

**ANTIPLASMODIAL ACTIVITY, CYTOTOXICITY AND PHYTOCHEMICAL
COMPOSITION OF *JUSTICIA BETONICA*, *VERNONIA DUMICOLA* AND
ROTHECA MYRICOIDES AND THEIR COMBINATIONS**

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the Degree of Master of Science (Applied Parasitology) in the School of Pure and
Applied Sciences of Kenyatta University**

MAY, 2019

DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other institution or university.

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DEDICATION

This work is dedicated to God Almighty, my family and friends for their support and encouragement in my education.

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ABBREVIATIONS AND ACRONYMS

CDC	Centre for Disease Control
FBS	Foetal bovine serum (newborn calf serum)
G6PD	Glucose-6-Phosphate Dehydrogenase
IC₅₀	Half Maximal Inhibitory Concentration
JB	<i>J. betonica</i> aerial parts
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
RBCs	Red blood cells
RDT	Rapid diagnostic test
RML	<i>R. myricoides</i> leaves
RPMI	Royal Park Memorial Institute (culture medium)
SI	Selectivity index
Spp	Species
VDL	<i>V. dumicola</i> eaves
WHO	World Health Organization

ABSTRACT

Malaria, a disease caused by *Plasmodium* species is currently the main cause of human sickness and death in the world, more so in the tropics. It mostly affects pregnant mothers and children below five years of age. The parasites develop resistance to antiparasmodial drugs and this has been a challenge in control and treatment of the disease through chemotherapy, something that necessitates the continuous search for alternative antiparasmodial agents. The use of plants to treat malaria and other ailments has been there since time immemorial. The plants: *J. betonica*, *V. dumicola* and *R. myricoides* are among the many that are used traditionally to treat malaria. In spite of this use, their antiparasmodial activity and toxicity has neither been scientifically confirmed nor evaluated. The World Health Organization suggests scientific evaluation of the safety of medicinal plants though they are generally regarded as safe. There are no reports on the scientific evaluation of the safe and effective concentration of these extracts. This study aimed at determining the antiparasmodial activity, cytotoxicity and qualitative phytochemical composition of aqueous extracts of *J. betonica* aerial parts, and leaves of *V. dumicola* and *R. myricoides*. The plant materials were collected from Gucha region, Kisii County and transported to Kenyatta University. *In vivo* antiparasmodial activity of the aqueous extracts of the plants was tested in a four day suppressive assay using *Plasmodium berghei* in mice. *In vitro* antiparasmodial activity was done using the D6 strain of *Plasmodium falciparum*. Cytotoxic evaluation was done using the vero cell-line *in vitro* using the MTT assay. Qualitative phytochemical screening was done according to standard protocols. The aqueous extract of *R. myricoides* leaves had the highest chemosuppression with an IC_{50} value of $1.32 \pm 0.03 \mu\text{g/ml}$ while lowest chemosuppression was in the combined aqueous extract of *J. betonica* aerial parts and leaves of *V. dumicola*. This recorded an IC_{50} value of $11.03 \pm 1.18 \mu\text{g/ml}$. For cytotoxicity studies, aqueous extract of *J. betonica* aerial parts recorded the lowest cytotoxic activity with IC_{50} value of $690 \pm 11.00 \mu\text{g/ml}$ whereas aqueous extract of *R. myricoides* leaves recorded highest cytotoxic activity with IC_{50} value of $106.00 \pm 8.00 \mu\text{g/ml}$. According to the selectivity index (SI) criteria, all the extracts showed SI value above 3 indicating the plant extracts were selective. All the plant extracts tested contained anthraquinones, phenols and tannins. They all showed antiparasmodial activity which could be attributed to the phytochemicals observed. The three plants analysed in this study are recommended for development of antimalarial agents.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria infection is caused by *Plasmodium* species parasites which are transmitted by female *Anopheles spp* of mosquitoes. The severity of the disease in human beings varies widely depending on the parasite species involved i.e. *P. falciparum*, *P. vivax*, *P. ovale* or *P. malariae*. Other factors include the genetics, immune status and age of the host (Pasvol, 2004). An estimate by the World Health Organization (WHO) indicates that there were nearly 219 million cases of malaria worldwide, causing more than 435,000 deaths in the year 2017 (WHO, 2018). Out of all the malaria cases, Sub-Saharan Africa had approximately 200 million (92%) causing 404,550 (93%) deaths. Pregnant mothers and children below 5 years of age contributed around 85% of the cases (KFF, 2013; WHO, 2018).

Malaria leads to increased financial expenses for the affected families and it hinders economic development of the affected communities and nations (WHO, 2012). It causes loss of nearly 170 million working days every year (Sarkar, 2016). It is the leading infectious disease in Kenya resulting in the highest morbidity and mortality in the country. Over 25 million people are prone to malaria in Kenya and thirty to fifty percent of all outpatients and 19 percent of all inpatients in health-care facilities in the country suffer from malaria (*Kioko et al.*, 2013). The disease also causes 20 percent of all mortalities in children aged below five years. Malarial parasites have over the years become resistant to the drugs in use, even artemisinin (Afonso *et al.*, 2006) which are currently being used. This necessitates the continuous search for other malaria treatments (*Nethengwe et al.*, 2012).

Many people prefer phytomedicine to conventional medicine particularly those with limited income. This is because the side effects and the cost of treatment are lower than for allopathic medicine (Bruno, 2013). The socio-economic status of any given society determines its capacity to control the parasitic diseases affecting it (Ukoli, 1992). The underdeveloped and impoverished nations in tropical Africa should explore cheaper alternatives to the conventional procedures which should be just as effective. Malaria is highly endemic in developing countries and is a leading cause of poverty. Countries having a low gross national income record the highest malaria-related deaths. Countries in which majority of the people are poor record more mortalities due to malaria. Children having the highest parasite prevalence rates are often found in poorer populations and in rural areas (WHO, 2012).

There is collaboration between the World Health Organization and its affiliate states to boost the use of traditional medicine in treatment and management of malaria. This is aimed at making national health systems incorporate use of traditional medicine in their policies (WHO, 2002). This will include policies to regulate products, practices and providers. Secondly, based on existing evidence, the policy should ensure that the products and practices used in traditional medicine are safe. The third goal is to recognize traditional therapies in mainstream medicine. The last goal is to make patients safe by equipping traditional medicine healers with the additional knowledge and skills needed to safeguard the indigenous knowledge and resources (WHO, 2002). However, there are no clear procedures to be used in clinically testing the traditional therapies as their development is overlooked. The clinical evaluation of herbal medicine should include efficacy, toxicity profiles, mutagenic effects, effect on

age and pregnancy, combined therapy with conventional anti-malarial drugs and determination of development of resistance (Ginsburg and Deharo, 2011).

Most people in developing countries treat many kinds of diseases using medicinal plants (Ganga, 2012). These countries have inferior health care systems and inadequate financial resources to run these facilities all because of poverty. This makes the people to revert to cheap and available traditional medicine to treat their ailments. The use of phytomedicine has been on the increase in developed countries with about 40% of the people using medicinal plants to treat medical conditions of diverse kinds (Mutuku *et al.*, 2013). About 90% of Kenyans have at one time treated their ailments with medicinal plants (Adongo *et al.*, 2012). However, ethnomedicine has been made unattractive by some practitioners who charge their clients and administer drugs for diseases they cannot treat (Kokwaro, 2009). For this reason, scientific evidence needs to be provided so as to demonstrate the efficacy and safety of medicinal plants as anti-disease agents.

There is a general perception that medicinal plants are very safe and have no adverse side effects: which is untrue and misleading (Canter and Ernst, 2004). Many of them contain compounds which have cytotoxic and genotoxic effects (Rietjens *et al.*, 2005). Others cause kidney failure and cancer (Bieler *et al.*, 1997), while some are hepatotoxic and neurotoxic (Varlibas *et al.*, 2009). The toxicity is mainly due to alkaloids such as aconitine, mesaconitine and hyaconitine in them (Xu *et al.*, 2005). Therefore the idea that plant medicines are efficacious (Ekor, 2013) may not suffice without assurance of their safety. The current study evaluated the antiplasmodial activity of the extracts as well as their cytotoxicity on vero cells.

1.2 Statement of the problem

In spite of many programs to eradicate malaria, the disease continues to be a big cause of sickness and death worldwide (Bruno, 2013). Bioscreening has not been done for quite a large number of the plants used to treat malaria in order to determine their antiparasmodial activity. *J. betonica*, *V. dumicola* and *R. myricoides* are examples of such plants. Further, development of resistance to available antimalarial drugs has been reported.

There is need to evaluate medicinal plants used in traditional malaria treatment to confirm their efficacy. There is also need to determine the safety of the herbal medicine.

The aim of the study was to evaluate the antiplasmodial activity, cytotoxic profile and qualitative phytochemical composition of aqueous extracts of *V. dumicola* and *R. myricoides* leaves and *J. betonica* aerial parts to confirm their potential for malaria treatment.

1.3 Justification of the study

There is urgency for search of antimalarial drugs due to the upsurge of malaria incidences globally. Application of conventional antimalarial drugs as a remedy for malaria is limited by the development of drug resistance in the parasites. Approximately, 75% of the population in the developing world resort to use of traditional medicine since they lack access to modern conventional drugs (Were, 2010; Njagi *et al.*, 2016).

However, most of the plants have not been bioscreened for their activity. *J. betonica*, *V. dumicola* and *R. myricoides* are some of the plants used in Kenya for treatment of malaria, though their antiplasmodial potential has not been evaluated and validated. They are readily available and affordable to local communities. Therefore there is a need to confirm the antiplasmodial potential scientifically. Given that malarial parasites resist existing drugs, it becomes necessary to evaluate alternative sources of treatment for development of new effective antimalarial drugs. Further, some antimalarial drugs used currently are costly and not adequately available.

1.4 Research Questions

- i. What are the effects of aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* on the growth of *P. berghei* and *P. falciparum*?
- ii. What are the cytotoxic effects of aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* against vero cells?
- iii. Which phytochemicals are found in the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides*?

1.5 Hypothesis

- i. Aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* do not have effects on the growth of *P. berghei* and *P. falciparum*.
- ii. Aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* have no cytotoxic effects on the proliferation of vero cells.

- iii. Aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* have no phytochemicals.

1.6 Objectives

1.6.1 General Objective

To determine the phytochemical composition, antiplasmodial and cytotoxic activity of aqueous extracts of the aerial parts of *J. betonica*, and leaves of *V. dumicola* and *R. myricoides*.

1.6.2 Specific Objectives

- i. To determine the antiplasmodial activity of aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* *in vitro* and *in vivo*.
- ii. To determine the cytotoxicity of aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides* against vero cells.
- iii. To determine the phytochemical composition of aqueous extracts of the aerial parts of *J. betonica* and leaves of *V. dumicola* and *R. myricoides*.

1.6.3 Significance of the study

The study results will help users develop confidence in the plants being studied as efficacious and safe antiplasmodial remedies. The results form the basis for further research in other aspects such as mutagenicity, age- and pregnancy-dependent effects and possible evolution of resistance. Also they will play a role in development of antiplasmodial agents from the plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of Malaria

The causal agent of malaria is a protozoan parasite belonging to the genus *Plasmodium*. The four species known to cause human malaria are *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The parasites are transmitted through the bites of female *Anopheles* mosquitoes. The severity of the disease in human beings is influenced by a number of factors. These are parasite species and host-related factors such as genetics, immune status and age (Pasvol, 2004). These factors have critical impacts on all aspects of the disease including epidemiology, pathogenesis, clinical presentation and treatment. *P. simiovale* and *P. knowlesi* are simian parasites that have been reported to infect humans, but on rare cases (Wiser, 2018).

2.2 The Life Cycle of *Plasmodium* species Affecting Humans

Human beings and female *Anopheles* mosquitoes are the two hosts involved in this cycle. An infected female *Anopheles* mosquito injects sporozoites (the infective stage) into a human host during feeding (Chang *et al.*, 2013). The sporozoites infect liver cells and grow into schizonts. Bursting of the liver cells releases merozoites that invade erythrocytes and multiply asexually. They differentiate into ring stage trophozoites that further transform into schizonts, which rupture to release merozoites (Wirth, 2002; Cowman and Crabb, 2006; Arnot *et al.*, 2011; Soulard *et al.*, 2015). In *P. vivax* and *P. ovale* a stage of dormancy known as hypnozoites may remain in the liver and cause relapses of malaria after a long time (Arnot *et al.*, 2011) (Fig 2.1).

