus* showed higher abundance of alkaloids and saponins.

With regard to *L. major* promastigotes, different quantities of the concentrates from *O. europaea, K. africana, T. mollis, C. macrostachyus, B. micrantha,* and a conventional medication (Amphotericin B) had different activity. After 24 hours of exposure, crude extracts from the plants strongly inhibited promastogote and amastigote growth. The highly efficacious anti-promastigote was the standard drug (amphotericin B), then, *T. mollis*, while *O. europeae* recorded the lowest potency in terms of optimal efficacy among the crude extracts of the herbal remedies. The LDU of the *L. major* inoculated mice that was administered *T. mollis* as well as Amphotericin B showed significant differences, with *T. mollis* and Amphotericin B having the lowest LDU and *O. europeae* having the highest.

In the mice model that received therapy from crude extracts of various plant species plus a conventional medication, there were disparities in lesion development. The mice that received amphotericin B therapy, showed the greatest reduction in lesion size after four weeks. Therapy with *T. mollis*, on the other hand, resulted in a substantially higher reduction of wound in mice than treatment with extracts from other plants. After 4 weeks of treatment, *O. europeae* treated mice had the least reduction in lesion diameters of all the drugs

examined. The livers of *L. major* parasitized mice exhibited microscopic lesions. Same patterns were seen in *L. major* parasitized mice which received *B. micrantha* as well as *O. europeae* therapy. After 24 hours of exposure, The various test compounds significantly affected mammalian cell viability, with percentage cell viability estimates of mammalian cells based on the gradient analysis of logistic regression of the crude extracts of the herbal drugs, *B. micrantha,* and *O. europeae* having the greatest harmful consequences, with less than 50% cell viability.

6.2 Recommendations

1. Over all, the results obtained from the crude extracts screening, especially all extracts with Leshmania activities, suggest that these may be promising sources for the development of new drugs for controlling leishmaniasis
2. The study recommends that *T. mollis* in the treatment of leishmaniasis in locations where it is found. However, more research into the active components that alter the efficiency of plant extracts is recommended. Purification of active ingredients to be done with the goal of removing components that have cytotoxic properties.
3. Leishmaniasis being a global health problem, coupled with drug-resistance and the side effects caused by current drugs, makes it necessary to redouble efforts to continue investigating other medicinal plant species, in order to find active compounds that contribute to the treatment of the disease or that serve as prototype molecules to develop drugs with mechanism of actions that treat the disease
4. Further study on the identification of the active plant metabolites in the plant species responsible for both antipromastigote and antiamastigote inhibitory effect is suggested.
5. Evaluation of the plants methanolic leaf extracts in the treatment of visceral leishmaniasis in non human primates is of important value.

REFERENCES

AbbasP, Z.M. (2015). Efficacy of Amphotericine B Ddrug Against Promastigote and Axenic Amastigote of Leishmania Dononvani in Vitro. *Iraqi Journal of Science*. **56(3B)**: 2210-2217.

Abdelhaleem, A., Elamin, E., Bakheit, S. and Mukhtar, M. (2019). Identification and characterization of Leishmania amastigote and axenic form antigens. *Tropical Biomedicine*. **36(4)**: 866-873.

Abera, T., Ashebir, R. and Basha, H. (2019). Phytochemical-constituents, safety and efficacy of commonly used medicinal plants for the treatment of malaria in Ethiopia-a review. *Pharmacy and Pharmacology International Journal*. **7(6)**: 284-295.

Adeeyo, A.O., Odiyo, J. and Odelade, K. (2018). Chemical profiling and antimicrobial properties of phyto-active extracts from *Terminalia glaucescens* stem against water microbial contaminants. *The Open Biotechnology Journal*. **12(1)**: 12-16.

Adefuye, A.O. and Ndip, R.N. (2013). Phytochemical analysis and antibacterial evaluation of the ethyl acetate extract of the stem bark of *Bridelia micrantha*. *Pharmacognosy Magazine*. **9(33)**: 45-53.

Afolabi, C., Akinmoladun, Ibujun, E.O., Emmanuel, A., Obuotor, E.M. and Farombi, E. (2007). Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum*. *Scientific Research and Essays*. **2(5)**: 163-166.

Agresti, A. (2018). *An introduction to categorical data analysis. John Wiley & Sons.* John Wiley & Sons.

Aguiar, M.G., Silva, D.L., Nunan, F.A.n., Nunan, E.A., Fernandes, A.P. and Ferreira, L.A.n.M. (2009). Combined topical paromomycin and oral miltefosine treatment of mice experimentally infected with *Leishmania (Leishmania) major* leads to reduction in both lesion size and systemic parasite burdens. *Journal of Antimicrobial Chemotherapy*. **64(6)**: 1234-1240.

Ahmed, E.H., Abadi, R.S. and Mohammed, A.M. (2018). Phytochemical screening, chemical composition and antioxidant activity of seeds essential oil of *Coriandrum sativum* L. from the Sudan. *International Journal of Herbal Medicine*. **6(1)**: 1-4.

Ahmed, S.S., Prakash, K.C., Tabassum, S., Salma, N. and Devi, K.H. (2019). Pharmacognostical and Pharmacological Review on *Tridax procumbens* Linn. *Research Journal of Pharmacology and Pharmacodynamics*. **11(1)**: 11-16.

Akbari, B., Baghaei-Yazdi, N., Bahmaie, M. and Mahdavi Abhari, F. (2022). The role of plant-derived natural antioxidants in reduction of oxidative stress. *BioFactors*. **1(2)**: 23-24.

Alemayehu, B. and Alemayehu, M. (2017). Leishmaniasis: a review on parasite, vector and reservoir host. *Health Science Journal*. **11(4)**: 1-9.

Amadi, C.N. and Orisakwe, O.E. (2018). Herb-induced liver injuries in developing nations: An update. *Toxics*. **6(2)**: 24-29.

Amro, A. and Hamarsheh, O. (2021). *Epidemiology of Leishmaniasis in Palestine*.

Andrade, M.A., Azevedo, C.d.S., Motta, F.N., Santos, M.L.d., Silva, C.L., Santana, J.M.d. and Bastos, I. (2016). Essential oils: in vitro activity against *Leishmania amazonensis*, cytotoxicity and chemical composition. *BMC Complementary and Alternative Medicine*. **16(1)**: 1-8.

Angelova, N., Kong, H.W., Van Der Heijden, R., Yang, S.Y., Choi, Y.H., Kim, H.K., Wang, M., Hankemeier, T., Van Der Greef, J. and Xu, G. (2008). Recent methodology in the phytochemical analysis of ginseng. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*. **19(1)**: 2-16.

Antwi, A., Owusu, K., Amoa-Bosompem, M., Williams, N., Ayertey, F., Akyeh, L., Agyapong, J., Tetteh, T., Djameh, G. and Botchie, S. (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*. **20(2)**: 1-14.

Anywar, G., Kakudidi, E., Byamukama, R., Mukonzo, J., Schubert, A., Oryem-Origa, H. and Jassoy, C. (2021). A review of the toxicity and phytochemistry of medicinal plant species used by herbalists in treating people living with HIV/AIDS in Uganda. *Frontiers in Pharmacology*. **12(**615147.

Aoun, K., Kalboussi, Y., Sghaier, I.B., Souissi, O., Hammami, H., Bellali, H. and Bouratbine, A. (2020). Assessment of incubation period of cutaneous leishmaniasis due to *Leishmania major* in Tunisia. *The American Journal of Tropical Medicine and Hygiene*. **103(5)**: 1934.

Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E.M., Ephros, M., Jeronimo, S. and Magill, A. (2016). Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clinical Infectious Diseases*. **63(12)**: e202-e264.

Aronson, N.E. and Joya, C.A. (2019). Cutaneous leishmaniasis: updates in diagnosis and management. *Infectious Disease Clinics*. **33(1)**: 101-117.

Asthana, S., Gupta, P.K., Chaurasia, M., Dube, A. and Chourasia, M.K. (2013). Polymeric colloidal particulate systems: intelligent tools for intracellular targeting of antileishmanial cargos. *Expert opinion on Drug Delivery*. **10(12)**: 1633-1651.

Atawodi, S.E.-O. and Olowoniyi, O.D. (2015). Pharmacological and therapeutic activities of *Kigelia africana* (Lam.) Benth. *Annual Research and Review in Biology*. **5(1)**: 1-17.

Azizi, K., Shahidi-Hakak, F., Asgari, Q., Hatam, G.R., Fakoorziba, M.R., Miri, R. and Moemenbellah-Fard, M.D. (2016). In vitro efficacy of ethanolic extract of *Artemisia absinthium* (Asteraceae) against *Leishmania major* L. using cell sensitivity and flow cytometry assays. *Journal of Parasitic Diseases*. **40(3)**: 735-740.

Azmir, J., Zaidul, I., Rahman, M., Sharif, K., Mohamed, A., Sahena, F., Jahurul, M., Ghafoor, K., Norulaini, N. and Omar, A. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of food engineering*. **117(4)**: 426-436.

Bahmani, M., Saki, K., Ezatpour, B., Shahsavari, S., Eftekhari, Z., Jelodari, M., Rafieian-Kopaei, M. and Sepahvand, R. (2015). Leishmaniosis phytotherapy: Review of plants used in Iranian traditional medicine on leishmaniasis. *Asian Pacific Journal of Tropical Biomedicine*. **5(9)**: 695-701.

Barbosa Gomes de Carvalho, Y.M., Shanmugam, S., Batista, M.S., Serafini, M.R., Araújo, A.A.d.S. and Quintans Júnior, L.J. (2020). Pharmaceutical agents for treatment of leishmaniasis: a patent landscape. *Expert Opinion on Therapeutic Patents*. **12(2)**: 34-43.

Barrett, M.P., Kyle, D.E., Sibley, L.D., Radke, J.B. and Tarleton, R.L. (2019). Protozoan persister-like cells and drug treatment failure. *Nature Reviews Microbiology*. **17(10)**: 607-620.

Bashir, S., Alam, M., Adhikari, A., Shrestha, R.L.S., Yousuf, S., Ahmad, B., Parveen, S., Aman, A. and Choudhary, M.I. (2014). New antileishmanial sesquiterpene coumarins from *Ferula narthex* Boiss. *Phytochemistry Letters*. **9(**46-50.

Bates, P.A. (2018). Revising Leishmania's life cycle. *Nature Microbiology*. **3(5)**: 529-530.

Beach, D.H., Goad, L.J. and Holz Jr, G.G. (1988). Effects of antimycotic azoles on growth and sterol biosynthesis of *Leishmania promastigotes*. *Molecular and Biochemical Parasitology*. **31(2)**: 149-162.

Bekhit, A.A., El-Agroudy, E., Helmy, A., Ibrahim, T.M., Shavandi, A. and Bekhit, A.E.-D.A. (2018). Leishmania treatment and prevention: Natural and synthesized drugs. *European Journal of Medicinal Chemistry*. **160(2)**: 229-244.

Bello, I., Shehu, M.W., Musa, M., Asmawi, M.Z. and Mahmud, R. (2016). *Kigelia africana* (Lam.) Benth.(Sausage tree): Phytochemistry and pharmacological review of a quintessential African traditional medicinal plant. *Journal of Ethnopharmacology*. **189(1)**: 253-276.

Benzie, I.F. and Devaki, M. (2018). The ferric reducing/antioxidant power (FRAP) assay for non-enzymatic antioxidant capacity: concepts, procedures, limitations and applications. *Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications*. **21(**77-106.

Bereksi, M.S., Hassaïne, H., Bekhechi, C. and Abdelouahid, D.E. (2018). Evaluation of antibacterial activity of some medicinal plants extracts commonly used in Algerian traditional medicine against some pathogenic bacteria. *Pharmacognosy Journal*. **10(3)**: 507-512.

Berman, J.D. and Lee, L.S. (1984). Activity of antileishmanial agents against amastigotes in human monocyte-derived macrophages and in mouse peritoneal macrophages. *The Journal of Parasitology*: 220-225.

Beshbishy, A.M., Batiha, G.E.-S., Adeyemi, O.S., Yokoyama, N. and Igarashi, I. (2019). Inhibitory effects of methanolic Olea europaea and acetonic Acacia laeta on growth of Babesia and Theileria. *Asian Pacific Journal of Tropical Medicine*. **12(9)**: 425.

Bettaieb, J., Toumi, A., Ghawar, W., Chlif, S., Nouira, M., Belhaj-Hamida, N., Gharbi, A., Ben-Alaya, N., Laouini, D. and Louzir, H. (2020). A prospective cohort study of cutaneous Leishmaniasis due to *Leishmania major*: Dynamics of the Leishmanin skin test and its predictive value for protection against infection and disease. *PLoS neglected Tropical Diseases*. **14(8)**: e0008550.

Bewick, V., Cheek, L. and Ball, J. (2005). Statistics review 14: Logistic regression. *Critical Care*. **9(1)**: 1-7.

Bhunia, G.S. and Shit, P.K. (2020). *Introduction of Visceral Leishmaniasis (Kala-azar), Spatial Mapping and Modelling for Kala-azar Disease*. Springer. pp. 1-18.

Bi, K., Chen, Y., Zhao, S., Kuang, Y. and John Wu, C.-H. (2018). Current visceral leishmaniasis research: a research review to inspire future study. *BioMed Research International*: Volume 2018 | Article ID 9872095 | https://doi.org/10.1155/2018/9872095.

Brito, N.C., Rabello, A. and Cota, G.F. (2017). Efficacy of pentavalent antimoniate intralesional infiltration therapy for cutaneous leishmaniasis: A systematic review. *PloS one*. **12(9)**: e0184777.

Cabrera-Serra, M.G., Valladares, B. and Piñero, J.E. (2008). In vivo activity of perifosine against *Leishmania amazonensis*. *Acta Tropica*. **108 (1)**: 20-25.

Capela, R., Moreira, R. and Lopes, F. (2019). An Overview of Drug Resistance in Protozoal Diseases. *International Journal of Molecular Sciences*. **20(22)**: 5748-5754.

Carnielli, J.B., Crouch, K., Forrester, S., Silva, V.C., Carvalho, S.F., Damasceno, J.D., Brown, E., Dickens, N.J., Costa, D.L. and Costa, C.H. (2018). A Leishmania infantum genetic marker associated with miltefosine treatment failure for visceral leishmaniasis. *EBioMedicine*. **36(**83-91.

Casulli, A. (2021). New global targets for NTDs in the WHO roadmap 2021–2030. Public Library of Science San Francisco, CA USA, pp. e0009373.

Cavalcante, F.R.A., Cavalcante, K.K.d.S., Florencio, C.M.G.D., Moreno, J.d.O., Correia, F.G.S. and Alencar, C.H. (2020). Human visceral leishmaniasis: epidemiological, temporal and spacial aspects in Northeast Brazil, 2003-2017. *Revista do Instituto de Medicina Tropical de São Paulo*. **62(**22-29.

Chakravarty, J., Hasker, E., Kansal, S., Singh, O.P., Malaviya, P., Singh, A.K., Chourasia, A., Singh, T., Sudarshan, M. and Singh, A.P. (2019). Determinants for progression from asymptomatic infection to symptomatic visceral leishmaniasis: A cohort study. *PLoS Neglected Tropical Diseases*. **13(3)**: e0007216.

Chakravarty, J. and Sundar, S. (2019). Current and emerging medications for the treatment of leishmaniasis. *Expert Opinion on Pharmacotherapy*. **20(10)**: 1251-1265.

Chang, Z., Zhang, Q., Liang, W., Zhou, K., Jian, P., She, G. and Zhang, L. (2019). A comprehensive review of the structure elucidation of Tannins from *Terminalia* Linn. *Evidence-Based Complementary and Alternative Medicine*. **2019(**129-138.

Chattopadhyay, A. and Jafurulla, M. (2011). A novel mechanism for an old drug: amphotericin B in the treatment of visceral leishmaniasis. *Biochemical and Biophysical Research Communications*. **416(1-2)**: 7-12.

Chávez-Fumagalli, M.A., Ribeiro, T.G., Castilho, R.O., Fernandes, S.O.A., Cardoso, V.N., Coelho, C.S.P., Mendonça, D.V.C., Soto, M., Tavares, C.A.P. and Faraco, A.A.G. (2015). New delivery systems for amphotericin B applied to the improvement of leishmaniasis treatment. *Revista da Sociedade Brasileira de Medicina Tropical*. **48(**235-242.

Chen, G. and Chen, H. (2011). Extraction and deglycosylation of flavonoids from sumac fruits using steam explosion. *Food chemistry*. **126(4)**: 1934-1938.

Chen, J., Shen, Y., Chen, C. and Wan, C. (2019). Inhibition of key citrus postharvest fungal strains by plant extracts in vitro and in vivo: A review. *Plants*. **8(2)**: 26.

Cheurfa, M., Abdallah, H., Allem, R., Noui, A., Picot-Allain, C. and Mahomoodally, F. (2019). Hypocholesterolaemic and antioxidant properties of *Olea europaea* L. leaves from Chlef province, Algeria using in vitro, in vivo and in silico approaches. *Food and Chemical Toxicology*. **123(**98-105.

Chouhan, G., Islamuddin, M., Sahal, D. and Afrin, F. (2014). Exploring the role of medicinal plant-based immunomodulators for effective therapy of leishmaniasis. *Frontiers in Immunology*. **5(**193.

Coria-Téllez, A.V., Montalvo-Gónzalez, E., Yahia, E.M. and 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.

Corral-Caridad, M.J., Moreno, I., Toraño, A., Domínguez, M. and Alunda, J.M. (2012). Effect of allicin on promastigotes and intracellular amastigotes of *Leishmania donovani* and *L. infantum*. *Experimental Parasitology*. **132(4)**: 475-482.

Cortes, S., Bruno de Sousa, C., Morais, T., Lago, J., and Campino, L. (2020). Potential of the natural products against leishmaniasis in Old World-a review of in-vitro studies. *Pathogens and Global Health*. **114(4)**: 170-182.

Costa, R., Albergamo, A., Pellizzeri, V. and Dugo, G. (2017). Phytochemical screening by LC-MS and LC-PDA of ethanolic extracts from the fruits of *Kigelia africana* (Lam.) Benth. *Natural Product Research*. **31(12)**: 1397-1402.

Cotton, J.A. (2017). The expanding world of human leishmaniasis. *Trends in Parasitology*. **33(5)**: 341-344.

Cruz, I., Millet, A., Carrillo, E., Chenik, M., Salotra, P., Verma, S., Veland, N., Jara, M., Adaui, V. and Castrillón, C. (2013). An approach for interlaboratory comparison of conventional and real-time PCR assays for diagnosis of human leishmaniasis. *Experimental Parasitology*. **134(3)**: 281-289.

Cunze, S., Kochmann, J., Koch, L.K., Hasselmann, K.J. and Klimpel, S. (2019). Leishmaniasis in Eurasia and Africa: geographical distribution of vector species and pathogens. *Royal Society Open Science*. **6(5)**: e190334.

Da Silva, B.J.M., Hage, A.A.P., Silva, E.O. and Rodrigues, A.P.D. (2018). Medicinal plants from the Brazilian Amazonian region and their antileishmanial activity: a review. *Journal of Integrative Medicine*. **16(4)**: 211-222.

da Silva, D.A., de Fátima Madeira, M., Abrantes, T.R., de Lima Barbosa Filho, C.J. and Figueiredo, F.B. (2013). Assessment of serological tests for the diagnosis of canine visceral leishmaniasis. *The Veterinary Journal*. **195(2)**: 252-253.

da Silva, E.R., Brogi, S., Grillo, A., Campiani, G., Gemma, S., Vieira, P.C. and Maquiaveli, C.d.C. (2019). Cinnamic acids derived compounds with antileishmanial activity target *Leishmania amazonensis* arginase. *Chemical Biology and Drug Design*. **93(2)**: 139-146.

da Silveira Regueira-Neto, M., Tintino, S.R., Rolón, M., Coronal, C., Vega, M.C., de Queiroz Balbino, V. and de Melo Coutinho, H.D. (2018). Antitrypanosomal, antileishmanial and cytotoxic activities of Brazilian red propolis and plant resin of *Dalbergia ecastaphyllum* (L) Taub. *Food and Chemical Toxicology*. **119(**215-221.

Das, A., Jawed, J.J., Das, M.C., Parveen, S., Ghosh, C., Majumdar, S., Saha, B. and Bhattacharjee, S. (2020). Lupeol and amphotericin B mediate synergistic anti-leishmanial immunomodulatory effects in *Leishmania donovani*-infected BALB/c mice. *Cytokine*. **137(**155319.

de Melo, J.O., Bitencourt, T.A., Fachin, A.L., Cruz, E.M.O., de Jesus, H.C.R., Alves, P.B., de Fátima Arrigoni-Blank, M., de Castro Franca, S., Beleboni, R.O. and Fernandes, R.P.M. (2013). Antidermatophytic and antileishmanial activities of essential oils from *Lippia gracilis* Schauer genotypes. *Acta Tropica*. **128(1)**: 110-115.

de Menezes, J.P.B., Guedes, C.E.S., Petersen, A.L.d.O.A., Fraga, D.B.M. and Veras, P.S.T. (2015). Advances in development of new treatment for leishmaniasis. *BioMed Research International*. **2015(**Volume 2015 | Article ID 815023 | https://doi.org/10.1155/2015/815023.

de Santana, F.R., de Paula Coelho, C., Cardoso, T.N., Hurtado, E.C.P., Benites, N.R., Laurenti, M.D. and Bonamin, L.V. (2014). Modulation of inflammation response to murine cutaneous Leishmaniasis by homeopathic medicines: Antimonium crudum 30cH. *Homeopathy*. **103(4)**: 264-274.

Dekebo, A. (2019). *Introductory Chapter: Plant Extracts*, *Plant Extracts*. IntechOpen.

Delorenzi, J.C., Attias, M., Gattass, C.R., Andrade, M., Rezende, C., da Cunha Pinto, Â., Henriques, A.T., Bou-Habib, D.C. and Saraiva, E.M. (2001). Antileishmanial activity of an indole alkaloid from *Peschiera australis*. *Antimicrobial Agents and Chemotherapy*. **45(5)**: 1349-1354.

Di Giulio, R., Benson, W., Sanders, B. and Van Veld, P. (2020). *Biochemical mechanisms: metabolism, adaptation, and toxicity*, *Fundamentals of aquatic toxicology*. CRC Press. pp. 523-561.

Difonzo, G., Russo, A., Trani, A., Paradiso, V.M., Ranieri, M., Pasqualone, A., Summo, C., Tamma, G., Silletti, R. and Caponio, F. (2017). Green extracts from Coratina olive cultivar leaves: Antioxidant characterization and biological activity. *Journal of Functional Foods*. **31(**63-70.

Dimas, K., Said, J., Sani, A. and Shuaibu, T. (2020). In vitro Antifungal, Antibacterial and Cytotoxicity Studies of Solvents Extracts of Some Medicinal Plants (*Echinaceae angustifolia*, *Myosotis scorpioides*, *Detarium microcarpum* and *Terminalia mollis*) collected in Girei Adamawa State–Nigeria. *FUDMA Records of Chemical Sciences*. **1(3)**: 19-27.

Dinesh, N., Neelagiri, S., Kumar, V. and Singh, S. (2017). Glycyrrhizic acid attenuates growth of *Leishmania donovani* by depleting ergosterol levels. *Experimental Parasitology*. **176(**21-29.

Djouwoug, C.N., Gounoue, R.K., Ngueguim, F.T., NankapTsakem, J.M., Gouni, C.D., Kandeda, A.C., Ngouela, S., Lenta, B.N., Sewald, N. and Fekam, F.B. (2020). In vitro and in vivo antiplasmodial activity of hydroethanolic bark extract of Bridelia atroviridis müll. Arg.(Euphorbiaceae) and lc-ms-based phytochemical analysis. *Journal of Ethnopharmacology*. **266(**113424.

do Nascimento, N.R.F., de Aguiar, F.L.N., Santos, C.F., Costa, A.M.L., de Jesus Hardoim, D., da Silva Calabrese, K., Almeida-Souza, F., de Sousa, E.H.S., de França Lopes, L.G. and Teixeira, M.J. (2019). In vitro and in vivo leishmanicidal activity of a ruthenium nitrosyl complex against *Leishmania (Viannia) braziliensis*. *Acta Tropica*. **192(**61-65.

do Vale Morais, A.R., Silva, A.L., Cojean, S., Balaraman, K., Bories, C., Pomel, S., Barratt, G., do Egito, E.S.T. and Loiseau, P.M. (2018). In-vitro and in-vivo antileishmanial activity of inexpensive Amphotericin B formulations: Heated Amphotericin B and Amphotericin B-loaded microemulsion. *Experimental parasitology*. **192(**85-92.