The parasites undergo differentiation during the sexual erythrocytic stages to form gametocytes; the infective stage for the mosquito. The mosquito ingests the gametocytes when it sucks blood from an infected human being. While in the mosquito's mid-gut, the microgametes generate zygotes which are motile and elongate and are known as ookinetes. The zygotes enter the mid-gut wall of the mosquito and grow into oocysts. Ruptured oocysts release sporozoites which invade the mosquito's salivary glands. When the mosquito takes a blood meal it inoculates the sporozoites into another human host thereby perpetuating the life cycle (Baton *et al.*, 2005). Other means for transmission of malaria parasites include blood transfusions (Owusu-Ofori *et al.*, 2010).

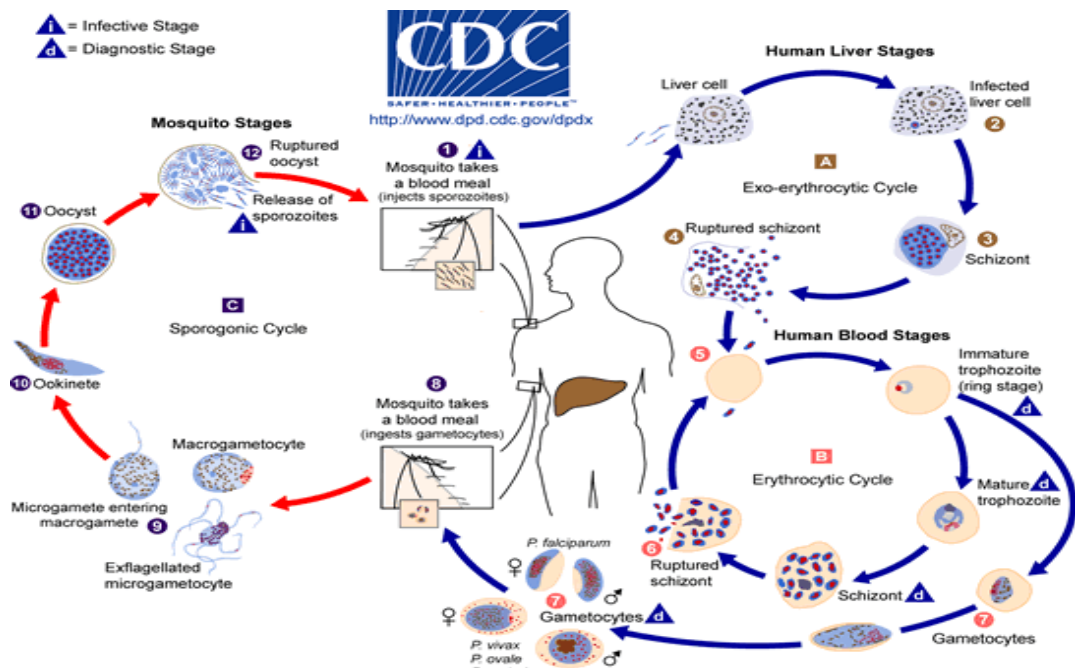


Figure 2.1: Life cycle of malaria parasite (CDC, 2012)

2.3 Epidemiology of Malaria

In 1990, there were 350 to 550 million malaria cases and 1 million deaths in the world (Murray *et al.*, 2012). In 2010, 219 million people were sick and 660,000 died of malaria (World Malaria Report, 2012). Children below five years are the most

affected group, accounting for 65% of the total cases (Murray *et al.*, 2012). The sub-Saharan region of Africa carries a risk population of about 125 million pregnant women and the annual infant mortality related to this is estimated at 200,000 (Hartman *et al.*, 2010). In Western Europe, there are approximately 10,000 malaria cases while in United States 1300–1500 are reported yearly. In Europe around 900 people died of malaria within the ten year period of 1993 to 2003 (Kajfasz, 2009). The worldwide occurrence of malaria has shown a drop in the past few years (WHO, 2012).

Regional distribution of malaria within broad geographical regions is complex. Areas having malaria and those where there is no malaria closely exist (Greenwood *et al.*, 2002). The disease commonly occurs in the tropics. This is partly favoured by the climate of these regions which is characterized by rainfall that is consistent with high temperatures and high humidity. Stagnant water is a common feature of this region that favours rapid maturity of mosquito larvae and continuous breeding (Jamieson *et al.*, 2006). Mapping rainfall has facilitated prediction of malaria outbreaks in drier areas with significant accuracy (Abeku, 2007).

In some regions malaria afflicts country side people more than those who dwell in big towns (Cui *et al.*, 2012). The case is different in Africa where malaria is common in all places, though on a higher scale in rural areas (Machault *et al.*, 2011).

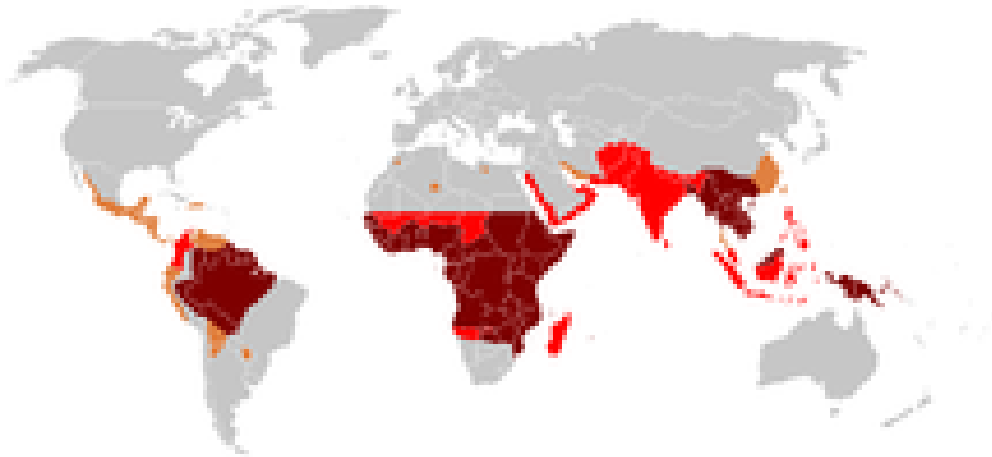


Figure 2.2: Global distribution of malaria (CDC, 2010)

◆ High occurrence ◆ Medium occurrence ◆ No chloroquine-resistance ◆ Malaria absent

Malarial parasites are variedly distributed geographically. *P. falciparum* is the most common in tropical Africa and some parts of Asia. *P. vivax* is widely spread mainly in India, South America, the Middle East and North Africa. *P. ovale* is found predominantly in West Africa but also in Asia. *P. malariae* is distributed worldwide, although most cases occur in Africa (Pasvol, 2004).

The epidemiology of malaria depends upon a complex interplay between the host (humans), vector (mosquito) and malarial parasite. Population density and prevalence of infection among children are important factors because children tend to have both high parasitaemia and burden of gametocytes, which are the infective stage. Paradoxically, in the 2 weeks after effective treatment of severe malaria, the number of gametocytes in the blood rises so that while the patient improves clinically, mosquitoes biting the patient during this time are more likely to transmit infection (Pasvol, 2004).

The longevity of the mosquito is also important in the spread of malaria because it needs to be of sufficient duration to allow full development of the parasite. Ambient temperatures have a major impact, because high temperatures significantly shorten this period of maturation in the mosquito (the extrinsic incubation period) and increases transmission. Seasonal rainfall dramatically increases the breeding of mosquitoes. Where malaria prospers, human societies prosper the least and malaria closely correlates with poverty. The effects of malaria are felt on diverse areas including fertility, population growth, economic burden, children and infant mortality, worker output, absenteeism, premature deaths and medical expenditure (Pasvol, 2004).

2.4 Diagnosis of Malaria

The symptoms in the patient presented at the time of examination form the basis of clinical diagnosis of malaria. Clinical findings for malaria infection have to be confirmed by a laboratory test. The most preferred method for laboratory confirmation of malaria is microscopic examination (Luxemburger *et al.*, 1998; Tangpukdee *et al.*, 2009). A patient's blood smear is prepared on a microscope slide then stained with Giemsa stain to make the parasites distinct and easy to observe. The smear is then viewed under the microscope to identify the distinctive parasite (Reyburn *et al.*, 2007).

There are other methods used to diagnose malaria besides microscopy and they involve detection of the parasite derived antigens. Molecular diagnosis technique such as polymerase chain reaction (PCR) can be used to detect parasite nucleic acids in the patient's blood sample. This method is used for confirmation the *Plasmodium* species

after microscopy or Rapid Diagnostic Test diagnosis (Nizamuddin, 2009; Global Health, 2012).

2.5 Clinical Manifestations

Patients having malaria infection present with acute febrile illness characterized by periodic fever paroxysms appearing at either 48 or 72 hour intervals. Non-specific prodromal symptoms can occur for all four species in the first days of infection before the first febrile attack. The symptoms include headache, anorexia, mild fever, nausea, muscle pain and lassitude. Next, febrile attacks ensue occurring at intervals of forty eight hours for *P. vivax*, *P. ovale*, and *P. falciparum* while for *P. malariae* they will appear at 72-hour periodicity. Splenomegaly, hepatomegaly and haemolytic anaemia may appear during malaria paroxysms. Malaria paroxysms last about four to eight hours. Chills suddenly set in whereby the patient feels very cold despite experiencing high temperatures. This condition is often known as the cold stage which is shown by vigorous shivering. The hot stage follows where the patient feels very hot with severe headache. Features of the hot stage include fatigue, dizziness, anorexia, myalgia and nausea. After these manifestations, a lot of sweating ensues and the fever starts to decrease (Wiser, 2018).

2.6 Pathogenesis and Severe Malaria

Pathogenesis of malaria is associated with the bursting of infected red blood cells and the release of parasite components and metabolites, and cellular debris. Malaria infection causes high activity of organs such as the liver and spleen. Severe malaria causes pulmonary oedema, acute renal failure, cerebral inflammation, severe anaemia and/or bleeding. The common metabolic complications include acidosis and hypoglycaemia. Cerebral malaria impairs the patient's conscious. There is severe

headache and confusion may follow. The patient feels drowsy, may convulse and go into coma. All these neurological presentations are caused by blockage of tiny blood vessels in vital organs such as the brain, gut, placenta, lungs and heart due to cytoadherence of trophozoite- and schizont-infected erythrocytes. Antigenic variation increases pathogenesis and disease severity (Clark *et al.*, 2003; Trampuz *et al.*, 2003; 2004; Idro *et al.*, 2005).

2.7 Prevention and Control of Malaria

2.7.1 Control of Malaria Vector

2.7.1.1 Insecticidal-treated Bed Net (ITNS)

Insecticidal treated nets are highly effective and cheap methods of controlling malaria. These nets are made in such a way that they repel or kill mosquitos that come into contact with them (WHO, 2007). Insecticide-treated nets come in two groups i.e. long- lasting insecticidal nets (LLINs) and conventionally treated nets (WHO, 2007). Conventionally treated nets are made by treating with insecticides recommended by WHO. Examples of such insecticides are pyrethroids like permethrin, deltamethrin, lambda-cyhalothrin and cypermethrin. These insecticides fast get degraded when exposed to UV-radiation but they relatively last longer when kept out in sunlight. Treatment should be repeated again after three washes to maintain insecticidal activity (Takken, 2002; Palmquist *et al.*, 2012).

Long-lasting insecticidal nets (LLINs) are mosquito nets treated in the factory by incorporating insecticide within the net fibres (WHO, 2007). They are recommended for use under field conditions for three years and should retain insecticidal activity with no more treatment for not less than twenty washes under laboratory conditions according to WHO protocols (WHO, 2007).

Expectant mothers and children are the most susceptible target in malaria-afflicted areas. Community based randomized controlled trials (RCT) of use of mosquito nets have been implemented in malaria endemic regions. These initiatives have reduced child mortality by 20% in duration of 2 years, causing up to 50% increase in use of ITNs (Binka *et al.*, 1996; Nevill *et al.*, 1996; Lengeler, 2000). This implies that insecticide treated nets used to control malaria have yielded good results. However, mosquitos have developed resistance to insecticides (Edi *et al.*, 2012).