Douglas, K. and Gitonga, A. (2016). Antimicrobial activity of *Bridelia micrantha* and *Grewia plagiophylla* leaf extracts. *Journal of Pharmaceutical Research International*. **12(3)**: 1-7.

Dulacha, D., Mwatha, S., Lomurukai, P., Owiny, M.O., Matini, W., Irura, Z., Odhiambo, F., Waititu, T., Matoke-Muhia, D. and Njenga, D. (2019). Epidemiological characteristics and factors associated with Visceral Leishmaniasis in Marsabit County, Northern Kenya. *Journal of Interventional Epidemiology and Public Health*. **2(3)**: 1-14.

Dzah, C.S., Duan, Y., Zhang, H., Boateng, N.A.S. and Ma, H. (2020). Latest developments in polyphenol recovery and purification from plant by-products: A review. *Trends in Food Science and Technology*. **99(**375-388.

Ebiloma, G.U., Igoli, J.O., Katsoulis, E., Donachie, A.-M., Eze, A., Gray, A.I. and De Koning, H.P. (2017). Bioassay-guided isolation of active principles from Nigerian medicinal plants identifies new trypanocides with low toxicity and no cross-resistance to diamidines and arsenicals. *Journal of Ethnopharmacology*. **202(**256-264.

Eddaikra, N., Boudjelal, A., Amine Sbabdji, M., Eddaikra, A., Boudrissa, A., Mounir Bouhenna, M., Chemat, S. and Harrat, Z. (2019). Leishmanicidal and cytotoxic activity of Algerian Medicinal plants on *Leishmania major* and *Leishmania infantum*. *Journal of Medical Microbiology and Infectious Diseases*. **7(3)**: 66-71.

Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in Pharmacology*. **4(**177-184.

Eksi, G., Kurbanoglu, S. and Erdem, S.A. (2020). *Analysis of diterpenes and diterpenoids*, *Recent Advances in Natural Products Analysis*. Elsevier. pp. 313-345.

El Aziz, M., Ashour, A. and Melad, A. (2019). A review on saponins from medicinal plants: chemistry, isolation, and determination. *Journal of Nanomedicine Research*. **8(1)**: 282-288.

El, S.N. and Karakaya, S. (2009). Olive tree (*Olea europaea*) leaves: potential beneficial effects on human health. *Nutrition Reviews*. **67(11)**: 632-638.

Elgorashi, E.E. and McGaw, L.J. (2019). African plants with in vitro anti-inflammatory activities: A review. . *South African Journal of Botany*. **126(**142-169.

Eskandari, E.G., Setorki, M. and Doudi, M. (2020). Medicinal plants with antileishmanial properties: A review study. *Pharmaceutical and Biomedical Research*. **6(1)**: 1-16.

Et-Touys, A., Hajiba Fellah, Meryem Mniouil, A. Bouyahya, N. Dakka, E. H. Abdennebi, A. Sadak and Bakri, Y. (2016). Screening of antioxidant, antibacterial and antileishmanial activities of *Salvia officinalis* L. extracts from Morocco. *Brazilian Microbiological Research Journal*. **16(**1-10.

Ezatpour, B., Saedi Dezaki, E., Mahmoudvand, H., Azadpour, M. and Ezzatkhah, F. (2015). In vitro and in vivo antileishmanial effects of *Pistacia khinjuk* against *Leishmania tropica* and *Leishmania major*. *Evidence-Based Complementary and Alternative Medicine*. **2015(**Volume 2015 | Article ID 149707 | https://doi.org/10.1155/2015/149707.

Fahmy, N., Al-Sayed, E. and Singab, A. (2015). Genus Terminalia: A phytochemical and biological review. *Medicinal and Aromatic Plants*. **4(5)**: 1-22.

Falode, J.A., Akinmoladun, A.C., Olaleye, M.T. and Akindahunsi, A.A. (2020). *Kigelia africana* (Lam.) Benth leaf extract inhibits rat brain and liver mitochondrial membrane permeability transition pore opening. *Drug and Chemical Toxicology*: 1-11.

Fanoudi, S., Rakhshandeh, H., Afshari, A.R., Mollazadeh, H. and Taher, M. (2017). Nephrotoxicity and hepatotoxicity of capparis spinosahydro-alcoholic extract in mice. *JOJ Uro & Nephron*. **4(2)**: 555638.

Farag, R.S., Abdel-Latif, M.S., Abd El Baky, H.H. and Tawfeek, L.S. (2020). Phytochemical screening and antioxidant activity of some medicinal plants' crude juices. *Biotechnology Reports* **28(**e00536.

Farash, B.R.H., Shamsian, S.A.A., Mohajery, M., Fata, A., Sadabadi, F., Berenji, F., Mastroeni, P., Poustchi, E., Moghaddas, E. and Sangani, G.S. (2020). Changes in the epidemiology of cutaneous leishmaniasis in northeastern Iran. *Turkiye Parazitolojii Dergisi*. **44(1)**: 52-57.

Faucher, J.-F., Morquin, D., Reynes, J., Chirouze, C., Hoen, B. and Le Moing, V. (2016). Serial use of pentamidine and miltefosine for treating *Leishmania infantum*-HIV coinfection. *Parasitology International*. **65(5)**: 444-446.

Felix-Cuencas, L., Delis-Hechavarria, E., Jarro, A., Parola-Contreras, I., Escamilla-García, A., Torres-Pacheco, I., García-Trejo, J.F., Soto-Zarazúa, G.M. and Guevara-González, R.G. (2022). *Bioactivity characterization of herbal molecules, Herbal Biomolecules in Healthcare Applications*. Elsevier. pp. 145-183.

Feres, M.V.C. (2019). Neglected and non-neglected diseases: contradictions in the patent legal system. *Revista Jurídica da Presidência*. **21(124)**: 264-288.

Flaih, M.H. (2022). *Geographical Distribution of Cutaneous Leishmaniasis and Pathogenesis, Leishmaniasis-General aspects of a stigmatized disease*. IntechOpen.

Fonseca-Silva, F., Inacio, J.D., Canto-Cavalheiro, M.M. and Almeida-Amaral, E.E. (2013). Reactive oxygen species production by quercetin causes the death of *Leishmania amazonensis* intracellular amastigotes. *Journal of Natural Products*. **76(8)**: 1505-1508.

Galluzzi, L., Ceccarelli, M., Diotallevi, A., Menotta, M. and Magnani, M. (2018). Real-time PCR applications for diagnosis of leishmaniasis. *Parasites and Vectors*. **11(1)**: 1-13.

Galvis, M.C.R., Franco, J.E.P., Vargas, M.Y.C. and Rubiano, M.F.O. (2020). Effectiveness and safety of amphotericin B deoxycholate, amphotericin B colloidal dispersion, and liposomal amphotericin B as third-line treatments for cutaneous and mucocutaneous leishmaniasis: a retrospective study. *The American journal of tropical medicine and hygiene*. **102(2)**: 274.

Garrido-Jareño, M., Sahuquillo-Torralba, A., Chouman-Arcas, R., Castro-Hernández, I., Molina-Moreno, J.M., Llavador-Ros, M., Gómez-Ruiz, M.D., López-Hontangas, J.L., Botella-Estrada, R. and Salavert-Lleti, M. (2020). Cutaneous and mucocutaneous leishmaniasis: experience of a Mediterranean hospital. *Parasites and Vectors*. **13(1)**: 1-7.

Ghatee, M.A., Taylor, W.R. and Karamian, M. (2020). The geographical distribution of cutaneous leishmaniasis causative agents in iran and its neighboring countries, a review. *Frontiers in Public Health*. **8:11 doi: 10.3389/fpubh.2020.00011(**

Ghodsian, S., Taghipour, N., Deravi, N., Behniafar, H. and Lasjerdi, Z. (2020). Recent researches in effective antileishmanial herbal compounds: narrative review. *Parasitology Research*. **119(12)**: 3929-3946.

Ghorbani, M. and Farhoudi, R. (2018). Leishmaniasis in humans: drug or vaccine therapy? *Drug design, development and therapy*. **12(**25-35.

Giovanny, H., Natalia, B., Luna, N., Martínez, D., Medina, J., Niño, S., Luisa, P., Ramírez, A., Vega, L. and Valeria, V. (2020). An interactive database of Leishmania species distribution in the Americas. *Scientific Data*. **7(1)**.

Giraud, E., Martin, O., Yakob, L. and Rogers, M. (2019). Quantifying Leishmania metacyclic promastigotes from individual sandfly bites reveals the efficiency of vector transmission. *Communications Biology*. **2(1)**: 1-12.

Gonçalves, G.A., Spillere, A.R., das Neves, G.M., Kagami, L.P., von Poser, G.L., Canto, R.F.S. and Eifler-Lima, V. (2020). Natural and synthetic coumarins as antileishmanial agents: A review. *European Journal of Medicinal Chemistry*. **203(**112514.

Gonelimali, F.D., Lin, J., Miao, W., Xuan, J., Charles, F., Chen, M. and Hatab, S.R. (2018). Antimicrobial properties and mechanism of action of some plant extracts against food pathogens and spoilage microorganisms. *Frontiers in Microbiology*. **9(**1639.

Gouri, V., Upreti, S. and Samant, M. (2022). Evaluation of target-specific natural compounds for drug discovery against Leishmaniasis. *Parasitology International*. **2(1)**: 102622.

Gouws, C. and Hamman, J.H. (2020). What are the dangers of drug interactions with herbal medicines? . *Expert Opinion on Drug Metabolism and Toxicology*. **16 (3)**: 165-167.

Guery, R., Henry, B., Martin-Blondel, G., Rouzaud, C., Cordoliani, F., Harms, G., Gangneux, J.-P., Foulet, F., Bourrat, E. and Baccard, M. (2017). Liposomal amphotericin B in travelers with cutaneous and muco-cutaneous leishmaniasis: Not a panacea. *PLoS Neglected Tropical Diseases*. **11(11)**: e0006094.

Guinda, Á., Castellano, J.M., Santos-Lozano, J.M., Delgado-Hervás, T., Gutiérrez-Adánez, P. and Rada, M. (2015). Determination of major bioactive compounds from olive leaf. *LWT-Food Science and Technology*. **64(1)**: 431-438.

Guldiken, B., Ozkan, G., Catalkaya, G., Ceylan, F.D., Yalcinkaya, I.E. and Capanoglu, E. (2018). Phytochemicals of herbs and spices: Health versus toxicological effects. . *Food and Chemical Toxicology*. **119(**37-49.

Gurnani, N., Gupta, M., Mehta, D. and Mehta, B.K. (2016). Chemical composition, total phenolic and flavonoid contents, and in vitro antimicrobial and antioxidant activities of crude extracts from red chilli seeds (*Capsicum frutescens* L.). *Journal of Taibah University for Science*. **10(4)**: 462-470.

Gutiérrez-Rebolledo, G.A., Drier-Jonas, S. and Jiménez-Arellanes, M.A. (2017). Natural compounds and extracts from Mexican medicinal plants with anti-leishmaniasis activity: An update. *Asian Pacific Journal of Tropical Medicine*. **10(12)**: 1105-1110.

Habtom, S. and Gebrehiwot, S. (2019). In vitro antimicrobial activities of crude extracts of *Vernonia amygdalina* and Croton macrostachyus against some bacterial and fungal test pathogens. *Journal of Phytopharmacology*. **8(2)**: 57-62.

Hailu, A., Dagne, D.A. and Boelaert, M. (2016). *Leishmaniasis, Neglected Tropical Diseases-Sub-Saharan Africa*. Springer. pp. 87-112.

Hajimehdipoor, H., Esmaeili, S., Shekarchi, M., Emrarian, T. and Naghibi, F. (2013). Investigation of some biologic activities of Swertia longifolia Boiss. *Research in Pharmaceutical Sciences*. **84(4)**: 253.

Hajimehdipoor, H., Shahrestani, R. and Shekarchi, M. (2014). Investigating the synergistic antioxidant effects of some flavonoid and phenolic compounds. *Research Journal of Pharmacognosy*. **1(3)**: 35-40.

Handler, M.Z., Patel, P.A., Kapila, R., Al-Qubati, Y. and Schwartz, R.A. (2015). Cutaneous and mucocutaneous leishmaniasis: differential diagnosis, diagnosis, histopathology, and management. *Journal of the American Academy of Dermatology*. **73(6)**: 911-926.

Hao, Y., Hu, X., Gong, Y., Xue, J., Zhou, Z., Li, Y., Wang, Q., Zhang, Y. and Li, S. (2021). Spatio-temporal clustering of mountain-type zoonotic visceral leishmaniasis in China between 2015 and 2019. *PLoS Neglected Tropical Diseases*. **15(3)**: e0009152.

Hashiguchi, Y., Cáceres, A.G., Velez, L.N., Villegas, N.V., Hashiguchi, K., Mimori, T., Uezato, H. and Kato, H. (2018). Andean cutaneous leishmaniasis (Andean-CL, uta) in Peru and Ecuador: the vector *Lutzomyia* sand flies and reservoir mammals. *Acta tropica*. **178(**264-275.

Hashmi, M.A., Khan, A., Hanif, M., Farooq, U. and Perveen, S. (2015). Traditional uses, phytochemistry, and pharmacology of *Olea europaea* (olive). *Evidence-Based Complementary and Alternative Medicine*. **Volume 2015 | Article ID 541591 | https://doi.org/10.1155/2015/541591(**

Heidari-Kharaji, M., Badirzadeh, A., Khadir, F., Soori, M. and Nilforoushzadeh, M.A. (2016). Herbal drugs with promising anti-leishmanial activity: new hope for leishmaniasis treatment. *Journal of Skin and Stem Cell*. **3(2)**: 12-20.

Hendrickx, S., Van Bockstal, L., Caljon, G. and Maes, L. (2019). In-depth comparison of cell-based methodological approaches to determine drug susceptibility of visceral Leishmania isolates. *PLoS Neglected Tropical Diseases*. **13(12)**: e0007885.

Hernández-Bojorge, S.E., Blass-Alfaro, G.G., Rickloff, M.A., Gómez-Guerrero, M.J. and Izurieta, R. (2020). Epidemiology of cutaneous and mucocutaneous leishmaniasis in Nicaragua. *Parasite Epidemiology and Control*. **11(**e00192.

Hide, M., Singh, R., Kumar, B., Banuls, A.L. and Sundar, S. (2007). A microculture technique for isolating live Leishmania parasites from peripheral blood of visceral leishmaniasis patients. *Acta Tropica*. **102(3)**: 197-200.

Ibarra-Meneses, A.V., Moreno, J. and Carrillo, E. (2020). New strategies and biomarkers for the control of visceral leishmaniasis. *Trends in Parasitology*. **36(1)**: 29-38.

Inceboz, T. (2019). *Epidemiology and Ecology of Leishmaniasis, Current Topics in Neglected Tropical Diseases*. IntechOpen.

Ingole, V.V., Mhaske, P.C. and Katade, S.R. (2021). Phytochemistry and pharmacological aspects of *Tridax procumbens* (L.): A systematic and comprehensive review. *Phytomedicine Plus*. **2(1)**: 100199.

Iorizzo, M., Ellison, S., Senalik, D., Zeng, P., Satapoomin, P., Huang, J., Bowman, M., Iovene, M., Sanseverino, W., Cavagnaro, P. and Yildiz, M. (2016). A high-quality carrot genome assembly provides new insights into carotenoid accumulation and asterid genome evolution. . *Nature Genetics*. **48(6)**: 657-666.

Iqbal, H., Ishfaq, M., Wahab, A., Abbas, M.N., Ahmad, I., Rehman, A. and Zakir, M. (2016). Therapeutic modalities to combat leishmaniasis, a review. *Asian Pacific Journal of Tropical Disease*. **6(1)**: 1-5.

Islamuddin, M., Farooque, A., Dwarakanath, B.S., Sahal, D. and Afrin, F. (2012). Extracts of *Artemisia annua* leaves and seeds mediate programmed cell death in *Leishmania donovani*. *Journal of Medical Microbiology*. **61(12)**: 1709-1718.

Ismail, H.F., Hashim, Z., Soon, W.T., Ab Rahman, N.S., Zainudin, A.N. and Majid, F.A.A. (2017). Comparative study of herbal plants on the phenolic and flavonoid content, antioxidant activities and toxicity on cells and zebrafish embryo. *Journal of Traditional and Complementary Medicine*. **7(4)**: 452-465.

Ivanović, M., Islamčević Razboršek, M. and Kolar, M. (2020). Innovative extraction techniques using deep eutectic solvents and analytical methods for the isolation and characterization of natural bioactive compounds from plant material. *Plants*. **9(11)**: 1428-1433.

Jagadesh, S., Combe, M., Ginouvès, M., Simon, S., Prévot, G., Couppié, P., Nacher, M. and Gozlan, R.E. (2021). Spatial variations in Leishmaniasis: A biogeographic approach to mapping the distribution of *Leishmania* species. *One Health*. **13(**100307.

Jain, K. and Jain, N.K. (2013). Novel therapeutic strategies for treatment of visceral leishmaniasis. *Drug Discovery Today*. **18(23-24)**: 1272-1281.

Jambwa, P. and Nyahangare, E.T. (2020). *Ethnoveterinary Medicine: A Zimbabwean Perspective*, *Ethnoveterinary Medicine*. Springer. pp. 269-283.

Jha, A.K. and Sit, N. (2021). Extraction of bioactive compounds from plant materials using combination of various novel methods: A review. *Trends in Food Science and Technology*. **119(**579-591.

Kahraman, C., Arituluk, Z.C., Irem, I. and Cankaya, T. (2020). The clinical importance of herb-drug interactions and toxicological risks of plants and herbal products *Medical Toxicology*. **1(1-31)**.

Kakooei, Z., Hajjaran, H., Akhoundi, B., Charehdar, S., Elikaee, S., Shafeghat, Z., Hassanpour, H., Satvat, M.T., Kazemi-Rad, E. and Mohebali, M. (2020). Treatment Failure in Cutaneous Leishmaniasis Patients Referred to the School of Public Health, Tehran University of Medical Sciences during 2008–2017. *Journal of Arthropod-Borne Diseases*. **14(4)**: 363-371.

Kamaraj, C., Rahuman, A.A., Mahapatra, A., Bagavan, A. and Elango, G. (2010). Insecticidal and larvicidal activities of medicinal plant extracts against mosquitoes. *Parasitology Research*. **107(6)**: 1337-1349.

Kamau, P.K., Njeruh, F.M. and Thuita, J. (2020). In vitro antiplasmodial, cytotoxicity assay and partial chemical characterization of Kenyan *Physalis peruviana* L.(Solanaceae family) extracts. *Journal of Medicinal Plants Research*. **14(2)**: 73-80.

Kanhar, S. and Sahoo, A.K. (2021). An insight into the bioactive compounds of genus Homalium with therapeutic potential in different diseases: Current applications and future prospects. *Biocatalysis and Agricultural Biotechnology*. **37(**102173.

Karaali, H.F., Fahmi, R.R. and Borjac, J.M. (2018). Effect of Ocimum basilicum leaves extract on acetaminophen-induced nephrotoxicity in BALB/c mice. *Journal of Complementary and Integrative Medicine*. **16(2)**.

Karen, K. (2022). *Chapter-9 Overview of Important Plant Secondary Metabolites and Their Therapeutic Applications*. in: Naresh, R.K. (Ed.), *Bioactivity of secondary metabolites of various plants: a review*. pp. 132.

Kaur, G. and Rajput, B. (2014). Comparative analysis of the omics technologies used to study antimonial, amphotericin B, and pentamidine resistance in leishmania. *Journal of parasitology research*. **Volume 2014 | Article ID 726328 | https://doi.org/10.1155/2014/726328(**

Kaur, R. and Kaur, S. (2018). Evaluation of in vitro and in vivo antileishmanial potential of bergenin rich *Bergenia ligulata* (Wall.) Engl. root extract against visceral leishmaniasis in inbred BALB/c mice through immunomodulation. *Journal of Traditional and Complementary Medicine*. **8(1)**: 251-260.

Kaye, P.M., Cruz, I., Picado, A., Van Bocxlaer, K. and Croft, S.L. (2020). Leishmaniasis immunopathology—impact on design and use of vaccines, diagnostics and drugs: *Seminars in immunopathology*. Springer, pp. 247-264.

Keshav, P., Goyal, D.K. and Kaur, S. (2021). Promastigotes of Leishmania donovani exhibited sensitivity towards the high altitudinal plant Cicer microphyllum. *Current Research in Parasitology & Vector-Borne Diseases*. **1(**100040.

Kevric, I., Cappel, M.A. and Keeling, J.H. (2015). New world and old world *Leishmania* infections: a practical review. *Dermatologic Clinics*. **33(3)**: 579-593.

Khademvatan, S., Saki, J., Gharavi, M.J. and Rahim, F. (2011). *Allium sativum* extract induces apoptosis in *Leishmania major* (MRHO/IR/75/ER) promastigotes. *Journal of Medicinal Plants Research*. **5(16)**: 3725-3732.

Khan, I., Ahmad, K., Khalil, A.T., Khan, J., Khan, Y.A., Saqib, M.S., Umar, M.N. and Ahmad, H. (2015). Evaluation of antileishmanial, antibacterial and brine shrimp cytotoxic potential of crude methanolic extract of Herb *Ocimum basilicum* (Lamiacea). *Journal of Traditional Chinese Medicine*. **35(3)**: 316-322.

Kharchoufa, L., Bouhrim, M., Bencheikh, N., Addi, M., Hano, C., Mechchate, H. and Elachouri, M. (2021). Potential toxicity of medicinal plants inventoried in northeastern Morocco: an ethnobotanical approach. *Plants*. **10(6)**: 1108-1115.

Khayeka–Wandabwa, C., Kutima, H.L., Nyambati, V., Ingonga, J., Oyoo–Okoth, E., Karani, L.W., Jumba, B., Githuku, K.S. and Anjili, C.O. (2013). Combination therapy using Pentostam and Praziquantel improves lesion healing and parasite resolution in BALB/c mice co-infected with *Leishmania major* and *Schistosoma mansoni*. *Parasites & Vectors*. **6(1)**: 1-10.

Kheiri Manjili, H., Jafari, H., Ramazani, A. and Davoudi, N. (2012). Anti-leishmanial and toxicity activities of some selected Iranian medicinal plants. *Parasitology Research*. **111(5)**: 2115-2121.

Kibret, B., Israel, A., Milkyas, E. and Demiss, A. (2018). Comparative study of the antibacterial activity of leaves of *Croton macrostachyus* and *Aloe vera*. *Advance in Life Science and Technology*. **54(**22-28.

Kiros, Y.K. and Regassa, B.F. (2017). The role of rk39 serologic test in the diagnosis of visceral leishmaniasis in a Tertiary Hospital, Northern Ethiopia. *BMC Research Notes*. **10(1)**: 1-5.

Koley, H., Howlader, D.R. and Bhaumik, U. (2019). *Assessment of antimicrobial activity of different phytochemicals against enteric diseases in different animal models*, *New look to phytomedicine*. Elsevier. pp. 563-580.

Kosari, N. and Khamesipour, F. (2022). *Dracocephalum kotschyi* Boiss. In *Vitro* efficacy on growth and apoptosis induction in *Leishmania major* promastigotes. . *BioMed Research International.* **Volme 22 Article ID 8109264, 8 pages(**

Kotb Elmahallawy, E. and Agil, A. (2015). Treatment of leishmaniasis: a review and assessment of recent research. *Current Pharmaceutical Design*. **21(17)**: 2259-2275.

Kühne, V., Rezaei, Z., Pitzinger, P. and Büscher, P. (2019). Systematic review on antigens for serodiagnosis of visceral leishmaniasis, with a focus on East Africa. *PLoS Neglected Tropical Diseases*. **13(8)**: e0007658.

Kumar, S. (2021). Characterization of priming induced memory T-cell generation in Leishmania infection, Vidyasagar University, Midnapore, West Bengal, India.