2.7.1.2 Indoor Residual Spraying (IRS)

Malaria vector control by use of insecticides has been revived of late, partly due to application of indoor residual spraying (IRS) technique. Indoor residual spraying includes controlled insecticide spraying inside the walls of the house to kill mosquitoes. Organophosphates (malathion, fenitrothion and pirimiphos-methyl), organochlorine (DDT: dichloro-diphenyl-trichloroethane), carbamates (bandiocarb and propoxur) and pyrethroids (lambda-cyhalothrin, alpha-cypermethrin, etofenprox, cyfluthrin, and bifenthrin) are some of the insecticides approved by WHO for indoor residual spraying (WHO, 2006; WHO, 2015). In 2009, this method contributed to reduction of malaria by offering protection to 75 million people (WHO, 2010; Kim *et al.*, 2012).

Popularity of use of some of IRS has reduced, partly due to safety of some of the insecticides. For instance, DDT toxicity profiles from animal models and epidemiologic information show that there could be previous unknown long-term unhealthy impacts due to exposure to these insecticides, especially DDT. This has led to limited government commitment and funding to propel IRS programs. Similarly,

some communities have rejected use of the insecticides (WHO, 2006; Jager *et al.*, 2006; Longnecker *et al.*, 2007; Bornman *et al.*, 2009). Therefore, it is imperative to search for safer antimosquito insecticides that are acceptable to society and less toxic or have no toxic effects.

2.7.1.3 Outdoor Fogging

This is a technique in which adulticides are applied to outdoor environment with high mosquito density where there is a risk of mosquito borne diseases. Fogging is majorly restricted to regions and situations of emergencies like epidemics. There are three types of fogging namely thermal fogging, cold fogging and residual fogging. In thermal fogging, the insecticide is heated and then spreads in the air. It diffuses and reaches air spaces that are blocked by heavy vegetation. The insecticide fog kills fleeing mosquitoes that encounter it. Thermal fogging is effective against biting adult mosquitoes but has no lasting residual activity.

Cold fogging involves production of cold fog by breaking the insecticide mechanically into microscopic droplets under pressure and microfine nozzle. It allows use of small amounts of the insecticide since it can produce highly concentrated fogs but its limitation is that it is not effective in penetrating dense vegetation and obstacles. Residual fogging involves formation of spray with big droplets that settle very quickly on the surface of the treatment area. Residual fogging has long residual activity that lasts several weeks depending on the insecticide used. For outdoor fogging to have an impact, proper timing and repeated application is required. It also requires well trained personnel and expensive equipment besides being costly to maintain (Tarwish, 2015). Therefore, it is necessary to explore new paths and ways to control the malaria pandemic.

2.7.2 Malaria Chemotherapy

2.7.2.1 Artemisinin-based Combination Therapy (ACT)

Due to resistance of *Plasmodium* species to existing drugs used to treat malaria, ACTs came into use as the drug of choice following recommendation by the World Health Organization (WHO, 2006). Artemisinins are highly effective, rapid acting and well-tolerated. They lower the spread of the disease besides slowing down development of resistance (WHO, 2001). Artemisinin chemical structure is a sesquiterpene lactone with a peroxy group. They are insoluble in water and oil with only oral and rectal formulations in clinical use (Price *et al.*, 1996). The peroxide bond of artemisinin is stable during some chemical reactions that make it possible to develop oil and water-soluble derivatives namely dihydroartemisinin, artemether and artesunic acid (Navaratnam *et al.*, 2000).

Being hydrophobic artemisinin easily penetrates biological membranes (Agustijns *et al.*, 1996). Its mechanism of activity includes binding to haem, either in haemoglobin or in haemozoin through an iron-mediated cleavage of the peroxide bridge, generating AR free radicals. These radicals destroy the cell membrane of the parasite among other membranes (Asawamahsakda *et al.*, 1994). Absorption lag-time of Artemisinin is 0.5-2 hours after oral administration and peaks in plasma concentrations at 1-3 hours after administration. It is enzymatically metabolized to inactive metabolites by removing the peroxide bridge (Lee *et al.*, 1988). Only trace amounts can be detected in the urine of healthy volunteers and malaria patients after administering the drugs orally (Navaratnam *et al.*, 2000). One of its major limitations is a relatively short half-life of 1-3 hours (Gordiet *et al.*, 2002).

2.7.2.2 Aminoquinolines

Aminoquinolines are a class of antiparasitic drugs with an amine group that substitutes a carbon atom in the molecular structure. They include a number of drugs namely chloroquine, primaquine and amodiaquine (Simooya *et al.*, 1998). The use of chloroquine as the standard drug in places endemic with malaria has been limited by resistance from these areas. It has no activity against sporozoites, hypnozoites and gametocytes, but it is very potent against erythrocytic stage of *Plasmodium* species (Ginsburg, 1999). Chloroquine is basic and has no charge at neutral pH but has a positive charge at acidic pH. It is a lysosomotropic drug that diffuses via the plasma and lysosomal membrane into the lysosomes of the *Plasmodium* species. In the lysosomes it is charged and remains inside the acidic compartment of the parasite lysosomes.

Haemoglobin is metabolized by intracellular trophozoite of the parasite, generating ferriprotoporphyrin IX (FP) and a free haem (Ginsburg, 1999). Ferriprotoporphyrin IX precipitates in the parasite's lysosomes appearing as black malaria pigment. In the process of pathogenesis, FP should be converted to haemozoin that is harmless, by plasmodial haem polymerase. The mechanism of activity of chloroquine is by blocking the activity of this enzyme resulting in accumulation of haem, which is toxic to the parasite. Side effects associated with chloroquine intake includes: nausea, blurring vision, headache, vomiting, diarrhoea, psychoses, rashes, pruritis and urticarial symptoms (Simooya *et al.*, 1998).

2.7.2.3 Folate Antagonists

Antifolates act by inhibiting the synthesis of folate that is an important cofactor in DNA replication. They act as competitive inhibitors of the dihydropteroate synthetase, dihydrofolate reductase and DHFR-thymidylate synthetase that are involved in synthesis of folate, resulting in inhibition of folate synthesis. This leads to decreased synthesis of pyrimidines, resulting to inhibition of DNA replication, reduced methionine synthesis and low conversion of glycine to serine, leading to cell cycle halt and eventually parasite death (Plowe *et al.*, 1998). Commonly used antifolate drugs against malaria are pyrimethamine, proguanil and the sulfa drugs namely sulfonamide and sulfadoxine (Müller, and Hyde, 2010).

2.7.2.4 Malaria Drug Resistance

Antimalarial drugs are one of the most popularly prescribed drugs in the tropics. The major cause of widespread antimalarial drug resistance has been due to the widespread use of these drugs. The incidence of malaria resurgent emergence is attributable to the fact that *P. falciparum* resists antmalarial drugs except artemisin. Development of resistance to artemisinin would mark an era of untreatable multidrug resistant malaria (Mark, 1998; Hastings and D'Alessandro, 2000; WHO, 2001). Other causes are lack of complete therapy due to poor compliance, long drug half-life and self-treatment. When the level of transmission is high, the patient can be re-infected when the drug is at a level that cannot treat the infection (Hyde, 2007; Wiser, 2018).

Resistance to antiplasmodial drugs basically is due to mutations in the parasite genome that may not be influenced by the drugs. The mutations might be as a result of single or multiple unlinked (multigenic) genetic events. *P. falciparum* resistance to

chloroquine can be described as multigenic occurring in gene encoding transporters *PfCRT* in the parasite (Plowe, 2003). Single point mutations in the genes encoding for cytochrome *b* (*cytB*) and dihydrofolate reductase (*dhfr*) confer resistance to atovaquone and pyrimethamine respectively (Ecker *et al.*, 2013). This resistance makes it necessary to explore new possibilities for development of new antiparasmodial drugs.

2.8 Role of herbal medicine in treatment of malaria

Medicinal plants continue to provide promising leads as sources of antimalarial drugs. A number of medicinal extracts have been screened for antiparasmodial activity and have shown promising results. Methanolic leaf and stem extracts of *Uvariopsis congolana* have demonstrated antiparasmodial activity with IC_{50} value of $4.47 \pm 0.45 \mu\text{g/mL}$ and $4.57 \pm 0.76 \mu\text{g/mL}$. Methanolic extract of *Buxus hyrcana* aerial parts has been shown to have antiparasmodial activity, recording IC_{50} value of $7.7 \mu\text{g/mL}$ (Boyom *et al.*, 2009; Esmaeili *et al.*, 2009). Chloroethane leaf extract of *Lantana camara* was active with an IC_{50} value of $5.70 \pm 1.60 \mu\text{g/mL}$. Ethylacetate *Siphonochilus aethiopicus* rhizomes give an antiparasmodial activity with an IC_{50} value of $1.4 \mu\text{g/ml}$ (Jonville *et al.*, 2008; Lategan *et al.*, 2009). Chloroform-soluble fraction from methanolic extracts of *Carpesium rosulatum* has been found to have antiparasmodial activity with IC_{50} of $8.2 \mu\text{g/ml}$ (Moon, 2007). Various studies have screened antiparasmodial activity of medicinal plants. However, enormous numbers of medicinal plants used in malaria management traditionally have not been screened to confirm their activity.

Quinine is one good example of malaria chemotherapeutic agents with medicinal plant origin utilized in conventional medicine. Quinine is an aminoquinoline alkaloid developed from *Cinchona* species (Rubiaceae) and is still in use for malaria

management though cases of resistance have been reported. *Artemisia annua* has been the sole source for commercial production of artemisinin since its discovery (El-Naggar *et al.*, 2013).

2.9 Phytochemicals in Medicinal Plants

Phytochemicals occur naturally in plants as secondary metabolites which offer protection to plants against pests and diseases. They are classified as non-essential nutrients to plants, suggesting that they are not required for plant growth but are necessary for survival as defense mechanisms (Doughari *et al.*, 2009).

Phytochemicals are classified into broad classes mainly alkaloids, phenolics, terpenes, flavonoids, anthraquinones and tannins, among others. Alkaloids are basic in nature and the level of basicity depends on the number and location of hydrogen atoms in the compound (Kennedy and Wightman, 2011). Alkaloids have contributed to a variety of compounds with significant health and social application namely atropine, hyoscyamine, scopolamine, nicotine, opiates, caffeine, ephedrine, cocaine, galantamine, physostigmine, huperzine and rivastigmine (Zenk and Juenger, 2007; Mukherjee *et al.*, 2007).

Flavonoids are naturally occurring water-soluble plant secondary metabolites. The flavonoid family comprises six main sub-groups namely chalcone, flavonol, flavone, flavanone, isoflavonoids and anthocyanins (Galeotti *et al.*, 2008). In plants, they are responsible for flower pigmentation to attract pollinators. They are also useful as UV filters, chemical messengers, cell cycle inhibitors and physiological regulators. They

have been demonstrated to possess antioxidant, anti-inflammatory, anti-allergic, antiviral and anticancer properties (Tapas *et al.*, 2008).

Tannins are water-soluble polyphenols naturally occurring in plants. They are characterized by high molecular weight and astringent properties, with the ability to bind and shrink biomolecules such as proteins and nucleic acids (Ashok and Upadhyaya, 2012). Attention to tannins medicinal value has heightened due to the rise in various human ailments such as malaria, cancer and HIV/AIDS (De Bruyne *et al.*, 1999; Dolara *et al.*, 2005). Tannins have antioxidant, anti-inflammatory, diuretic, antitumor, antiseptic and haemostatic properties (Dolara *et al.*, 2005).

Research has shown that some phytonutrients have medicinal significance towards treatment of various human ailments. Herbal plants are viewed as an emerging solution to a number of human ailments majorly due to their pharmacological activities. The pharmacological activities are conferred to them by the presence of the phytochemicals (Doughari *et al.*, 2009). With the increasing human diseases, medicinal plants are important in making therapeutics. Some conventional antimalarial drugs are not accessible, unaffordable and adversely affect the users. The resistance of *Plasmodium* species to drugs has threatened the future of current antiplasmodial drugs (Cui *et al.*, 2015). Therefore, there is essence in bioscreening of herbal plants for development of new antiplasmodial therapeutics.

2.10 Experimental Plants Used in the Study

2.10.1 *Justicia betonica* (L.)

J. betonica (L.) (Acanthaceae) is commonly known as squirrel's tail. It is a perennial herb natively found in Africa and tropical Asia (Plate 2.1). It grows fast and may reach

a height of 5 or more feet (Plate 2.1) (Kinsey, 2013). *J. betonica* is regarded as an environmental weed that has escaped cultivation. It grows along roadsides, urban bushlands and waste areas with preference to damp sites and waterways (Deedi, 2016). It is a native of eastern and southern Africa, including Mozambique, Namibia, Kenya, South Africa, Lesotho and Swaziland. The plant has become naturalized to coastal areas of Australia and Wales. Also, it has become an inhabitant of pacific islands namely Hawaii, French Polynesia, New Caledonia and Niue through naturalization. Other common names are White Shrimp Plant and Paper Plume (Glover *et al.*, 1966; Deedi, 2016).



Plate 2.1: *Justicia betonica* aerial parts

Leaves and flowers of *J. betonica* are used by the Nandi people to treat cough, orchitis and stomach upsets (Jeruto *et al.*, 2008). The Luhya and Kipsigis use it to treat snake bites, orchitis and venereal diseases (Kokwaro, 2009). It is used to treat malaria by the Gusii people (Siso, 2007) and also in some parts of southwestern Uganda (Stangeland *et al.*, 2011). Reports have indicated that *J. betonica* has previously been used in Uganda for treatment of fever, soar throat, malaria and intestinal parasites (Bukonya *et al.*, 1997; Katuura *et al.*, 2007).

2.10.2 *Rothea myricoides*

R. myricoides (Hochst) R.Br. ex Vatke (Lamiaceae) is commonly referred to as “Blue cat’s whiskers”. It is a shrub whose leaves are opposite or 3-4-whorled. The leaves are usually crowded near the branch ends, ovate to broadly elliptic (Plate 2.2). The decurrent leaf base obscures the petiole and the margin is serrated. Flowers are found in few-flowered terminal and axillary heads; they are purple-blue and white with long exerted stamens. Fruits are 2-4 lobed, surrounded by the spreading calyx lobes when young and red to black when ripe. It grows on rocky places, along streams and at the edges of evergreen forests. It is found in many African countries such as Angola, Botswana, Namibia, Burundi, DRC, Rwanda, Kenya and South Africa (Hyde *et al.*, 2014). Other common names include butterfly-bush, losstrauch and afrikansklerodendrum (Fernandes and Verdcourt, 2000).



Plate 2.2: *Rothea myricoides* leaves

People use different parts of *R. myricoides* to treat ailments. The bark, roots and leaves have been traditionally used to treat malaria, rabies, gonorrhoea, abdominal pains, snake bites, glandular TB, measles, colic, asthma, eye disease, wound dressings, hemorrhoids and as an aphrodisiac (Abebe *et al.*, 2003; Kebede *et al.*,

2011). Bathing over the steam from boiled leaves of *Clusia abyssinia* and *R. myricoides* can be useful in treatment of epilepsy. Generally the plant is useful in management of toothache and mental disorder (Dessissa and Binggeli, 2000).

2.10.3 *Vernonia dumicola*

V. dumicola (S. Moore) (Asteraceae) is a small shrub 60–250 cm tall with an unbranched stem having velvety-tomentose and dark spots (Plate 2.3). It grows within an altitude range of between 1100–2200 m. Leaves have short petioloids and toothed margins (Global Plants, 2007). The plant has intense purple flowers (Tarwish, 2015). It is found in many African countries including Kenya, Tanzania, Uganda, Sudan, Rwanda and Congo. In Tanzania the plant is found in Mwanza District while in Uganda it is found in Masaka District. In Kenya, the plant is a natural inhabitant of Trans-Nzoia and Kisii Counties (Beentje, 2000). Common names include brownie and kaessnan (Beentje, 2000).



Plate 2.3: *Vernonia dumicola* leaves

V. dunicola leaves have been used to treat wounds, malaria, epilepsy, hepatitis, bacterial and viral skin diseases, gastro-intestinal tract parasites and as an aphrodisiac (Toyang and Verpoorte, 2013). Ethnopharmacological studies have documented that several species of the genus *Vernonia* including *V. dunicola* contain many medicinal properties such as wound healing, antidiabetic, diuretic and boosting of immunity. Ailments treated include leprosy, scabies, rheumatic pain, hysteria, epilepsy, gastric and intestinal ulcers, respiratory tract disorders, hepatitis and cancer. (Toyang and Verpoorte, 2013; Ahmed *et al.*, 2014). Therefore, this suggests that the genus has enormous potential for development of therapeutic agents. The ethno-botanical information about the three plants: *J. betonica*, *R. myricoides* and *V. dunicola* shows that they have medicinal properties, including use as antiplasmodial remedies. This needed to be verified scientifically.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and Preparation of Sample Materials

The leaves of *V. dumicola*, *R. myricoides* and aerial parts (stem, leaves and flowers) of *J. betonica* are the parts used by local herbalists to treat malaria. The plants were collected from Gucha region, Kisii County with the aid of a local herbalist. They were packaged and transported to Kenyatta University. They were identified by Mr. Mwadime Nyange at the East Africa Herbarium at National Museums of Kenya, Nairobi, where voucher specimens were deposited (Appendix VIII and IX). The fresh plant materials were chopped into small pieces and ground into homogeneous matter with an electric mill and packaged. Map showing Gucha region in Kisii County is shown (Appendix VII).

3.2 Extraction of the Plant Materials

Extraction was done at the Centre for Traditional Medicine and Drug Research, KEMRI, Nairobi. The method used by Gitua *et al.* (2012) was adopted. From each plant material, 20g of the wet ground plant material were separately heated in 200 ml of water for 2 hours at 90 °C with regular stirring. Heating was then stopped, the extract covered and allowed to cool at room temperature. Subsequently, the extracts were decanted and filtered using Whatman No. 1 paper until they were clear. A similar procedure was used for combined plants (*J. betonica* and *V. dumicola*) extraction at the ratio of 1:1. The combination of *J. betonica* and *V. dumicola* was done to reflect the ethnomedical practice. The resulting filtrates were concentrated by freeze drying and then kept in airtight containers at 4 °C in a refrigerator for future use.

3.3 Parasites for Infection

Chloroquine sensitive *P. berghei*, ANKA strain and *P. falciparum*, D6 strain were used for *in vivo* and *in vitro* antiplasmodial assays of the plant extracts respectively. These strains were obtained courtesy of Dr. Festus Tolo of the Centre for Traditional Medicine and Drug Research (CTMDR) – KEMRI, Nairobi.

3.4 Experimental Design

3.4.1 Laboratory Animals

Nuliparous female swiss albino mice 4 weeks old, 20-30g bred at the animal house in the Department of Zoological Sciences, Kenyatta University, were used in this study. The animals were placed in Macrolon type II cages in the experimental room. They were fed on rodent pellets and supplied with water. Time was given for acclimatization to the experimental room conditions of room temperature 25°C and 12 hour daylight for two weeks before being used. The animals were used for *in vivo* antiplasmodial assay. All ethical guidelines and procedures were observed when addressing the animals (Gitua *et al.*, 2012).

3.4.2 *In Vitro* Determination of Antiplasmodial Activity of the Plant Extracts

This assay was carried out at KEMRI malaria laboratory. Chloroquine-sensitive *P. falciparum* was cultured in culture flasks at 37°C, in a 3% O₂, 5% CO₂ and 92% N₂ atmosphere. The culture medium used was RPMI 1640, 25 mM HEPES, pH 7.4 supplemented with 5% Sodium Hydrogen Carbonate (NaHCO₃), 40µg/ml gentamycin sulphate, heat inactivated 10% AB+ human serum and human erythrocytes. Parasites were synchronized in the ring stage by addition of 5% sorbitol. The stock was diluted with RPMI 1640 media to achieve 0.4% parasitaemia level. A volume of 100µl of the

culture was transferred to the 96 well culture plates. The plant extracts were added at the volume of 50µl at a starting concentration of 100µg/ml and serially diluted to achieve various dilutions namely 100µg/ml, 50µg/ml, 25µg/ml, 12.50µg/ml, 6.25µg/ml, 3.13µg/ml and 1.56µg/ml. The reference drug used was chloroquine. They were then incubated for 48 hours after which parasitaemia levels were determined spectrophotometrically in syberI assay using multi-well plate reader (Fidock *et al.*, 2004; Bbosa *et al.*, 2013) (Table3.1).

Table 3.1: *In vitro* antiplasmodial protocol of aqueous extracts of leaves of *V. duminicola* and *R. myricoides* and aerial parts of *J. betonica*

Group	Status	Treatment (µg/ml)
I.	Negative control (RPMI 1640)	100µl
II.	Positive control (Chloroquine)	25
III.	Experimental group A	1.56
IV.	Experimental group B	3.13
V.	Experimental group C	6.25
VI.	Experimental group D	12.50
VII.	Experimental group E	25.00
VIII.	Experimental group F	50.00
IX.	Experimental group G	100.00

3.4.3 Determination of Antiplasmodial Activity of the Plant Extracts against *P. berghei* In Vivo

Antiplasmodial activity of the plant samples was tested in a four-day suppressive test as described by Peters & Robinson (1991), Gitua *et al.* (2012) and Muregi *et al.* (2007). Blood parasitized with *P. berghei* was obtained from a donor mouse then diluted with PBS to reduce the parasitaemia level to 1% for infection. Intraperitoneal route was used to infect healthy experimental mice with 0.2ml of the diluted blood containing 1×10^7 parasitized (*P. berghei*) red blood cells and randomly divided into 6

groups of 5 mice. The sample size of 5 mice was derived from the protocol in Appendix XII.

The infected mice were then treated with the vehicle and plant extracts administered orally using mouse gavage needle and Chloroquine (reference drug) administered intraperitoneally (Table 3.2). The vehicle (water) was administered to the negative control group, the reference drug (Chloroquine) was given to the positive control group and sample extracts were given to their respective experimental groups. The samples were prepared in distilled water on the day of administration.

Table 3.2: *In vivo* antiplasmodial protocol of aqueous extracts of *V. dumicola*, *R. myricoides* leaves and aerial parts of *J. betonica*

Group	Status	Treatment (mg/kg)
Negative control	Water	0.1ml
Positive control	Chloroquine	25
Experimental group A	<i>J. betonica</i> (JB)	500
Experimental group B	<i>V. dumicola</i> (VD)	500
Experimental group C	<i>R. myricoides</i> (RML)	500
Experimental group D	JB & VD	500

The dosage of extracts used (500mg/kg body weight) was adapted from Muregi *et al.* (2007) and Gitua *et al.* (2012). The animals were treated with the extracts daily for four consecutive days. On the fourth day blood was drawn from the tail vein of each mouse and thin smears made, fixed with methanol and stained with Giemsa. The prepared slides were packed in a slide box. Reading of the slides was done at Chiromo Campus, University of Nairobi (courtesy of Mr. Micheni Ndi) using a compound photomicroscope (LEICA DM 500) fitted with a camera and projected on a computer screen (Appendix V). The number of parasitized erythrocytes out of 1000 red blood cells was determined on random fields under the microscope at a magnification of

×1000. Reading of the The parasitaemia and chemosuppression were calculated as described by Gessler *et al.* (1995).

$$\text{Percentage Parasitaemia } \% P = \left(\frac{\text{PRBC}_s}{\text{TRBC}_s} \right) \times 100$$

Where;

%P= Percentage parasitaemia

PRBCs= Parasitized RedBlood Cells

TRBCs= Total Red Blood Cells

$$\text{Percentage Chemosuppression was estimated as } \% CP = \frac{P(\text{NC}) - P(\text{PE})}{P(\text{NC})} \times 100$$

Where;

%CP = Percentage Chemosuppression

P(NC) = Parasitaemia level of negative control

P(PE) = Parasitaemia level of experimental groups.

3.4.4 Determination of Cytotoxicity of the Plant Extracts

3.4.4.1 Cell Culture

Vero E6 cell line was used to determine cytotoxicity of the plant extracts. The cell line was grown in monolayer cultures in RPMI 1640 media augmented with 10% heat inactivated foetal bovine serum (FBS), L-Glutamine, 100µg/ml penicillin and 100µg/ml streptomycin. It was incubated at 37°C, and 5% CO₂ in a humidified incubator.