Kumari, P., Kumari, C. and Singh, P.S. (2017). Phytochemical screening of selected medicinal plants for secondary metabolites. . *International Journal of Life Science and Scientific Research*. **3(4)**: 1151-1157.

Kumari, S., Kumar, V., Tiwari, R.K., Ravidas, V., Pandey, K. and Kumar, A. (2022). Amphotericin B: A drug of choice for Visceral Leishmaniasis. *Acta Tropica*. **235(**106661.

Kundishora, A., Sithole, S. and Mukanganyama, S. (2020). Determination of the cytotoxic effect of different leaf extracts from *Parinari curatellifolia* (Chrysobalanaceae). . *Journal of Toxicology*. **Volume 2020 | Article ID 8831545 | https://doi.org/10.1155/2020/8831545(**

Kwan, Y.P., Saito, T., Ibrahim, D.,, Al-Hassan, F.M.S., Ein Oon, C., Chen, Y., Jothy S.L, Kanwar J.R. and Sasidharan, S. (2016). Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by Euphorbia hirta in MCF-7 breast cancer cells.

. *Pharmaceutical Biology*. **54(7)**: 1223-1236.

Lagat, Z.O., Christopher, K.W., Jumba, B.N., Waihenya, R.W., Karanja, R. and Ong'ondo, B.O. (2014). Efficacy of glucocorticoids in controlling leishmania major infecting Balb/c mice. *Journal of Infectious Diseases and Immunity*. **6(2)**: 10-18.

Lambert, R. and Pearson, J. (2000). Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. *Journal of applied microbiology*. **88(5)**: 784-790.

Lanza, J.S., Pomel, S., Loiseau, P.M. and Frézard, F. (2019). Recent advances in amphotericin B delivery strategies for the treatment of leishmaniases. *Expert Opinion on Drug Delivery*. **16(10)**: 1063-1079.

Liaqat, I. and Asgar, A. (2021). Antimicrobial potential of eucalyptus oil and lemongrass oil alone and in combination against clinical bacteria in planktonic and biofilm mode. *World Journal of Biology and Biotechnology*. **6(2)**: 21-26.

Liévin-Le Moal, V. and Loiseau, P.M. (2016). Leishmania hijacking of the macrophage intracellular compartments. *The FEBS Journal*. **283(4)**: 598-607.

Lima, Á.L., de Lima, I.D., Coutinho, J.F., de Sousa, Ú.P., Rodrigues, M.A., Wilson, M.E., Pearson, R.D., Queiroz, J.W. and Jerônimo, S.M. (2017). Changing epidemiology of visceral leishmaniasis in northeastern Brazil: a 25-year follow-up of an urban outbreak. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **111(10)**: 440-447.

Lima, I.D., Lima, A.L., Mendes-Aguiar, C.d.O., Coutinho, J.F., Wilson, M.E., Pearson, R.D., Queiroz, J.W. and Jeronimo, S.M. (2018). Changing demographics of visceral leishmaniasis in northeast Brazil: Lessons for the future. *PLoS Neglected Tropical Diseases*. **12(3)**: e0006164.

Lugo-Huitrón, R., Blanco-Ayala, T., Ugalde-Muñiz, P., Carrillo-Mora, P., Pedraza-Chaverrí, J., Slver-Adaya, D., Maldonado, P.D., Torres, I., Pinzón, E., Ortiz-Islas, E. and Pérez-De La Cruz, V. (2011). On the antioxidant properties of kynurenic acid: free radical scavenging activity and inhibition of oxidative stress. *Neurotoxicology and Teratology*. **33(5)**: 538-547.

Luize, P.S., Tiuman, T.S., Morello, L.G., Maza, P.K., Ueda-Nakamura, T., Dias Filho, B.P., Cortez, D.A.G., Mello, J.C.P.d. and Nakamura, C.V. (2005). Effects of medicinal plant extracts on growth of *Leishmania (L.) amazonensis* and *Trypanosoma cruzi*. *Revista Brasileira de Ciências Farmacêuticas*. **41(**85-94.

Luyckx, V.A. and Naicker, S. (2008). Acute kidney injury associated with the use of traditional medicines. . *Nature Clinical Practice Nephrology*. **4(12)**: 664-671.

Luyckx, V.A., Steenkamp, V. and Stewart, M.J. (2005). Acute renal failure associated with the use of traditional folk remedies in South Africa *Renal Failure*. **27(1)**: 35-43.

Maaroufi, Z., Cojean, S., Loiseau, P.M., Yahyaoui, M., Agnely, F., Abderraba, M. and Mekhloufi, G. (2021). In vitro antileishmanial potentialities of essential oils from *Citrus limon* and *Pistacia lentiscus* harvested in Tunisia. *Parasitology Research*. **120(4)**: 1455-1469.

Macêdo, C.G., Fonseca, M.Y.N., Caldeira, A.D., Castro, S.P., Pacienza-Lima, W., Borsodi, M.P.G., Sartoratto, A., da Silva, M.N., Salgado, C.G. and Rossi-Bergmann, B. (2020). Leishmanicidal activity of *Piper marginatum* Jacq. from Santarém-PA against *Leishmania amazonensis*. *Experimental Parasitology*. **210(**107847-107855.

MacKinnon, D.P., Lockwood, C.M., Brown, C.H., Wang, W. and Hoffman, J.M. (2007). The intermediate endpoint effect in logistic and probit regression. *Clinical Trial*. **4(5)**: 499-513.

Mahdy, M.A., Al-Mekhlafi, A.M., Abdul-Ghani, R., Saif-Ali, R., Al-Mekhlafi, H.M., Al-Eryani, S.M., Lim, Y.A. and Mahmud, R. (2016). First molecular characterization of Leishmania species causing visceral leishmaniasis among children in Yemen. *PloS One*. **11(3)**: e0151265.

Mahmoudvand, H., Dezaki, E.S., Ezatpour, B., Sharifi, I., Kheirandish, F. and Rashidipour, M. (2016). In vitro and in vivo antileishmanial activities of *Pistacia vera* essential oil. *Planta Medica*. **82(04)**: 279-284.

Mahmoudvand, H., Harandi, M.F., Shakibaie, M., Aflatoonian, M.R., ZiaAli, N., Makki, M.S. and Jahanbakhsh, S. (2014). Scolicidal effects of biogenic selenium nanoparticles against protoscolices of hydatid cysts. *International Journal of Surgery*. **12(5)**: 399-403.

Makwali, J.A., Wanjala, F.M., Kaburi, J.C., Ingonga, J., Byrum, W.W. and Anjili, C.O. (2012). Combination and monotherapy of *Leishmania major* infection in BALB/c mice using plant extracts and herbicides. *Journal of Vector Borne Diseases*. **49(3)**: 123-134.

Marango, S.N., Khayeka-Wandabwa, C., Makwali, J.A., Jumba, B.N., Choge, J.K., Adino, E.O. and Anjili, C.O. (2017). Experimental therapeutic assays of *Tephrosia vogelii* against *Leishmania major* infection in murine model: in vitro and in vivo. *BMC Research Notes*. **10(1)**: 1-12.

Maroyi, A. (2017). Ethnopharmacology and therapeutic value of *Bridelia micrantha* (Hochst.) Baill. in tropical Africa: a comprehensive review. *Molecules*. **22(9)**: 1493-1503.

Marshall, S., Kelly, P.H., Singh, B.K., Pope, R.M., Kim, P., Zhanbolat, B., Wilson, M.E. and Yao, C. (2018). Extracellular release of virulence factor major surface protease via exosomes in *Leishmania infantum* promastigotes. *Parasites and Vectors*. **11(1)**: 1-10.

Martínez-Valencia, A.J., Daza-Rivera, C.F., Rosales-Chilama, M., Cossio, A., Casadiego Rincon, E.J., Desai, M.M., Saravia, N.G. and Gómez, M.A. (2017). Clinical and parasitological factors in parasite persistence after treatment and clinical cure of cutaneous leishmaniasis. *PLoS Neglected Tropical Diseases*. **11(7)**: e0005713.

Mathew, N.S. and Negi, P.S. (2019). *Plant-derived compounds against protozoan neglected diseases: toward sustainable drug development. , In Discovery and Development of Therapeutics from Natural Products against Neglected Tropical Diseases*. Elsevier. pp. 241-292.

Matysiak, J. (2015). Biological and pharmacological activities of 1, 3, 4-thiadiazole based compounds. *Mini Reviews in Medicinal Chemistry*. **15(9)**: 762-775.

Maurício, I.L. (2018). *Leishmania taxonomy, The Leishmaniases: Old Neglected Tropical Diseases*. Springer. pp. 15-30.

Mbui, J., Wasunna, M., Balasegaram, M., Laussermayer, A., Juma, R., Njenga, S.N., Kirigi, G., Riongoita, M., de la Tour, R. and van Peteghem, J. (2013). Validation of two rapid diagnostic tests for visceral leishmaniasis in Kenya. *PLoS Neglected Tropical Diseases*. **7(9)**: e2441.

Mendonça, L.S.O., Santos, J.M., Kaneto, C.M., de Carvalho, L.D., Lima-Santos, J., Augusto, D.G., Carvalho, S.M.S., Soares-Martins, J.A.P. and Silva-Jardim, I. (2020). Characterization of serum cytokines and circulating microRNAs that are predicted to regulate inflammasome genes in cutaneous leishmaniasis patients. *Experimental Parasitology*. **210(**107846.

Mensah, M.L., Komlaga, G., Forkuo, A.D., Firempong, C., Anning, A.K. and Dickson, R.A. (2019). Toxicity and safety implications of herbal medicines used in Africa. *Herbal Medicine*. **63(**1992-0849.

Meresa, A. (2019). Ethno medicinal uses, phytochemistry and anti-malarial effect of *Croton macrostachyus* (Bisana): A review. *Journal of Medicinal Plants*. **7(2)**: 79-88.

Mesa, L.E., Vasquez, D., Lutgen, P., Vélez, I.D., Restrepo, A.M., Ortiz, I. and Robledo, S.M. (2017). In vitro and in vivo antileishmanial activity of *Artemisia annua* L. leaf powder and its potential usefulness in the treatment of uncomplicated cutaneous leishmaniasis in humans. *Revista da Sociedade Brasileira de Medicina Tropical*. **50(**52-60.

Milad, R., El-Ahmady, S. and Singab, A.N. (2014). Genus *Kalanchoe* (Crassulaceae): a review of its ethnomedicinal, botanical, chemical and pharmacological properties. *European Journal of Medicinal Plants*. **4(1)**: 86-94.

Mishra, P., Pandey, C.M., Singh, U., Gupta, A., Sahu, C. and Keshri, A. (2019). Descriptive statistics and normality tests for statistical data. . *Annals of Cardiac Anaesthesia*. **22(1)**: 67-71.

Mitropoulos, P., Konidas, P. and Durkin-Konidas, M. (2010). New World cutaneous leishmaniasis: updated review of current and future diagnosis and treatment. *Journal of the American Academy of Dermatology*. **63(2)**: 309-322.

Mkaouar, S., Krichen, F., Bahloul, N., Allaf, K. and Kechaou, N. (2018). Enhancement of bioactive compounds and antioxidant activities of olive (*Olea europaea* L.) leaf extract by instant controlled pressure drop. *Food and Bioprocess Technology*. **11(6)**: 1222-1229.

Monzote, L. (2009). Current treatment of leishmaniasis: a review. *The Open Antimicrobial Agents Journal*. **1(1)**: 21-29.

Monzote, L., García, M., Scull, R., Cuellar, A. and Setzer, W.N. (2014). Antileishmanial activity of the essential oil from *Bixa orellana*. *Phytotherapy Research*. **28(5)**: 753-758.

Moraes Neto, R.N., Setúbal, R.F.B., Higino, T.M.M., Brelaz-de-Castro, M.C.A., da Silva, L.C.N. and Aliança, A.S.d.S. (2019). Asteraceae plants as sources of compounds against leishmaniasis and Chagas disease. *Frontiers in Pharmacology*. **10(**477-491.