3.4.4.2 Cell Treatment

The cell culture was trypsinized and maintained in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS). To neutralize the trypsin enzyme, 5ml of growth media was added to the cell culture. To remove dead cells, the

suspension was centrifuged at 3000 rpm for 5 minutes. The emerging supernatant was poured out and cells resuspended in 5ml of culture media. Trypan blue exclusion assay was used to determine cell density. The cells were checked for confluence using an inverted phase contrast microscope (Appendix IV). A Volume of 0.1ml at a density of 2×10^4 cells was added to each well of 96 well microtitre plates. The cells were incubated at 37 °C in 5% CO₂ for 24 hours to allow attachment. The supernatant media was discarded and 100µl of culture media added to the cells in microtitre plates. Cells were then treated with 50µl of each plant extract and incubated further at 37 °C in 5% CO₂ for 72 hours. The extracts were serially diluted to make the following concentration; 1000µg, 333.33µg, 111.11µg, 37.04µg, 12.35µg, 4.12µg, 1.37µg and the last row of the 96 well plates was left untreated. The reference drug used in the study was chloroquine. After 72 hours the MTT assay was carried out (Table 3.3).

Table 3.3: *In vitro* cytotoxicity protocol for aqueous extracts of *V. dumicola*, *R. myricoides* leaves and aerial parts of *J. betonica* against vero cells

Groups	Treatments Concentration (µg/ml)
Positive control	Chloroquine
Negative control	Untreated cells
Experimental Groups	Extracts
I	1.37
II	4.12
III	12.35
IV	37.04
V	111.11
VI	333.33
VII	1000.00

3.4.4.3 MTT Assay

The microculture tetrazolium assay is a colourimetric test based on the ability of viable cells to metabolise the MTT dye into an insoluble coloured substance (formazan) that is spectrophotometrically measured. Enzyme succinate

dehydrogenase in the mitochondria of living cells converts tetrazolium salt into a formazan salt. The amount of formazan produced is directly proportional to the number of viable cells and indirectly proportional to cell inhibition (Patel *et al.*, 2009). After 72 hours of incubation, the media and extract supernatant in the well was discarded and 50µl of MTT dye added to each well. The plates were gently shaken and incubated for 4 hours and then 50µl of dimethyl sulfoxide (DMSO) added. Subsequently, the plates were gently shaken to solubilize the formed formazan. The optical density of the solution was measured spectrophotometrically at a wavelength 562nm using microplate reader. The proliferation of vero cells was calculated using the following formula:

$$\text{Proliferation rate} = \frac{At - Ab}{Ac - Ab}$$

$$\text{Percentage viability} = \frac{At - Ab}{Ac - Ab} \times 100$$

$$\text{Percentage inhibition} = 100 - \frac{At - Ab}{Ac - Ab} \times 100$$

Where,

At = Absorbance value of test compound

Ab = Absorbance value of blank

Ac = Absorbance value of negative control (cells plus media)

Selectivity index of the plant extracts was determined by calculating the ratio between IC₅₀ of vero cells and IC₅₀ of antiplasmodial activity of the plant extracts as described by Pinmai *et al.*, (2010) and Nabende *et al.*, (2015). This was calculated as:

$$SI = \frac{IC_{50} \text{ (Vero cells)}}{IC_{50} \text{ (Antiplasmodial)}}$$

Where,

SI = Selectivity index

IC_{50} (vero) = IC_{50} of the plant extracts against vero cells

IC_{50} (Antiplasmodial) = IC_{50} of the plants extract against *P. falciparum*

3.5 Determination of Qualitative Phytochemical Composition of the Plant Extracts

The plant extracts were screened for secondary metabolites such as alkaloids, flavonoids, saponins and phenolics. The qualitative phytochemical screening was carried out according to standard protocols (Sofowora, 1993; Trease and Evans, 2002).

3.5.1 Test for Alkaloids

For each plant extract, 1g was added to 5ml of 1% aqueous hydrochloric acid (HCl) and heated in a water bath for 5 minutes. Dragendorff's reagent was added dropwise to the mixture. Appearance of an orange-red precipitate was indicative of presence of alkaloids (Sofowora, 1993).

3.5.2 Test for Phenolics

About 0.5 of each plant extract was boiled with distilled water and then filtered. To 2 ml of the filtrate, a few drops of 10% ferric chloride solution were added. A green-blue or violet colouration indicated the presence of phenolic compounds (Trease and Evans, 2002).

3.5.3 Test for Tannins

1g each plant extract was stirred with 10 ml of distilled water and then filtered. A few drops of 1% ferric chloride solution were added to 2 ml of the filtrate occurrence of a blue-black, green or blue-green precipitate indicated the presence of tannins (Trease and Evans, 2002).

3.5.4 Test for Saponins

1g each plant extract was boiled with 5ml of distilled water and filtered. To the filtrate, 3ml of distilled water was further added and shaken vigorously. Frothing which persisted on warming was taken as an evidence for the presence of saponins (Sofowora, 1993).

3.5.5 Test for Flavonoids

0.5g of each plant extract was dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips were then added to the filtrate followed by few drops of concentrated hydrochloric acid. A pink, orange, red or purple colouration indicated the presence of flavonoids (Trease and Evans, 2002).

3.5.6 Test for Terpenoids

Two grams of each portion was dissolved in ethanol. To the mixture, 1ml of acetic anhydride was added followed by the addition of concentrated sulphuric acid. A change in colour from pink to violet showed the presence of terpenoids (Sofowora, 1993).

3.5.7 Test for Anthraquinones

About 0.2 g of each plant extract to be tested was shaken with 10ml of benzene and then filtered. Five millilitres of 10% ammonia solution was then added to the filtrate and shaken. Appearance of a pink, red or violet colour in the ammoniacal (lower) phase was taken as the presence of free anthraquinones (Sofowora, 1993).

3.6 Statistical Analysis

The data generated from this study was both quantitative and qualitative and were analysed using Minitab Version 17. The quantitative values were expressed as mean \pm SEM. One-way ANOVA was used for comparison of means and the Tukey's post-hoc test was used to pairwise separate and compare the means. *P*-value \leq 0.05 was considered significant. Different dose response curves were used to determine IC₅₀ values.

CHAPTER FOUR

RESULTS

4.1 Antiplasmodial Activity of *J. betonica*, *V. dumicola* and *R. myricoides* Plant Extracts *In Vitro*

Aqueous extracts of *J. betonica* aerial parts, and leaves of *V. dumicola* and *R. myricoides* and combination of the extracts were tested for antiplasmodial activity *in vitro* against chloroquine-sensitive *P. falciparum*. Generally the plant extracts showed *in vitro* antiplasmodial activity against chloroquine sensitive *P. falciparum* (Table 4.1). Aqueous extracts of *J. betonica* aerial parts generally suppressed growth of *P. falciparum in vitro* at all concentrations. There was no significant difference in chemosuppression of *P. falciparum* across various concentrations of aqueous extracts of *J. betonica* aerial parts ($p>0.05$; Table 4.1). However, significant difference in chemosuppression was observed between negative control and various treatments of aqueous extracts of *J. betonica* (JB) aerial parts against *P. falciparum* ($p<0.05$; Table 4.1).

Similarly, aqueous extract of *R. myricoides* (RML) leaves reported antiplasmodial activity by reducing percentage parasitaemia against chloroquine sensitive *P. falciparum*. It was observed that there was significant difference between various concentrations of aqueous extract of *R. myricoides* leaves in chemosuppression of *P. falciparum* ($p<0.05$; Table 4.1). Further significant difference was observed in percentage chemosuppression of *P. falciparum* between the negative control and all concentration of aqueous extract of *R. myricoides* leaves ($p<0.05$; Table 4.1).

Aqueous extract of *V. dumicola* leaves reported antiplasmodial activity against *P. falciparum*. The extract reduced parasitaemia across all the concentrations tested.

There was significant difference in chemosuppression at all the concentrations as compared with the negative control ($p < 0.05$, Table 4.1).

The combination of 1:1 of aqueous extracts of *J. betonica* (JB) aerial parts and *V. dumicola* (VDL) leaves was observed to possess antiplasmodial activity (Table 4.1). The various concentrations of the combination significantly reduced percentage parasitaemia as compared with the negative control ($p < 0.05$; Table 4.1). Furthermore, there was significant difference in percentage chemosuppression of the combination extract concentrations as compared to the negative control. The combination also recorded significant differences among various concentrations in percentage chemosuppression ($p < 0.05$; Table 4.1).

The highest and lowest values of percentage chemosuppression of the plant extracts are shown in Table 4.1. Percentage chemosuppression of the highest concentration of *R. myricoides* (100mg/ml) was 99.04%. It was comparable to chloroquine (25mg/ml) the reference drug which had a chemosuppression of 100%). *J. betonica* (JB), *V. dumicola* (VDL) and *J. betonica* and *V. dumicola* combination were not comparable to chloroquine chemosuppression since there was significant difference in their antiplasmodial activity ($p < 0.05$; Table 4.1).

Table 4.1: *In vitro* antiplasmodial activity of *J. betonica*, *V. dumicola* and *R. myricoides* aqueous extracton percentage parasitaemia and percentage chemosuppression on *P. falciparum*

Treatment ($\mu\text{g/ml}$)	<i>J. betonica</i>		<i>R. myricoides</i>		<i>V. dumicola</i>		J B & VDL	
	% paras	%Chemosup	% para	%Chemosup	%para	%Chemosup	% para	%Chemosup
NC	100 \pm 0.00 ^a	0.00 \pm 0.00 ^a	100.0 \pm 0.00 ^a	0.00 \pm 0.00 ^c	100 \pm 0.00 ^a	0.00 \pm 0.00 ^a	100.00 \pm 0.00 ^a	0.00 \pm 0.00 ^c
1.56	39.76 \pm 4.39 ^b	60.24 \pm 4.39 ^b	6.87 \pm 0.42 ^{bc}	92.08 \pm 0.40 ^d	38.50 \pm 4.39 ^b	29.60 \pm 4.39 ^b	35.23 \pm 0.70 ^c	64.77 \pm 0.70 ^a
3.13	41.10 \pm 16.10 ^b	58.90 \pm 16.10 ^b	7.92 \pm 0.40 ^b	93.13 \pm 0.42 ^{cd}	33.86 \pm 16.10 ^b	31.77 \pm 16.10 ^b	33.91 \pm 0.18 ^{bc}	66.62 \pm 0.18 ^{ab}
6.25	33.05 \pm 1.02 ^b	66.95 \pm 7.72 ^b	4.82 \pm 0.70 ^{cd}	93.26 \pm 0.04 ^{cd}	29.29 \pm 1.02 ^b	39.83 \pm 7.72 ^b	33.38 \pm 0.05 ^{bc}	66.62 \pm 0.05 ^{ab}
12.5	33.79 \pm 2.91 ^b	66.21 \pm 1.02 ^b	6.74 \pm 0.03 ^{bc}	95.18 \pm 0.70 ^{bc}	26.57 \pm 2.12 ^b	41.83 \pm 1.93 ^b	31.60 \pm 0.09 ^{bc}	68.40 \pm 0.09 ^{ab}
25	32.86 \pm 2.12 ^b	67.14 \pm 1.93 ^b	3.25 \pm 0.91 ^{de}	96.38 \pm 0.04 ^b	25.32 \pm 1.93 ^b	44.44 \pm 2.91 ^b	30.60 \pm 2.64 ^b	69.40 \pm 2.64 ^b
50	32.57 \pm 7.72 ^b	67.43 \pm 2.12 ^b	3.62 \pm 0.04 ^d	96.76 \pm 0.91 ^{cd}	22.21 \pm 7.72 ^b	45.44 \pm 2.12 ^b	29.15 \pm 0.09 ^{bc}	70.85 \pm 0.08 ^{ab}
100	30.33 \pm 1.93 ^b	69.67 \pm 2.91 ^b	0.96 \pm 0.22 ^e	99.04 \pm 0.23 ^a	20.24 \pm 2.91 ^b	61.97 \pm 1.02 ^b	25.92 \pm 1.08 ^b	74.08 \pm 1.08 ^b
PC	0 \pm 0.00 ^c	100 \pm 0.00 ^c	0 \pm 0.00 ^e	100 \pm 0.00 ^a	0 \pm 0.00 ^c	100 \pm 0.00 ^c	0 \pm 0.00 ^d	100 \pm 0.00 ^d

Values are expressed as Mean \pm SEM. Values followed by the same superscript are not significantly different ($P>0.05$)

Key: **Para** means parasitaemia, **Chemosup** means chemosuppression, **NC** is Negative Control, **PC** is Positive Control

4.2 IC₅₀ Values of Aqueous Extracts of *J. betonica* aerial Parts, and Leaves of *V. dumicola* and *R. myricoides* Against *P. falciparum* In Vitro

Drug-response curve plotted on MS Excel was used to determine the IC₅₀ values of the plant extracts against *P. falciparum* through linear regression analysis. Aqueous extract of *R. myricoides* leaves recorded the highest antiplasmodial activity against *P. falciparum*, recording IC₅₀ values of 1.32±0.03µg/ml while the combination of JB and VDL had the least antiplasmodial activity with an IC₅₀ value of 11.03±1.18µg/ml (Table 4.2). There was significant difference between IC₅₀ values of all the plant extracts against *P. falciparum* (p<0.05; Table 4.2).

Table 4.2: IC₅₀ values of aqueous extracts of *J. betonica* (JB) aerial parts, and leaves of *V. dumicola* (VD) and *R. myricoides* (RM) against *P. falciparum*

Chemosuppression of <i>P. falciparum</i>	
Plant Extracts	IC ₅₀ (µg/ml)
<i>J. betonica</i> Aerial Parts (JB)	2.01±0.69 ^a
<i>V. dumicola</i> Leaves (VD)	9.28±1.21 ^c
<i>R. myricoides</i> Leaves (RM)	1.32±0.03 ^b
JB & VDL	11.03±1.18 ^d

Values are expressed as Mean±SEM. Values followed by different superscripts are statistically different ($P<0.05$)

4.3 Antiplasmodial Activity of *J. betonica*, *V. dumicola* and *R. myricoides* Plant Extracts In Vivo

Generally the plant extracts showed antiplasmodial activity (Table 4.3). The lowest percentage parasitaemia levels of 4.11±0.21 were observed in the activity of the combination of aqueous extract of JB and VDL against *P. berghei*, showing chemosuppression of 92.12±0.49 while the highest parasitaemia level of 29.29±2.99

was recorded in the activity of the aqueous extract of *J.betonica* aerial parts reporting percentage chemosuppression of 44.17 ± 4.98 . There was significant difference between percentage parasitaemia of negative control and all the plant extracts ($p<0.05$; Table 4.3). The antiplasmodial activity for the combination of JB and VD was comparable to that of the reference drug (Table 4.3).

Table 4.3: Effect of 500mg/kg of *J. betonica* (JB), *V. dumicola* (VD) and *R. myricoides* (RM) aqueous extract against *P. berghei* in vivo

Plant Extracts	Percentage Parasitaemia	Percentage Chemosuppression
RML	17.26 ± 0.35^{bc}	66.87 ± 1.82^{bc}
JB	29.29 ± 2.99^b	44.17 ± 4.98^c
JB andVDL	4.11 ± 0.21^d	92.12 ± 0.49^a
VDL	25.63 ± 6.26^b	49.8 ± 12.6^c
Chloroquine	0.67 ± 0.19^d	98.77 ± 0.325^a
Negative	52.72 ± 3.08^a	0.00^d

All values are expressed as Mean \pm SEM. Values followed by the same superscript are not significantly different ($P>0.05$).

4.4 Cytotoxicity of *V. dumicola*, *R. myricoides* and *J. betonica* on Vero Cells in vitro

Generally, the plant extracts were observed to inhibit proliferation rate of vero cells at various concentrations. The proliferation rate of vero cells reduced as the concentration of the plant extracts increased and the proliferation rates increased with decrease in extracts concentration (Table 4.4). It was observed that percentage proliferation decreased with increase in concentration of the extracts and percentage cell inhibition increased with increase in the concentration of the plant extracts (Figure 4.1). Proliferation rates of the vero cells was highest at negative control where

the cells were untreated. There was significant difference in proliferation rates between untreated cells and cells treated with highest concentration of the plant extracts across all the plant extracts tested ($p < 0.05$, Table 4.4).

Table 4.4: *In vitro* cytotoxic activity of *J. betonica*, *V. dumicola* and *R. myricoides* on proliferation rate of vero cells

Proliferation Rates of Vero Cells				
Concentration $\mu\text{g/ml}$	JB	RML	VDL	JB & VDL
0	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a	1.00 \pm 0.00 ^a
1.37	0.97 \pm 0.01 ^{ab}	0.89 \pm 0.02 ^{ab}	0.96 \pm 0.03 ^a	0.97 \pm 0.02 ^a
4.12	0.89 \pm 0.03 ^{ab}	0.79 \pm 0.03 ^{ab}	0.92 \pm 0.00 ^a	0.94 \pm 0.04 ^a
12.35	0.82 \pm 0.03 ^{bc}	0.74 \pm 0.01 ^b	0.89 \pm 0.02 ^{ab}	0.93 \pm 0.04 ^a
37.04	0.70 \pm 0.05 ^{cd}	0.65 \pm 0.05 ^b	0.84 \pm 0.02 ^b	0.92 \pm 0.04 ^a
111.11	0.67 \pm 0.04 ^{cd}	0.23 \pm 0.08 ^c	0.73 \pm 0.01 ^{ab}	0.86 \pm 0.00 ^{ab}
333.33	0.63 \pm 0.03 ^d	0.17 \pm 0.05 ^c	0.37 \pm 0.08 ^c	0.67 \pm 0.08 ^c
1000.00	0.15 \pm 0.03 ^e	0.13 \pm 0.03 ^c	0.16 \pm 0.01 ^d	0.34 \pm 0.00 ^d

Values are expressed as Mean \pm SEM. Values followed by different superscripts are statistically different ($P < 0.05$)

Percentage proliferation of the cells increased as the concentration of the plant extracts decreased. On the other hand, percentage inhibition of the cells increased as the plant extract increased. The highest percentage proliferation was observed in the untreated cells while the highest percentage inhibition was observed in the cells treated with the highest concentration of the plant extracts (Table 4.5).

Table 4.5: Percentage inhibition and percentage viability for the activity of *J. betonica*, *V. dumicola* and *R. myricoides* aqueous extracts on vero cells

Treatment ($\mu\text{g/ml}$)	JB		RML		VDL		J B & VDL	
	PP	PI	PP	PI	PP	PI	PP	PI
0.00	100	0	100	0	100	0	100	0
1.37	97	3	89	11	96	4	97	3
4.12	89	11	79	21	92	8	94	6
12.35	82	18	74	26	89	11	93	7
37.04	70	30	65	35	84	16	92	8
111.11	67	33	23	77	73	27	86	14
333.33	63	37	17	83	37	63	67	33
1000	15	85	13	87	16	84	34	66

Key PP is Percentage Proliferation, **PI** is Percentage Inhibition

4.5 IC₅₀ Values of Aqueous Extracts of *J. betonica* Aerial Parts and leaves of *V. dumicola* and *R. myricoides* Against Vero Cells *in vitro*

The IC₅₀ values of the plant extracts were determined from the dose response curve plotted on MS Excel through linear regression analysis. There was significant difference between IC₅₀ values of all the plant extracts against vero cells ($p < 0.05$; Table 4.6).

Table 4.6: IC₅₀ values of aqueous extracts of *J. betonica* aerial parts leaves of *V. dumicola* and *R. myricoides* against vero cells

Vero cell line	
Plant Extracts	IC ₅₀ ($\mu\text{g/ml}$)
JB	690.56 \pm 8.00 ^a
VDL	346.04 \pm 4.00 ^d
RML	106.00 \pm 11.00 ^c
JB andVDL	504.70 \pm 13.10 ^b

All values are expressed as Mean \pm SEM. Values followed by the same superscript are not significantly different ($P > 0.05$).

4.6 Selectivity Indexes (SI) of *J. betonica*, *V. dumicola* and *R. myricoides*

Selectivity indexes of aqueous extracts of *J. betonica* (JB) aerial parts, *V. dumicola* (VDL) and *R. myricoides* (RML) leaves were determined by calculating the ratio between IC₅₀ of vero cells and IC₅₀ of antiplasmodial activity of the plant extracts. Significant difference was observed in selectivity indexes of all the plant extracts ($p < 0.05$; Table 4.7).

Table 4.7: Selectivity indexes of aqueous extracts of *J. betonica* aerial parts, *V. dunicola* and *R. myricoides* leaves against vero cells

Plant Extracts	Selectivity index
JB	343.56±8.22 ^a
VDL	37.28±2.79 ^b
RML	80.30±3.03 ^c
JB andVDL	45.75±7.03 ^d

Values are expressed as Mean±SEM. Values not sharing a superscript are considered significantly different (p<0.05)

4.7 Qualitative Phytochemical Composition of *J. betonica*, *V. dunicola* and *R. myricoides*

The aqueous extracts of *J. betonica* aerial parts, *V. dunicola* and *R. myricoides* leaves revealed presence of tannins, phenols and anthraquinones. The presence or absence of other phytochemicals varied across the extracts (Table 4.8).

Table 4.8: Qualitative phytochemistry of aqueous extracts of *J. betonica* aerial parts, *V. dunicola* and *R. myricoides* leaves

Test	<i>J. betonica</i> (aerial parts)	<i>R. myricoides</i> (leaves)	<i>V. dunicola</i> (leaves)
Saponins (Frothing test)	+	-	+
Alkaloids	+	+	-
Tannins	+	+	+
Flavonoids	-	+	+
Phenols	+	+	+
Triterpenes	+	+	-
Anthraquinones	+	+	+
*Free	-	-	-
*Bound	-	-	-
Cardiac glycosides	-	-	-
Resins	-	-	-

Key: + indicates low present, - indicates absent

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

5.1 Discussion

5.1.1 Antiplasmodial Activity of *J. betonica*, *V. dumicola* and *R. myricoides* Plant Extracts

Many plants have been used for antimalaria treatment traditionally but their efficacy is yet to be scientifically proven. This study determined antiplasmodial activity of aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves. Aqueous extracts of *J. betonica* (JB) aerial parts, *V. dumicola* (VDL), *R. myricoides* (RML) leaves, JB and VDL combination recorded IC₅₀ values of 2.01±0.69µg/ml, 9.28±1.21µg/ml, 1.32±0.03µg/ml and 11.03±1.18µg/ml respectively against *P. falciparum* *in vitro*. *R. myricoides* leaves demonstrated the best antiplasmodial activity followed by *J. betonica* aerial parts, *V. dumicola* leaves, the combination of JB and VDL respectively.

Based on WHO guidelines for classification of antiplasmodial activity, extracts are classified as highly active, active, moderately active and inactive at IC₅₀ values of < 5µg/ml, 5-15µg/ml, 15-50µg/ml and > 50µg/ml, respectively (Philippe *et al.*, 2005; Jonville *et al.*, 2008). Therefore, the aqueous extracts of *J. betonica* aerial parts and *R. myricoides* leaves showed high antiplasmodial activity, while aqueous extracts of *V. dumicola*, the combination of JB and VDL were active against *P. falciparum*.

The combination of *J. betonica* aerial parts and *V. dumicola* leaves demonstrated the best antiplasmodial activity *in vivo* followed by *R. myricoides*, *V. dumicola* and *J. betonica* in that order. There was significant difference in the *in vivo* antiplasmodial

activity of *J. betonica* aerial parts and *V. duminicola* leaves combination and other plants extracts. This suggests that there was a difference in the activity of various plants extracts tested. *R. myricoides* extract demonstrated the best *in vitro* antiplasmodial activity whereas the combination of *J. betonica* and *V. duminicola* extract demonstrated the best antiplasmodial activity *in vivo*. This suggests that there was a difference in *in vitro* antiplasmodial and *in vivo* antiplasmodial activities of the plant extracts. This could be attributed to possible difference in mechanisms of action of the plant extracts, variation in concentration of phytochemicals and pharmacokinetics (Bryant and Knights, 2002; Pharmacokinetics, 2006).

The current study reports for the first time antiplasmodial activity of aqueous extracts of *J. betonica* aerial parts, *V. duminicola* and *R. myricoides* leaves. Previous studies have demonstrated antiplasmodial potential of medicinal plants with some extracts being highly active, active, moderately active or inactive. Pinmai *et al.* (2010) demonstrated *in vivo* antiplasmodial activity of water extracts of *Phyllanthus emblica*, *Terminalia chebula*, and *Terminalia bellerica* whose IC_{50} values ranged from 14.33 ± 0.25 to $15.41 \pm 0.61 \mu\text{g/ml}$ against chloroquine susceptible strain *P. berghei* in mice model. In a study to determine chemotherapeutic potential of selected medicinal plants against *P. berghei* it was noted that extracts of *W. ugandensis* and *Z. usambarensis* had chemosuppressive activity against *P. berghei*. The extracts maintained low parasitaemia levels in swiss mice models with chloroform, methanolic and ethylacetate extracts of *W. ugandensis* recording IC_{50} values of 3.4g/ml, 6.8g/ml and 12.5g/ml (Were, 2010).