Moreira, R.R.D., Santos, A.G.d., Carvalho, F.A., Perego, C.H., Crevelin, E.J., Crotti, A.E.M., Cogo, J., Cardoso, M.L.C. and Nakamura, C.V. (2019). Antileishmanial activity of *Melampodium divaricatum* and *Casearia sylvestris* essential oils on *Leishmania amazonensis*. *Revista do Instituto de Medicina Tropical de São Paulo*. **61:e33(**

Moremi, M.P., Makolo, F., Viljoen, A.M. and Kamatou, G.P. (2021). A review of biological activities and phytochemistry of six ethnomedicinally important south African croton species. *Journal of Ethnopharmacology*. **280(**114416.

Moreno, I., Álvarez, J., García, N., de la Fuente, S., Martínez, I., Marino, E., Toraño, A., Goyache, J., Vilas, F. and Domínguez, L. (2014). Detection of anti-*Leishmania infantum* antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. *Veterinary Parasitology*. **199(3-4)**: 264-267.

Mozaffari, E., Vatandoost, H., Rassi, Y., Mohebali, M., Akhavan, A.A., Moradi-Asl, E., Zarei, Z., Zahrai-Ramazani, A. and Ghorbani, E. (2020). Epidemiology of visceral leishmaniasis with emphasis on the dynamic activity of sand flies in an important endemic focus of disease in Northwestern Iran. *Journal of Arthropod-Borne Diseases*. **14(1)**: 97-102.

Muganga, R., Bero, J., Quetin-Leclercq, J., Angenot, L., Tits, M., Mouithys-Mickalad, A., Franck, T. and Frédérich, M. (2020). In vitro Antileishmanial, antitrypanosomal, and anti-inflammatory-like activity of *Terminalia mollis* root Bark. *Planta Medica*. **87(9)**: 724-731.

Musonda, C.C., Whitlock, G.A., Witty, M.J., Brun, R. and Kaiser, M. (2009). Synthesis and evaluation of 2-pyridyl pyrimidines with in vitro antiplasmodial and antileishmanial activity. *Bioorganic and Medicinal Chemistry Letters*. **19(2)**: 401-405.

Mutoro, C.N., Kinyua, J., Kariuki, D., Ingonga, J.M. and Anjili, C.O. (2018). Efficacy of the combination of crude extracts of *Solanum nigrum* and *Plumbago capensis* on *Leishmania major*. *F1000Research*. **7(1556)**: 1556.

Muzitano, M.F., Tinoco, L.W., Guette, C., Kaiser, C.R., Rossi-Bergmann, B. and Costa, S.S. (2006). The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*. *Phytochemistry*. **67(18)**: 2071-2077.

Nabatanzi, A., Nkadimeng, S.M., Lall, N., Kabasa, J.D. and McGaw, L.J. (2020a). Antioxidant and anti-inflammatory activities of *Kigelia africana* (Lam.) Benth. *Evidence-Based Complementary and Alternative Medicine*. **Volume 2020 | Article ID 4352084 | https://doi.org/10.1155/2020/4352084(**

Nabatanzi, A., Nkadimeng, S.M., Lall, N., Kabasa, J.D. and McGaw, L.J. (2020b). Ethnobotany, Phytochemistry and Pharmacological Activity of *Kigelia africana* (Lam.) Benth.(Bignoniaceae). *Plants*. **9(6)**: 753-763.

Naicker, S., Aboud, O. and Gharbi, M.B. (2008). Epidemiology of acute kidney injury in Africa. *Seminars in Nephrology* **28(**348-353.

Nassif, P.W., De Mello, T.F.P., Navasconi, T.R., Mota, C.A., Demarchi, I.G., Aristides, S.M.A., Lonardoni, M.V.C., Teixeira, J.J.V. and Silveira, T.G.V. (2017). Safety and efficacy of current alternatives in the topical treatment of cutaneous leishmaniasis: a systematic review. *Parasitology*. **144(8)**: 995-1002.

Neves, L.O., Talhari, A.C., Gadelha, E.P.N., Silva Júnior, R.M.d., Guerra, J.A.d.O., Ferreira, L.C.d.L. and Talhari, S. (2011). A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by *Leishmania guyanensis*. *Anais Brasileiros de Dermatologia*. **86(**1092-1101.

Ngane, R.A.N. (2019). Antibacterial activity of methanol extract and fractions from stem bark of *Bridelia micrantha* (Hochst.) Baill.(Phyllanthaceae). *EC Pharmacology and Toxicology*. **7(**609-616.

Ngure, P.K., Tonui, W.K., Ingonga, J., Mutai, C., Kigondu, E., Ngrsquo, Z., Rukunga, G. and Kimutai, A. (2009). In vitro antileishmanial activity of extracts of *Warburgia ugandensis* (Canellaceae), a Kenyan medicinal plant. *Journal of Medicinal Plants Research*. **3(2)**: 61-066.

Nicolaus, C., Junghanns, S., Hartmann, A., Murillo, R., Ganzera, M. and Merfort, I. (2017). In vitro studies to evaluate the wound healing properties of Calendula officinalis extracts. *Journal of ethnopharmacology*. **196(**94-103.

Nigatu, H., Belay, A., Ayalew, H., Abebe, B., Tadesse, A., Tewabe, Y. and Degu, A. (2021). In vitro antileishmanial activity of some ethiopian medicinal plants. *Journal of Experimental Pharmacology*. **13(**15-24.

Noli, C. and Saridomichelakis, M.N. (2014). An update on the diagnosis and treatment of canine leishmaniosis caused by *Leishmania infantum* (syn. *L. chagasi*). *The Veterinary Journal*. **202(3)**: 425-435.

Obey, J.K., von Wright, A., Orjala, J., Kauhanen, J. and Tikkanen-Kaukanen, C. (2016). Antimicrobial activity of *Croton macrostachyus* stem bark extracts against several human pathogenic bacteria. *Journal of Pathogens*: Volume 2016 | Article ID 1453428 | https://doi.org/10.1155/2016/1453428.

Odiwuor, S., Veland, N., Maes, I., Arévalo, J., Dujardin, J.-C. and Van der Auwera, G. (2012). Evolution of the *Leishmania braziliensis* species complex from amplified fragment length polymorphisms, and clinical implications. *Infection, Genetics and Evolution*. **12(8)**: 1994-2002.

Ohashi, M., Amoa-Bosompem, M., Kwofie, K.D., Agyapong, J., Adegle, R., Sakyiamah, M.M., Ayertey, F., Owusu, K.B.A., Tuffour, I. and Atchoglo, P. (2018). In vitro antiprotozoan activity and mechanisms of action of selected Ghanaian medicinal plants against trypanosoma, leishmania, and plasmodium parasites. *Phytotherapy Research*. **32(8)**: 1617-1630.

Olías-Molero, A.I., Fontán-Matilla, E., Cuquerella, M. and Alunda, J.M. (2021). Scientometric analysis of chemotherapy of canine leishmaniasis (2000–2020). *Parasites & Vectors*. **14(1)**: 1-8.

Oloya, B., Namukobe, J., Ssengooba, W., Afayoa, M. and Byamukama, R. (2022). Phytochemical screening, antimycobacterial activity and acute toxicity of crude extracts of selected medicinal plant species used locally in the treatment of tuberculosis in Uganda. . *Tropical Medicine and Health*. **50(1)**: 1-13.

Omar, F., Tareq, A.M., Alqahtani, A.M., Dhama, K., Sayeed, M.A., Emran, T.B. and Simal-Gandara, J. (2021). Plant-based indole alkaloids: A comprehensive overview from a pharmacological perspective. *Molecules*. **26(8)**: 2297.

Oryan, A. (2015). Plant-derived compounds in treatment of leishmaniasis. *Iranian Journal of Veterinary Research*. **16(1)**: 1-7.

Oskuee, R., Jafari, M., Farzad, S.A. and Ramezani, M. (2012). In vitro Leishmanicidal activity of *Calotropis gigantea* and its fractions against *Leishmania major*. *Journal of Medicinal Plants Research*. **6(23)**: 3977-3983.

Ostyn, B., Hasker, E., Dorlo, T.P., Rijal, S., Sundar, S., Dujardin, J.-C. and Boelaert, M. (2014). Failure of miltefosine treatment for visceral leishmaniasis in children and men in South-East Asia. *PloS One*. **9(6)**: e100220.

Padilha Ferreira, F.B., Pereira Cabral, M.R., Sarragiotto, M.H., Fernandez, M., Maria, C., Cristiani Gazim, Z., Piau Junior, R., Galhardo Demarchi, I., Verzignassi Silveira, T.G. and Campana Lonardoni, M.V. (2021). Screening of six medicinal plant species for antileishmanial activity. *Acta Pharmaceutica*. **71(3)**: 399-414.

Pansera, M.R., Silvestre, W.P., Gonzatti, F., Pauletti, G.F. and Sartori, V.C. (2021). Chemical composition and antifungal activity of the essential oils from native species of the 'Campos de Cima da Serra'region, South Brazil. *Journal of Essential Oil Research*. **33(5)**: 488-501.

Parvez, M.K. and Rishi, V. (2019). Herb-drug interactions and hepatotoxicity. *Current Drug Metabolism*. **20(4)**: 275-282.

Parvizi, M.M., Zare, F., Handjani, F., Nimrouzi, M. and Zarshenas, M.M. (2020). Overview of herbal and traditional remedies in the treatment of cutaneous leishmaniasis based on Traditional Persian Medicine. *Dermatologic Therapy*. **33(4)**: e13566.

Pengelly, A. (2021). *The constituents of medicinal plants*. Cabi.

Pereira, K.L., Vasconcelos, N.B., Braz, J.V., Inacio, J.D., Estevam, C.S., Correa, C.B., Fernandes, R.P., Almeida-Amaral, E.E. and Scher, R. (2020). Ethanolic extract of Croton blanchetianus Ball induces mitochondrial defects in Leishmania amazonensis promastigotes. *Anais da Academia Brasileira de Ciências*. **92(**

Podolsky, S.H. and Lie, A.K. (2016). *1. Futures and their Uses, Therapeutic Revolutions*. University of Chicago Press. pp. 18-42.

Ponte-Sucre, A., Gamarro, F., Dujardin, J.-C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R. and Papadopoulou, B. (2017). Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLoS Neglected Tropical Diseases*. **11(12)**: e0006052.

Pospíšil, J., Konrádová, D. and Strnad, M. (2021). Antileishmanial activity of lignans, neolignans, and other plant phenols. *Progress in the Chemistry of Organic Natural Products 115*. **11(**115-176.

Prasad, P.R.C., Reddy, C.S., Raza, S. and Dutt, C. (2008). Folklore medicinal plants of north Andaman Islands, India. *Fitoterapia*. **79(6)**: 458-464.

Prpa, E., Bajka, B., Ellis, P., Butterworth, P., Corpe, C. and Hall, W. (2020). A systematic review of in vitro studies evaluating the inhibitory effects of polyphenol-rich fruit extracts on carbohydrate digestive enzymes activity: a focus on culinary fruits consumed in Europe. *Critical Reviews in Food Science and Nutrition*: 1-21.

Rabeta, M.S. and Faraniza, R.N. (2013). Total phenolic content and ferric reducing antioxidant power of the leaves and fruits of *Garcinia atrovirdis* and *Cynometra cauliflora*. . *International Food Research Journal*. **20(4)**: 1691-1699.

Rahman, M.A., Saha, S.K., Rahman, M.S., Uddin, M.J., Uddin, M.S., Pang, M.-G., Rhim, H. and Cho, S.-G. (2020). Molecular insights into therapeutic potential of autophagy modulation by natural products for cancer stem cells. *Frontiers in Cell and Developmental Biology*. **8(**283.

Rangel, H., Dagger, F., Hernandez, A., Liendo, A. and Urbina, J.A. (1996). Naturally azole-resistant *Leishmania braziliensis* promastigotes are rendered susceptible in the presence of terbinafine: comparative study with azole-susceptible *Leishmania mexicana* promastigotes. *Antimicrobial Agents and Chemotherapy*. **40(12)**: 2785-2791.

Rebouças-Silva, J., Tadini, M.C., Devequi-Nunes, D., Mansur, A.L., Silveira-Mattos, P.S., de Oliveira, C.I., Formiga, F.R., Berretta, A.A., Marquele-Oliveira, F. and Borges, V.M. (2020). Evaluation of in vitro and in vivo efficacy of a novel amphotericin B-loaded nanostructured lipid carrier in the treatment of *Leishmania braziliensis* Infection. *International Journal of Nanomedicine*. **15(**8659-8669.