Methanolic extracts of *Phyllanthus niruri* exhibited high antiplasmodial activity against *P. berghei* with IC_{50} values range of 2.3 to $3.9 \mu\text{g/ml}$ confirming its

antiplasmodial activity (Sholikhah and Wahyuono, 2007). A previous study has demonstrated antiplasmodial activity of ether extract of *J. betonica* leaves *in vitro* against *P. falciparum* reporting IC₅₀ values of 13.36µg/ml (Bbosa *et al.*, 2013). Methanolic leaf extract of *R. myricoides* has been shown to induce 82.50% chemosuppression of *P. berghei* at the dose of 600mg/kg (Deressa *et al.*, 2010). In a study to determine antiplasmodial activity of selected medicinal plants from Kenya methanolic rootbark extract of *R. myricoides* was shown to significantly induce parasitaemia suppressions ranging from 31.7-59.3% (Muregi *et al.*, 2007). Therefore, this study adds to the pool of knowledge of scientifically evaluated medicinal plants with antiparasmodial activity.

5.1.2 Cytotoxicity of *V. dumicola*, *R. myricoides* and *J. betonica* Against Vero Cells

Current conventional antimalarial drugs are associated with drug resistance and toxicity (Pinmai *et al.*, 2010). Findings show that communities have resorted to herbal remedies for malaria treatment since they are accessible, inexpensive and regarded as safe. Plants have always been considered as an alternative natural and rich source of drugs (Basco *et al.*, 1994; Milliken, 1997). However, most of herbal extracts being used in traditional medicine have not been evaluated for their safety standards. The safety profile of aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves was evaluated by determining the cytotoxicity of the extracts against vero cell. Selectivity indexes and IC₅₀ values were used to classify cytotoxicity of the plant extracts.

Zirich *et al.* (2005) classified plants extracts with IC₅₀ ≥ 20µg/ml on normal cells as safe. In current study, aqueous extracts of *J. betonica* aerial parts, *V. dumicola* leaves, *R. myricoides* leaves and *J. betonica* and *V. dumicola* combination had IC₅₀ values of

690.56±8.00µg/ml, 346.04±4.00µg/ml, 106.00±11.00µg/ml and 504.70±13.10µg/ml respectively against vero cell. Therefore, these extracts would be regarded as safe based on their IC₅₀ values. The extracts had selectivity index values of 343.56±8.22, 37.28±2.79, 80.30±3.03, 45.75±7.03 with aqueous extracts of *J. betonica* aerial parts, *V. dunicola* leaves, *R. myricoides* leaves and *J. betonica* and *V. dunicola* combination respectively. Extracts with selectivity index values above 3 are classified as selective hence safe against normal cells whereas selectivity index values below 3 are defined as non-selective and toxic (Nabende *et al.*, 2015). All the plant extracts recorded selectivity index values above 3 and are therefore regarded as safe for management of malaria. The selectivity indexes and IC₅₀ values of all the plant extracts were significantly different suggesting the plant extracts had different levels of effects to the vero cells.

Previously, medicinal plants have been tested for their cytotoxicity against vero cells and their findings reported. Nabende *et al.* (2015) reported that methanolic extract of *Prunus africana* leaves and aqueous extract of *Wambugia stuhlmannii* stem bark had IC₅₀ value above 1000µg/ml respectively against vero cells with selectivity index values above 3, suggesting that the extracts were selective. A study on antiproliferative activity of aqueous root bark extract of *Cassia abbreviata* reported an IC₅₀ value of 128.38µg/ml against vero cells (Njagi *et al.*, 2016). Treatment of vero cells with ethanolic and hydro-ethanol of *Jatropha tanjorensis* observed IC₅₀ values of 86.8±4.8 µg/ml and 547±9.4 µg/ml with selectivity indexes of 8 and 11.4, respectively. Stem bark extract of *T. robusta* and leaves of *T. nilotica* have been reported to have moderate cytotoxic activity against vero cells (Irungu *et al.*, 2015). A large body of information is available for safety of medicinal plants. This study adds

the cytotoxic activity of aqueous extracts of *J. betonica* aerial parts, *V. dumicola* leaves, *R. myricoides* leaves and *J. betonica* and *V. dumicola* combination to the available pool of knowledge.

5.1.3 Phytochemistry of Aqueous Extracts of *J. betonica*, *V. dumicola* and *R. myricoides*

Phytonutrient compounds present in medicinal plant extracts are responsible for their various pharmacological properties. These plant phytochemicals have been useful in development of various antimalarial drugs used in modern medicine (Kennedy and Wightman, 2011). The three plants extracts were screened for their phytonutrients to suggest possible therapeutic leads. Tannins, alkaloids, saponins, flavonoids, phenols, triterpenes and anthraquinones were observed in aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves. Aqueous extracts of *J. betonica* aerial parts and *V. dumicola* leaves contained saponins which were absent in *R. myricoides* leaves.

The antiplasmodial activity of the plant extracts could be attributed to the plant phytochemicals confirmed present in the extracts. Evidence of medicinal potentials of phytochemicals is available in publications. It has been documented that various antimalarial herbs used for traditional management of malaria contain phytocompounds such as alkaloids, terpenoids and flavonoids (Dharani *et al.*, 2010; Selamawit, 2015). Studies have reported that phenolics are implicated in antiplasmodial activity (Alexandru *et al.*, 2007; Ayoola *et al.*, 2008). It has also been reported that terpenoids and alkaloids have anti-plasmodial activity (Okokon and Nwafor, 2009). Aqueous extract of *Lecaniodiscus cupanioides* root that exhibited antiplasmodial activity revealed presence of saponins, alkaloids, tannins, phenols and

anthraquinones (Nafiu *et al.*, 2013). Its antiplasmodial activity was attributed to these phytochemicals (Delmas *et al.*, 2000; Christenzen and Kharazmi, 2001; Abdulelah and Zainal-Abidin, 2007).

Antiplasmodial activity of ethanolic extracts of *Momordica charantia*, *Caricapapaya*, *Tinosporacrispa* and *Annona muricata* has been reported. The plant extracts were found to contain alkaloids, flavonoids, tannins and saponins to which their antiplasmodial activity was attributed to (Abdillah *et al.*, 2015). Ethanolic root bark and dichloromethane leaf extracts of *Cassia abbreviata* have been reported to contain polyphenols, tannins, anthranoids and anthraquinones. Antiplasmodial activity of *Cassia abbreviata* has been confirmed and the activity is attributed to the phytochemicals found in the extracts. Therefore, antiplasmodial activity of aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves could be associated to the phytochemical confirmed to be present present in the plant extracts.

Flavonoids isolated from various medicinal plants have been shown to suppress influx of *L*-glutamine and myoinositol into infected erythrocytes and biosynthesis of fatty acids in the parasite (Yenesew *et al.*, 2004). Presence of phenolics in the plant extracts could have contributed to less cytotoxic and antiplasmodial activities of the extracts. Phenolics are known antioxidants that scavenge free radicals that contribute to oxidative damage associated with cytotoxicity and death of malaria parasites (Becker *et al.*, 2004; Nethengwe *et al.*, 2012). Phenolic compounds demonstrated antiplasmodial activity by inhibiting parasites protein synthesis and increasing red blood cells oxidation (Kim *et al.*, 2003; Batista *et al.*, 2009). Various tannins have been suggested to have antiplasmodial activity through antioxidation mechanism and

inhibition of protein synthesis (Peter and Anatoli, 1998). Tannins and anthraquinones have been demonstrated in previous studies to have antiplasmodial activity through antioxidant action. Therefore, the phytochemicals present in particular plant extracts could have a proportional relationship to the antiparasitic activity of the extracts (Hilou *et al.*, 2006; Valdes *et al.*, 2010; Ravikumar *et al.*, 2012).

The plant extracts may also act by prevention of free oxidized haem (FP – Fe III) detoxification through intercalation with the iron-carboxylate bond that joins haem units of hemozoin, inhibiting haem polymerization. Haem is a by-product of haemoglobin digestion (Becker *et al.*, 2004; Ravikumar *et al.*, 2012). The phytochemicals may be acting as metalchelators that chelate the parasite essential inner cations including Fe²⁺, Ca²⁺ and Mg²⁺. The cations act as cofactors of the *Plasmodium* enzyme ribonucleotide reductase (RNR). Ribonucleotide reductase is responsible for DNA synthesis in the parasite (Munro and Silva, 2012). Once the cofactors are inhibited the RNR becomes unstable and genetic material replication forestalls, which results to inhibition of parasite cell division, with the ultimate consequence of death (Munro and Silva, 2012). The phytochemicals possibly could cause inhibition of choline intracellular transport required for phosphatidylcholines biosynthesis essential for *Plasmodium* hepatocytes invasion and survival (Hilou *et al.*, 2006; Itoe *et al.*, 2014). Therefore, the plant extracts could be implementing their antiplasmodial activity via one or more of the possible mechanisms of activity. However the mechanism of action of the extracts should be empirically determined.

5.2 Conclusions

- i. Crude aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves have antiplasmodial activity *in vitro* and *in vivo*. *J. betonica* and *V. dumicola* had the highest *in vivo* antiplasmodial activity while *R. myricoides* had the best *in vitro* antiplasmodial activity.
- ii. Based on the selectivity index values, aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves had low cytotoxicity. The study found that these extracts were selective and safe against normal cell line, the vero cells. The aqueous extract of *J. betonica* was the most safe (least toxic) while the extract of *R. mycorides* was the least safe.
- iii. Aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves contained tannins, flavonoids, phenols, terpenoids and anthraquinones. Saponins were contained in aqueous extracts of *J. betonica* aerial parts and *V. dumicola* leaves but were absent in *R. myricoides* leaves. The antiplasmodial activity of the extracts could be attributed to the presence of these phytochemicals. High activity of *R. myricoides* crude extract can be attributed to the presence of flavanoids in the plant.

5.3 Recommendations

- i. Aqueous extracts of *J. betonica* aerial parts, *V. dumicola* and *R. myricoides* leaves have antiplasmodial activity and could be used for development of antimalarial drugs.

5.3.1 Areas for further studies

- i. Safety profiles of the plant extracts should be determined *in vivo*
- ii. Phytochemicals with antiplasmodial activity should be isolated and quantified.

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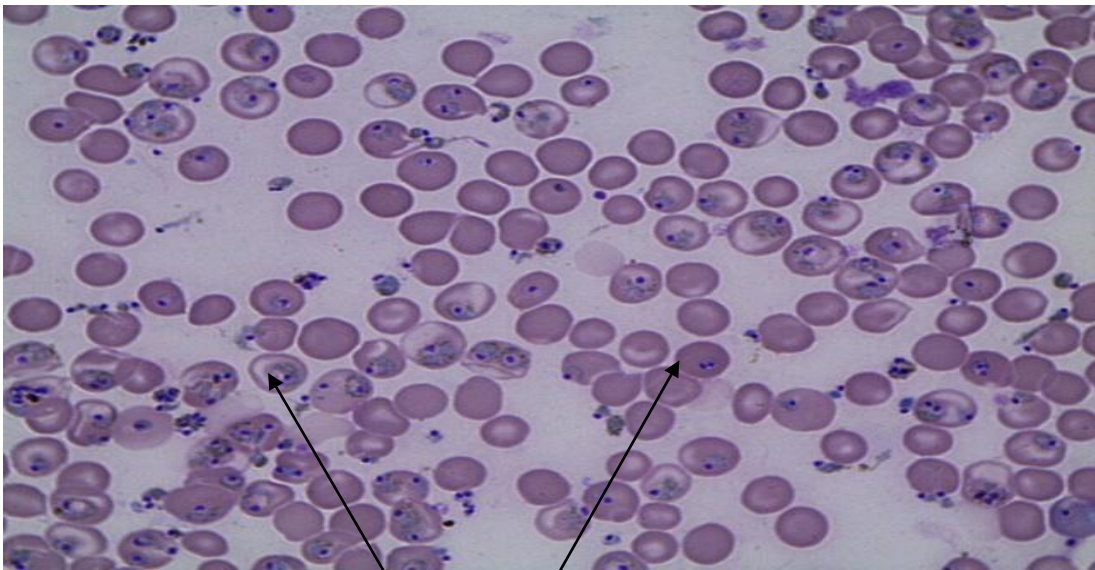
APPENDICES