Rivera-Fernández, I., Argueta-Donohué, J., Wilkins-Rodríguez, A.A. and Gutiérrez-Kobeh, L. (2019). Effect of two different isolates of Leishmania mexicana in the production of cytokines and phagocytosis by murine dendritic cells. *Journal of Parasitology*. **105(2)**: 359-370.

Roatt, B.M., de Oliveira Cardoso, J.M., De Brito, R.C.F., Coura-Vital, W., de Oliveira Aguiar-Soares, R.D. and Reis, A.B. (2020). Recent advances and new strategies on leishmaniasis treatment. *Applied Microbiology and Biotechnology*. **104(21)**: 8965-8977.

Rocha, L., Almeida, J., Macedo, R. and Barbosa-Filho, J. (2005). A review of natural products with antileishmanial activity. *Phytomedicine*. **12(6-7)**: 514-535.

Rodrigues, I.A., Azevedo, M., Chaves, F., Bizzo, H.R., Corte-Real, S., Alviano, D.S., Alviano, C.S., Rosa, M.S. and Vermelho, A.B. (2013). In vitro cytocidal effects of the essential oil from *Croton cajucara* (red sacaca) and its major constituent 7-hydroxycalamenene against *Leishmania chagasi*. *BMC Complementary and Alternative Medicine*. **13(1)**: 1-9.

Rojas-Jaimes, J., Rojas-Palomino, N., Pence, J. and Lescano, A.G. (2019). Leishmania species in biopsies of patients with different clinical manifestations identified by high resolution melting and nested PCR in an Endemic district in Peru. *Parasite Epidemiology and Control*. **4(**e00095.

Ruiz-Postigo, J.A., Jain, S., Mikhailov, A., Maia-Elkhoury, A.N., Valadas, S., Warusavithana, S., Osman, M., Lin, Z., Beshah, A. and Yajima, A. (2021). Global leishmaniasis surveillance: 2019-2020, a baseline for the 2030 roadmap/Surveillance mondiale de la leishmaniose: 2019-2020, une periode de reference pour la feuille de route a l'horizon 2030. *Weekly Epidemiological Record*. **96(35)**: 401-420.

Saeidnia, S., Manayi, A. and Abdollahi, M. (2015). From in vitro experiments to in vivo and clinical studies; pros and cons. *Current Drug Discovery Technologies*. **12(4)**: 218-224.

Sahne, F., Mohammadi, M., Najafpour, G.D. and Moghadamnia, A.A. (2017). Enzyme-assisted ionic liquid extraction of bioactive compound from turmeric (*Curcuma longa* L.): Isolation, purification and analysis of curcumin. *Industrial Crops and Products*. **95(**686-694.

Salah, I., Abbasi, I., Warburg, A., Davidovitch, N. and Kotler, B. (2020). Ecology of Leishmaniasis in an urbanized landscape: Relationship of sand fly densities, and *Leishmania tropica* infection rates with reservoir host colonies. *Acta Tropica*. **204(**105332.

Salari, S., Bamorovat, M., Sharifi, I. and Almani, P.G.N. (2022). Global distribution of treatment resistance gene markers for leishmaniasis. *Journal of Clinical Laboratory Analysis*. **36(8)**: e24599.

Sarwar, M. (2015). Microbial insecticides-an ecofriendly effective line of attack for insect pests management. *International Journal of Engineering and Advanced Research Technology*. **1(2)**: 4-9.

Schulz, R., Bub, S., Petschick, L.L., Stehle, S. and Wolfram, J. (2021). Applied pesticide toxicity shifts toward plants and invertebrates, even in GM crops. *Science*. **372(6537)(**81-84.

Seifert, K. and Croft, S.L. (2006). In vitro and in vivo interactions between miltefosine and other antileishmanial drugs. *Antimicrobial Agents and Chemotherapy*. **50 (1)**: 73-79.

Seow, E.S. (2021). Analysis of the Interaction between Pentraxins and Leishmania Phosphoglycans, London School of Hygiene & Tropical Medicine.

Serafim, T.D., Coutinho-Abreu, I.V., Oliveira, F., Meneses, C., Kamhawi, S. and Valenzuela, J.G. (2018). Sequential blood meals promote *Leishmania* replication and reverse metacyclogenesis augmenting vector infectivity. *Nature Microbiology*. **3(5)**: 548-555.

Seremet, O.C., Olaru, O.T., Gutu, C.M., Nitulescu, G.M., Ilie, M., Negres, S., Zbarcea, C.E., Purdel, C.N., Spandidos, D.A. and Tsatsakis, A.M. (2018). Toxicity of plant extracts containing pyrrolizidine alkaloids using alternative invertebrate models. *Molecular medicine reports*. **17(6)**: 7757-7763.

Sharwan, G., Jain, P., Pandey, R. and Shukla, S.S. (2015). Toxicity profile of traditional herbal medicine. . *Journal of Ayurdevich Herbal Medicine*. **1 (3)**: 81-90.

Shibata, T., Takahashi, T., Yamada, E., Kimura, A., Nishikawa, H., Hayakawa, H., Nomura, N. and Mitsuyama, J. (2012). T-2307 causes collapse of mitochondrial membrane potential in yeast. *Antimicrobial Agents and Chemotherapy*. **56(11)**: 5892-5897.

Siangu, B.N., Sauda, S., John, M.K. and Njue, W.M. (2019). Antioxidant activity, total phenolic and flavonoid content of selected Kenyan medicinal plants, sea algae and medicinal wild mushrooms. *African Journal of Pure and Applied Chemistry*. **13(3)**: 43-48.

Simoben, C.V., Ntie-Kang, F., Akone, S.H. and Sippl, W. (2018). Compounds from African medicinal plants with activities against selected parasitic diseases: schistosomiasis, trypanosomiasis and leishmaniasis. *Natural Products and Bioprospecting*. **8(3)**: 151-169.

Singh, A., Mishra, A., Chaudhary, R. and Kumar, V. (2020). Dear role of herbal plants in prevention and treatment of parasitic diseases. *Journal of Scientific Research*. **64(1)**: 21-29.

Singh, M.K., Das, A., Saha, R.P., Paul, J. and Nandi, D. (2022). Leishmaniasis: Plants as a source of antileishmanial agents. *Journal of Experimental Biology***1)**: 227-247.

Singh, S., Mandal, M.K., Masih, A., Saha, A., Ghosh, S.K., Bhat, H.R. and Singh, U.P. (2021). 1, 3, 5-Triazine: A versatile pharmacophore with diverse biological activities. *Archiv der Pharmazie*. **354(6)**: 2000363.

Sioud, F., Toumia, I.B., Lahmer, A., Khlifi, R., Dhaouefi, Z., Maatouk, M., Ghedira, K. and Chekir-Ghedira, L. (2020). Methanolic extract of Ephedra alata ameliorates cisplatin-induced nephrotoxicity and hepatotoxicity through reducing oxidative stress and genotoxicity. *Environmental Science and Pollution Research*: 1-10.

Soni, V., Chandel, S., Pandey, V., Asati, S., Jain, P., Jain, A. and Tekade, R.K. (2019). *Novel therapeutic approaches for the treatment of leishmaniasis*. Academic Press.

Soosaraei, M., Fakhar, M., Teshnizi, S.H., Hezarjaribi, H.Z. and Banimostafavi, E.S. (2017). Medicinal plants with promising antileishmanial activity in Iran: a systematic review and meta-analysis. *Annals of Medicine and Surgery*. **21(**63-80.

Sosa, A.M., Amaya, S., Salamanca Capusiri, E., Gilabert, M., Bardón, A., Giménez, A., Vera, N.R. and Borkosky, S.A. (2016). Active sesquiterpene lactones against *Leishmania amazonensis* and *Leishmania braziliensis*. *Natural Product Research*. **30(22)**: 2611-2615.

Sousa-Batista, A.J., Pacienza-Lima, W., Ré, M.I. and Rossi-Bergmann, B. (2019a). Novel and safe single-dose treatment of cutaneous leishmaniasis with implantable amphotericin B-loaded microparticles. *International Journal for Parasitology: Drugs and Drug Resistance*. **11(**148-155.

Sousa-Batista, A.J., Pacienza-Lima, W., Ré, M.I. and Rossi-Bergmann, B. (2019b). Novel and safe single-dose treatment of cutaneous leishmaniasis with implantable amphotericin B-loaded microparticles. *International Journal for Parasitology: Drugs and Drug Resistance*. **11 (**148-155.

Souto, E.B., Dias-Ferreira, J., Craveiro, S.A., Severino, P., Sanchez-Lopez, E., Garcia, M.L., Silva, A.M., Souto, S.B. and Mahant, S. (2019). Therapeutic interventions for countering leishmaniasis and chagas's disease: From traditional sources to nanotechnological systems. *Pathogens*. **8(3)**: 119-129.

Sridhar, K., Inbaraj, B.S. and Chen, B.-H. (2021). Recent developments on production, purification and biological activity of marine peptides. *Food Research International*. **147(**110468.

Steverding, D. (2017). The history of leishmaniasis. *Parasites & vectors*. **10(1)**: 82.

Sullivan, R.J. and Weber, J.S. (2022). Immune-related toxicities of checkpoint inhibitors: mechanisms and mitigation strategies. *Nature Reviews Drug Discovery*. **21(7)**: 495-508.

Sundar, S. and Chakravarty, J. (2015). An update on pharmacotherapy for leishmaniasis. *Expert opinion on Pharmacotherapy*. **16(2)**: 237-252.

Sundar, S., Chakravarty, J. and Meena, L.P. (2019a). Leishmaniasis: treatment, drug resistance and emerging therapies. *Expert Opinion on Orphan Drugs*. **7(1)**: 1-10.

Sundar, S. and Singh, A. (2106). Recent developments and future prospects in the treatment of visceral leishmaniasis. *Therapeutic Advances in Infectious Disease*. **3 (3-4)**: 98-109.

Sundar, S., Singh, A., Agrawal, N. and Chakravarty, J. (2019b). Effectiveness of single-dose liposomal amphotericin B in visceral leishmaniasis in Bihar. *The American Journal of Tropical Medicine and Hygiene*. **101(4)**: 795-810.

Sunyoto, T., Potet, J. and Boelaert, M. (2018). Why miltefosine—a life-saving drug for leishmaniasis—is unavailable to people who need it the most. *BMJ Global Health*. **3(3)**: e000709.

Tadele, M., Abay, S.M., Makonnen, E. and Hailu, A. (2020). *Leishmania donovani* growth inhibitors from pathogen box compounds of medicine for malaria venture. *Drug Design, Development and Therapy*. **14(**1307.

Tahir, A.E., Ibrahim, A.M., Satti, G.M., Theander, T.G., Kharazmi, A. and Khalid, S.A. (1998). The potential antileishmanial activity of some Sudanese medicinal plants. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives*. **12 (8)**: 576-579.

Tajbakhsh, E., Khamesipour, A., Hosseini, S.R., Kosari, N., Shantiae, S. and Khamesipour, F. (2021). The effects of medicinal herbs and marine natural products on wound healing of cutaneous leishmaniasis: A systematic review. *Microbial Pathogenesis*. **161(**105235.

Taran, M., Mohebali, M. and Esmaeli, J. (2010). In vivo efficacy of gum obtained *Pistacia atlantica* in experimental treatment of cutaneous leishmaniasis. *Iranian Journal of Public Health*. **39(1)**: 36-45.

Tariku, Y., Hymete, A., Hailu, A. and Rohloff, J. (2010). Constituents, antileishmanial activity and toxicity profile of volatile oil from berries of *Croton macrostachyus*. *Natural Product Communications*. **5(6)**: 1934578X1000500635.

Tariq, A., Adnan, M., Amber, R., Pan, K., Mussarat, S. and Shinwari, Z.K. (2016). Ethnomedicines and anti-parasitic activities of Pakistani medicinal plants against *Plasmodia* and *Leishmania* parasites. *Annals of Clinical Microbiology and Antimicrobials*. **15(1)**: 52-64.