Appendix I: Phytochemical screening of the plant extracts (Frothing test)



V. dumicola *J. betonica* *R. myricoides* (roots) *R. myricoides* (leaves)

Appendix II: Giemsa stained red blood cells



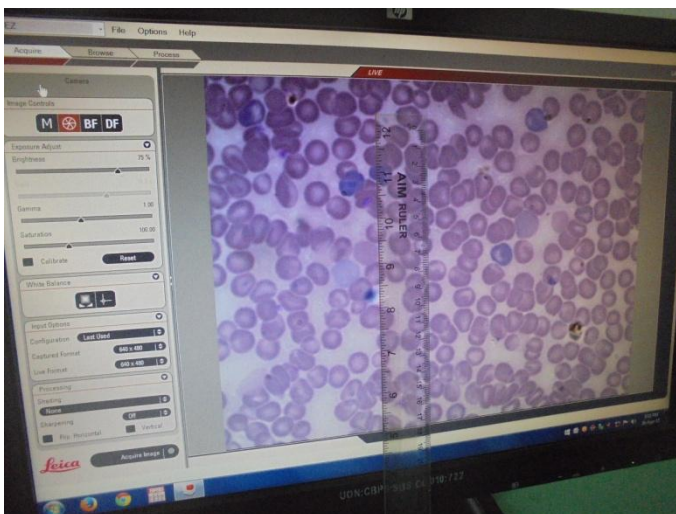
Red blood cells parasitized with *P. berghei*

Appendix III: Swiss albino mice from Department of Zoological Sciences



Appendix IV: Inverted phase contrast microscope



Appendix V: Compound photomicroscope**Appendix VI: Slide projected on a computer screen**

Appendix VII: Map showing site of plant collection

Retrieved from <https://www.researchgate.net/figure/Map-of-Kenya-and-district-level-map-of-Nyanza-Province-The-highland-ar>



Appendix VIII: First letter of plant identification

The East African Herbarium
P.O. Box 45166 00100 Nairobi, Kenya
Telephone: 3743513, 3742131/4 ext 2274
Fax: 3741424
E-Mail: botany@museums.or.ke

REF: NMK/BOT/CTX/1/2

10th February 2014

Lazarus Matini Nyachwaya
P.O. Box
Tel: 0735765390

Dear Mr. Matini

PLANT IDENTIFICATION

The plant specimens that you brought to us for identification have been determined as follows:

1. *Vernonia dumicola* S.Moore Hutch (Family: Asteraceae)
2. *Vernonia hymenolepis* A.Rich. (Family: Asteraceae)
3. *Justicia betonica* L. (Family: Acanthaceae)

Yours sincerely,

Mwadime Nyange
For: Head, Botany Department

Appendix IX: Second letter of plant identification

REF: NMK/BOT/CTX

28th August, 2014

Lazarus Nyachwaya

PLANT IDENTIFICATION

The plant specimen that you brought for identification has been determined as follows:

Botanical name: *Rothea myricoides* (Hochst.) Steane & Mabb,

Family: Lamiaceae

Yours faithfully,


Mwadime Nyange

For: Head, Botany Department

Appendix X: Letter of approval of research proposal

**KENYATTA UNIVERSITY
GRADUATE SCHOOL**

E-mail: dean-graduate@ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 810901 Ext. 57530

Website: www.ku.ac.ke

Internal Memo

FROM: Dean, Graduate School

DATE: 25th October, 2014

TO: **Lazarus Matini Nyachwaya**
C/o Department of Zoological Science
Kenyatta University

REF: 156/21120/12

SUBJECT: **APPROVAL OF RESEARCH PROPOSAL**

=====

This is to inform you that Graduate School Board, at its meeting of 22nd October 2014, approved your Research Proposal for the M.Sc. Degree Entitled, **“Determination of the Antimalarial Efficacy and Safety of Justicia Betonica, Vernonia Dumicola and Clerodendrum Myricoides in Vitro and in Mice”**.

You may now proceed with data collection, subject to clearance with the Permanent Secretary, Ministry of Higher Education, Science and Technology.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking forms per semester. The form has been developed to replace the progress report forms. The supervision Tracking Forms are available at the University's website under Graduate School webpage downloads.

Thank you

REUBEN MURIUKI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Department of Zoological Sciences

Supervisors:

1. Dr. Michael Gicheru
C/o Department of Zoological Sciences
Kenyatta University
2. Dr. Syprine Otieno
C/o Department of Zoological Sciences
Kenyatta University

Appendix XI: Protocol for *in vitro* antiplasmodial efficacy screening

The protocol for antiplasmodial efficacy screening is largely borrowed from Fidock et al., (2004)

This protocol for assessing compound efficacy against *Plasmodium falciparum in vitro* uses [³H]- hypoxanthine as a marker for inhibition of parasite growth. Many alternative protocols exist, including ones based on microscopic detection of Giemsa-stained slides, assays based on production of parasite lactate dehydrogenase, and the use of flow cytometry.

Parasite strain: Several well-characterized strains are available, either from academic laboratories or from www.malaria.mr4.org (reagents available to registered users). One recommendation would be to test activity against a drug-sensitive line such as 3D7 (West Africa), D6 (Sierra Leone) or D10 (Papua New Guinea), as well as a drug-resistant line such as W2 or Dd2 (both from Indochina), FCB (SE Asia), 7G8 (Brazil) or K1 (Thailand).

Malaria Culture Media: RPMI 1640 medium containing L-glutamine (Catalog number 31800, *In vitro* gen), 50mg/litre hypoxanthine, 25mM HEPES, 10µg/ml gentamicin, 0.225% NaHCO₃ and either 10% human serum or 0.5% Albumax I or II (purified lipid-rich bovine serum albumin). Medium is typically adjusted to a pH of 7.3 to 7.4.

Low Hypoxanthine Media: Same as above except that the hypoxanthine concentration is reduced to 2.5 mg/liter.

Serum (as opposed to Albumax) is important for culturing fresh isolates, and for maintaining properties of cytoadherence and gametocyte production. Some strains also prefer to propagate in serum. Batch-to-batch variation is nonetheless a problem, with occasional batches not supporting robust parasite growth. Accordingly, many laboratory lines have been adapted to propagate in the presence of Albumax, which typically gives more consistent growth between batches (variation was a problem in the past, but now appears to have been resolved). Albumax appears to reduce both the rate at which erythrocytes deteriorate *in vitro*, and pH drift when cultures are exposed to ambient air (i.e. during tissue culture hood manipulations).

Preparation of host erythrocytes: Human erythrocytes for parasite culture are prepared by drawing blood into heparin-treated tubes and washing several times in RPMI 1640 medium to separate the erythrocytes from the plasma and buffy coat. Separation can be achieved by centrifuging the blood at 00 ×g for 5 minutes in a swing-out rotor. Leukocyte-free erythrocytes are typically stored at 50 % hematocrit (i.e. 1 volume of malaria culture media for 1 volume of packed erythrocytes, corresponding to approximately 5 × 10⁹ cells/ml).

Parasite Culture Conditions: *P. falciparum* asexual blood stage parasites are propagated at 37°C in malariaculturemedia at 3-5% hematocrit in a reduced oxygen environment (e.g. a custom mixture of 5% CO₂, 5% O₂ and 90% N₂). Lines can be conveniently cultured in 6-24 well tissue culture plates in a modular chamber (Billups-Rothenberg, DelMar, CA, www.brincubator.com), with plates containing sterile water on the bottom to increase humidity and minimized desiccation. These chambers can be suffused with the low O₂ gas and maintained at 37°C in an incubator designed to minimize temperature fluctuations. Parasites can also be cultured in flasks that are individually gassed, or alternatively placed in flasks that permit gas exchange through the cap (in which case the incubator needs to be continuously infused with low O₂ gas mixture). Depending on the line, parasites typically propagate 3-8 fold every 48 hour, thus care must be taken to avoid parasite cultures attaining too high a parasitaemia (i.e. percentage of erythrocytes that are parasitized) for healthy growth. Most lines grow optimally at 0.5-4% parasitaemia. Parasites are most suitable for drug assays when they are 2-5% parasitaemia, and mostly ring stages with few or no gametocytes.

Compounds: Compounds can often be dissolved in 100% dimethyl sulfoxide (DMSO) and stored at -80°C (or -20°C). Particle size of insoluble compounds can be reduced by ball-milling or sonication. For the drug assays, serial drug dilutions (either 2× or customized) are made in low hypoxanthine medium (see above) and added to 96-well culture plates at 100µl per well. Drugs are added to columns 3-12 (test samples), with columns 1 and 2 reserved for wells with low hypoxanthine medium without compound. All drugs are typically tested in duplicate or triplicate for each parasite line. Once completed, plates are placed into their own modular chamber, gassed and placed at 37°C. These plates should be set up no more than a few hours prior to addition of the parasites.

Drug Assay Conditions: Parasites are diluted to a 2× stock consisting of 0.6% to 0.9% parasitaemia (depending on the growth rate of the line) and 3.2 % hematocrit in low hypoxanthine medium, and 100µl are added per well already containing 100 µl of low hypoxanthine medium with or without compound (present at different concentrations). Plates are then incubated in a gassed modular chamber at 37°C for 48 hour (some labs prefer 24hr). After this time, 100µl of culture supernatant from each well is removed and replaced with 100 µl of low hypoxanthine medium.

Appendix XII: Protocol for *in vivo* antiplasmodial efficacy screening.

The assay protocol is based on the 4-day suppressive test as described by Peters *et al.*, 1970; Peters *et al.*, 1975 and Peters *et al.*, 1992.

Parasite: *Plasmodium berghei* strain ANKA

Test animals: Male or female mice of weight 18-21g. Strain: BALB/C, Swiss (OFI) or Ddy 6-8 weeks old.

Standard drug (positive control): Chloroquine diphosphate – 20mg/kg/day.

Preparation of inoculum

- Parasite strain is maintained by serial passage of blood from mouse to mouse.
- Parasitized erythrocytes are obtained from an infected mouse donor with rising parasitaemia (20%) by cardiac puncture in heparin and the infected red blood cells diluted with sterile saline (0.9% NaCl) to a working titer. The blood is diluted to 1 % for infection.

Infection of mice

Each mouse is inoculated intraperitoneally with 1×10^7 erythrocytes contained in 0.2ml parasitized with *Plasmodium berghei* strain ANKA contained in 0.9% saline, on day 0.

The infected mice are divided into groups of 5 mice consisting of:

Negative control – receiving placebo

Positive control – receiving control drug

Test - receiving test drug (extracts)

Note:- Each concentration of test extract should its own group of 5 mice.

Preparation of test extracts

Oral administration

The extracts should be prepared in a suitable vehicle so that the concentration to be administered is contained in 0.2ml of solution.

Intraperitoneal administration

4. Evaluation of blood schizonticidal activity on early infection (4-day test) of the extracts.

Oral administration

The mice receiving test extracts are treated daily from day 0 (immediately after infection) to day 3, with an oral dose of extract (e.g. 200 or 800mg/kg/day).

On the same days, mice in the control-positive are given chloroquine diphosphate (dissolved in water to give a 0.2ml dose volume) at 20mg/kg/day.

Those in the negative control group are given placebo (e.g. water) at 0.2ml/kg/day.

Each day from day 0 to day 4, a thin film is made from the tail blood sample from each mouse and stained with Giemsa and used to estimate the level of parasitaemia (%).

Note:- the % of parasitized red blood cells is determined using a microscope slide containing a blood smear from the tail of each mouse and stained with Giemsa. The % parasitaemia of each mouse is recorded and the mean % parasitaemia for each group is calculated.

On day 4, the mean parasitaemia in each group of mice is determined so that the percent chemosuppression of each dose of each extract is calculated using the formula: $[(A-B)/A] \times 100$ (Tona *et al.*, 2001)

Where A – is the mean parasitaemia in the negative control group

B – the parasitaemia in the test group.

The statistical significance of differences in parasitaemias between control and test groups is assessed using the Student's t-test. P-values of 0.05 or less are considered significant.

Doses resulting in survival times greater than that of infected non-treated mice are considered active.

Intraperitoneal administration

Each dose is given in 0.2ml solution i/p.

All the other procedures remain the same as in the oral experiment.