Tchatat Tali, M.B., Jiatsa Mbouna, C.D., Yamthe Tchokouaha, L.R., Tsouh Fokou, P.V., Tsakem Nangap, J.M., Keumoe, R., Ngoutane Mfopa, A., Bakarnga-Via, I., Gounoue Kamkumo, R. and Fekam Boyom, F. (2020). In Vivo antiplasmodial activity of *Terminalia mantaly* stem bark aqueous extract in mice infected by *Plasmodium berghei*. *Journal of Parasitology Research*. **Volume 2020 | Article ID 4580526 | https://doi.org/10.1155/2020/4580526(**

Tekalign, S., Adera, C., Den Boer, M., Miecha, H., Zewde, A., Mulugeta, D., Bishawu, T., Birru, W., Lema, A. and Sahlu, T. (2020). Clinical features and treatment outcomes of visceral leishmaniasis patients admitted to three centers in Oromia, Ethiopia. *The Journal of Infection in Developing Countries*. **14(06.1)**: 42-47.

Thakur, S., Joshi, J. and Kaur, S. (2020a). Leishmaniasis diagnosis: an update on the use of parasitological, immunological and molecular methods. *Journal of Parasitic Diseases*. **12(**1-20.

Thakur, S., Joshi, J. and Kaur, S. (2020b). Leishmaniasis diagnosis: An update on the use of parasitological, immunological and molecular methods. *Journal of Parasitic Diseases*. **44(2)**: 253-272.

Tiuman, T.S., Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P. and Nakamura, C.V. (2011). Recent advances in leishmaniasis treatment. *International Journal of Infectious Diseases*. **15(8)**: 525-532.

Tiuman, T.S., Ueda-Nakamura, T., Filho, B.P.D., Cortez, D.A.G. and Nakamura, C.V. (2005). Studies on the effectiveness of *Tanacetum parthenium* against *Leishmania amazonensis*. *Acta Protozoologica*. **44(3)**: 245-253.

Tiwari, N., Gedda, M.R., Tiwari, V.K., Singh, S.P. and Singh, R.K. (2018). Limitations of current therapeutic options, possible drug targets and scope of natural products in control of leishmaniasis. *Mini Reviews in Medicinal Chemistry*. **18(1)**: 26-41.

Tomiotto-Pellissier, F., da Silva Bortoleti, B.T., Concato, V.M., Ganaza, A.F.M., Quasne, A.C., Ricci, B., e Carvalho, P.V.D., Della Colleta, G.H., Lazarin-Bidóia, D. and Silva, T.F. (2022). The cytotoxic and anti-leishmanial activity of Oregano (*Origanum vulgare*) essential oil: An in vitro, in vivo, and in silico study. *Industrial Crops and Products*. **187(**115367.

Tonui, K. (2006). Estimation of the minimumnumber of *Leishmania major* amastigotes required for infecting *Phlebotomus duiboscqi* (Diptera: Psychodidae). . *East African Medical Journal*. **83(2)**: 11-19.

Torres-Guerrero, E., Quintanilla-Cedillo, M.R., Ruiz-Esmenjaud, J. and Arenas, R. (2017). Leishmaniasis: a review. *F1000Research*. **6(**21-29.

Trinh, T.A., Park, J., Oh, J.H., Park, J.S., Lee, D., Kim, C.E., Choi, H.-S., Kim, S.-B., Hwang, G.S. and Koo, B.A. (2020). Effect of herbal formulation on immune response enhancement in RAW 264.7 macrophages. *Biomolecules*. **10(3)**: 424-435.

Ullah, N., Nadhman, A., Siddiq, S., Mehwish, S., Islam, A., Jafri, L. and Hamayun, M. (2016). Plants as antileishmanial agents: current scenario. *Phytotherapy Research*. **30(12)**: 1905-1925.

Valencia, B.M., Miller, D., Witzig, R.S., Boggild, A.K. and Llanos-Cuentas, A. (2013). Novel low-cost thermotherapy for cutaneous leishmaniasis in Peru. *PLoS Neglected Tropical Diseases*. **7(5)**: e2196.

Van de Vel, E., Sampers, I. and Raes, K. (2019). A review on influencing factors on the minimum inhibitory concentration of essential oils. *Critical reviews in food science and nutrition*. **59(3)**: 357-378.

van Griensven, J. and Diro, E. (2019). Visceral leishmaniasis: recent advances in diagnostics and treatment regimens. *Infectious Disease Clinics*. **33(1)**: 79-99.

Vandeputte, M., van Henten, S., Van Griensven, J., Huits, R., Van Esbroeck, M., Van der Auwera, G., Cnops, L. and Bottieau, E. (2020). Epidemiology, clinical pattern and impact of species-specific molecular diagnosis on management of leishmaniasis in Belgium, 2010–2018: A retrospective study. *Travel Medicine and Infectious Disease*. **38(**101885.

Vemula, R., Pachipala, G., Reddy, P., Kota, S., Raman, M., Allam, U.S., Manikanta, K.N. and Chadipiralla, K. (2022). In Vitro anti-protozoan activity of methanolic extracts of *Caralluma procumbens* against *Tritrichomonas foetus*. *Current Trends in Biotechnology and Pharmacy*. **16(2)**: 144-154.

Vieira-Gonçalves, R., Fagundes-Silva, G.A., Heringer, J.F., Fantinatti, M., Da-Cruz, A.M., Oliveira-Neto, M.P., Guerra, J.A.O. and Gomes-Silva, A. (2019). First report of treatment failure in a patient with cutaneous leishmaniasis infected by *Leishmania (Viannia) naiffi* carrying Leishmania RNA virus: a fortuitous combination? *Revista da Sociedade Brasileira de Medicina Tropical*. **Volume 2020 | Article ID 4580526 | https://doi.org/10.1155/2020/4580526(**

Vila-Nova, N.S., de Morais, S.M., Falcão, M.J.C., Alcantara, T.T.N., Ferreira, P.A.T., Cavalcanti, E.S.B., Vieira, I.G.P., Campello, C.C. and Wilson, M. (2013). Different susceptibilities of Leishmania spp. promastigotes to the *Annona muricata* acetogenins annonacinone and corossolone, and the *Platymiscium floribundum* coumarin scoparone. *Experimental Parasitology*. **133(3)**: 334-338.

Vilegas, W., Tangerina, M.M.P., Ascêncio, S.D., Soares, I.M., Pavan, E., Damazo, A.S., Ribeiro, R.V. and de Oliveira Martins, D.T. (2018). Chemical characterisation and toxicity assessment in vitro and in vivo of the hydroethanolic extract of *Terminalia argentea* Mart. leaves. *Journal of Ethnopharmacology*. **227(**56-68.

Wafula, M.D. (2020). Evaluation of the knowledge, attitudes and prevention practices with regard to cutaneous Leishmaniasis caused by *Leishmania aethiopica* in Mount Elgon Focus, Kenya. *International Journal of Tropical Disease and Health* **41(7)**: 92-101.

Waiganjo, B., Moriasi, G., Onyancha, J., Elias, N. and Muregi, F. (2020). Antiplasmodial and cytotoxic activities of extracts of selected medicinal plants used to treat malaria in Embu County, Kenya. *Journal of Parasitology Research*. **Volume 2020 | Article ID 8871375 | https://doi.org/10.1155/2020/8871375(**

Wamai, R.G., Kahn, J., McGloin, J. and Ziaggi, G. (2020). Visceral leishmaniasis: a global overview. *Journal of Global Health Science*. **2(1)**: 23-42.

Wang, Z. and Tang, M. (2020). Research progress on toxicity, function, and mechanism of metal oxide nanoparticles on vascular endothelial cells. *Journal of Applied Toxicology*. **41(5)**: 683-700.

Wiart, C. (2020). *Medicinal Plants in Asia and Pacific for Parasitic Infections: Botany, Ethnopharmacology, Molecular Basis, and Future Prospect*. Academic Press.

World Health Organization. (2020). Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators–Surveillance mondiale de la leishmaniose, 2017-2018, et premier rapport sur 5 indicateurs supplémentaires. *Weekly Epidemiological Record= Relevé épidémiologique hebdomadaire*. **95(25)**: 265-279.

Yadav, R. and Agarwala, M. (2011). Phytochemical analysis of some medicinal plants. *Journal of phytology*.

Yang, B.-Y., Yin, X., Liu, Y., Zhao, D.-Y. and Kuang, H.-X. (2018). New steroidal saponins from the roots of *Solanum melongena* L. *Fitoterapia*. **128(**12-19.

Zeouk, I., Et-Touys, A.,, Balouiri, M., Fellah, H., Lalami, A.E.O. and Bekhti, K. (2019). Leishmanicidal activity of plant extracts from Sefrou, a Moroccan focus of Leishmaniasis, against various Leishmania parasites in the promastigote stage. . *Phytothérapie*. **17(2)**: 83-89.

Zeouk, I., Lalami, A.E.O., Ezzoubi, Y., Derraz, K., Balouiri, M. and Bekhti, K. (2020). Cutaneous Leishmaniasis: Medicinal plants used in Sefrou City (Center of Morocco), a focus of Leishmaniasis. . *Phytothérapie*. **18(3-4)**: 187-194.

Zhang, K. (2021). Balancing de novo synthesis and salvage of lipids by Leishmania amastigotes. *Current Opinion in Microbiology*. **63(**98-103.

Zhao, Y.L., Su, M., Shang, J.H., Wang, X., Bao, G.L., Ma, J. and Luo, X.D. (2020). Genotoxicity and safety pharmacology studies of indole alkaloids extract from leaves of *Alstonia scholaris* (L.) R. Br. *Natural Products and Bioprospecting*. **10(3)**: 119-129.

Zhou, X., Li, C. G., Chang, D., & Bensoussan, A. (2019). . (2019). Current status and major challenges to the safety and efficacy presented by Chinese herbal medicine. *Medicines*. **6(1)**: 14-22.

APPENDICES

Appendix I: Images of *Olea europae*

Appendix II: Image of *Kigelia africana*

Appendix III: Image of *Terminalia mollis*

Appendix IV: Image of *Croton macrostachyus*

Appendix V: Image of *Bridella micrantha*

Appendix VI: NACOST

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **345913**

Date of Issue: **22/January/2021**

RESEARCH LICENSE

This is to Certify that Mr.. Dennis WAFULA Mukhwana of Maseno University, has been licensed to conduct research in Baringo on the topic: IN VITRO AND IN VIVO EVALUATION OF ANTILEISHMANIAL ACTIVITIES OF OLEA EUROPAEA, KIGELIA AFRICANA, TERMINALIA MOLLIS, CROTON MACROSTACHYUS AND BRIDELLA MICRANTHA for the period ending : 22/January/2022.

License No: **NACOSTI/P/21/8451**

345913

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR
SCIENCE,TECHNOLOGY &
INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

Appendix VII: KEMR-SERU

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) 2722541, 2713349, 0722-205901,0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **October 29, 2020**

TO: **DENNIS WAFULA MUKHWANA,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CBRD,**
NAIROBI.

Dear Sir,

Re: **PROTOCOL NO. KEMR/SERU/CBRD/205/3968 *(RESUBMISSION II OF INITIAL SUBMISSION)*: *IN VITRO* AND *IN VIVO* EVALUATION OF ANTILEISHMANIAL ACTIVITIES OF *OLEA EUROPAEA, KIGELIA AFRICANA, TERMINALIA MOLLIS, CROTOLN MACRSTACHYUS* AND *BRIDELLA MICRANTHA. (VERSION 3.0 DATED 12 OCTOBER 2020)***

Reference is made to your letter dated October 14, 2020. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on October 14, 2020.

This is to inform you that the Committee notes that the following issues raised during the 295th Committee B meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on **January 22, 2020** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **October 29, 2020** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **October 28, 2021.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **September 16, 2021.**

Please note that only approved documents including (informed consents, study instruments, Material Transfer Agreement) will be used. You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

Appendix VIII: APPROVAL LETTER

M

ASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PHD/SC/00045/018

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 18th December, 2020

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR DENNIS WAFULA MUKHWANA - PHD/SC/00045/2018

The above named is registered in the Doctor of Philosophy Programme in the School of Physical and Biological Science, Maseno University. This is to confirm that his research proposal titled "*In Vitro and in Vivo Evaluation of Antileishmanial Activities of Olea europaea, Kigelia Africana, Terminalia mollis, Croton macrostachyus and Bridella micrantha.*" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

SCHOOL OF GRADUATE STUDIES * MASENO UNIVERSITY * 18 DEC 2020 * P.O. BOX BAG, MASENO

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES